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A Study on the Role of Histone Modifiers in Arabidopsis Developmental Transitions

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Abstract

A Study on the Role of Histone Modifiers in Arabidopsis Developmental Transitions

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Plants undergo a number of developmental transitions throughout their life cycle. Developmental phase transitions of plants are largely controlled by a combination of endogenous and environmental signals such as hormones, age, photoperiod, temperature, and nutrient. Recent studies have demonstrated that epigenetic control of phase-specific genes is one of major mechanisms allowing developmental transitions in plants. Epigenetic regulation is made possible by DNA methylation, histone modifications, non-coding RNAs, and crosstalks among them. These epigenetic mechanisms control transcriptional activity of target genes via changes

of their chromatin structure. Flowering and seed germination are the best-known developmental transitions controlled by epigenetic mechanisms. Thereby, I focused on understanding epigenetic mechanisms acting in the regulation of flowering and germination.

Flowering is a critical transition from vegetative to reproductive development. Flowering is genetically controlled by networks of flowering genes. FLOWERING LOCUS T (FT) plays a key role as a mobile floral induction signal during the floral transition. Deposition of a repressive mark, trimethylation at histone H3 lysine 27 (H3K27me3), has been reported as one of the mechanisms allowing for *FT* repression in *Arabidopsis*. However, the role of an active mark, H3K4me3, in *FT* regulation has not been addressed, nor have the components affecting this mark been identified. Mutations in *Arabidopsis thaliana Jumonji 4* (*AtJmj4*) and *EARLY FLOWERING 6* (*ELF6*) caused an additive early flowering correlated with increased expression of *FT* mRNA and H3K4me3 levels within *FT* chromatin. AtJmj4 protein possessed specific demethylase activity for mono-, di-, and trimethylated H3K4. AtJmj4 and ELF6 associated directly with the *FT* transcription initiation region, where H3K4me3 levels increased most significantly in the mutants. Thus, the study in the Chapter II demonstrates the roles of AtJmj4 and ELF6 as H3K4 demethylases directly repressing *FT* chromatin and preventing precocious flowering in *Arabidopsis*.

Seed germination is another pivotal transition from embryonic to post-embryonic development. Phytohormones, namely GA and ABA, play central but antagonistic roles in the regulation of seed germination. Seed germination is positively controlled by active GA and negatively controlled by ABA levels and

ABA signaling. However, ABA-dependent regulatory pathway of seed germination is not fully understood. Several *ABA INSENSITIVE* (*ABI*) genes are involved in ABA signaling. Arabidopsis SNL proteins, orthologs of yeast Swi-independent 3 (Sin3), are putative transcriptional co-repressors possibly involved in gene-silencing by acting in concert with histone deacetylases (HDACs). In the Chapter III, it is demonstrated that seed germination and early seedling establishment of *SNL*quadruple mutant (*snl1234*) mimics the germination process under hyperactive ABA signaling conditions. Dormancy released *snl1234* seeds and embryos showed delayed germination and this germination phenotype was exaggerated with exogenous ABA treatment. Moreover, different expression patterns of the ABA-signaling genes, *ABI3*, *ABI4*, and *ABI5*, were observed in the *snl1234* mutant during germination and early seedling establishment. Altered *ABI* expression in *snl1234* was associated with enriched histone acetylation within *ABI* chromatin. Further, SNL3 protein directly targets *ABI3* and *ABI5* loci. Taken together, SNLs play an important role during seed germination and early seedling establishment as bedrock for histone deacetylation-based mechanism of *ABI* gene regulation.

Keywords: epigenetic regulation, flowering, seed germination, *Arabidopsis thaliana Jumonji 4* (*AtJmj4*), *EARLY FLOWERING6* (*ELF6*), *FLOWERING LOCUS T* (*FT*), *SIN3-LIKE* (*SNL*), *ABA INSENSITIVE* (*ABI*)

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Abbreviations

ABA	Abscisic acid
ABI	ABA-INSENSITIVE
ABI3	ABA INSENSITIVE 3
ACO	ACC OXIDASE
ADA1	Alteration Deficiency in Activation 1
AFR1	SAP30 FUNCTION-RELATED 1
AG	AGAMOUS
AGL15	AGAMOUS-like15
AGL18	AGAMOUS-LIKE 18
AGO4	ARGONAUTE 4
AP1	APETALA1
AR	After-ripened
ARHs	ADP-ribosyl hydrolases
ARP6	ACITN-RELATED PROTEIN 6
ARTs	ADP-ribosyltransferases
AS1	ASYMMETRIC LEAVES1
Ash1	Absent, Small, or Homeotic Disc 1
ASHH	ASH1 homologs of which four ASH1 HOMOLOG
ASHR	ASH1-RELATED
AtJmj4	Arabidopsis thaliana Jumonji4
AtSWR1c	Arabidopsis SWR1 complex
ATXRs	ARABIDOPSIS TRITHORAX RELATED PROTEINS

ATXs	ARABIDOPSIS TRITHORAX PROTEINS
BES1	BRASSINAZOLE-RESISTANT 2
bHLH	Basic helix-loop-helix
BiFC	Bimolecular fluorescence complementation
bp	base pair
BR	Brassinosteroid
BTD	Biotinidase
$CaMV35S_{pro}$	Cauliflower Mosaic Virus 35S promoter
CBF1	C-REPEAT/DRE BINDING FACTOR 1
ChIP	Chromatin immunoprecipitation
CIB1	CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX1
CLF	CURLY LEAF
CLSY1	CLASSY1
CMT3	CHROMOMETHYLASE 3
CO	CONSTANS
COLDAIR	COLD ASSISTED INTRONIC NONCODING RNA
COMPASS	COMPLEX PROTEINS ASSOCIATED WITH SET 1
COOLAIR	COLD INDUCED LONG ANTISENSE INTRAGENIC RNA
CRY	CRYTOCHROMES
Ct	Threshold cycle
CYP707As	ABA 8’-hydroxylases
DAG1	DOF AFFECTING GERMINATION 1

DCL1	DICER-LIKE 1
DCL3	DICER-LIKE 3
DIC	Differential interference contrast
DME	DEMETER
DML2	DEMETER-LIKE proteins
DML3	DNA GLYCOSYLASE DEMETER-LIKE PROTEIN 3
DMS3	DEFECTIVE IN MERISTEM SILENCING 3
DNMTs	DNA methyltransferases
DOF	DNA-BINDING ONE ZINC FINGER
DOG1	DELAY OF GERMINATION 1
DRD1	DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1
DRM2	DOMAINS REARRANGED METHYLTRANSFERASE 2
DTT	Dithiothreitol
E(z)	Enhancer of zeste
EBS	EARLY BOLTING IN SHORT DAYS
EDTA	Ethylenediaminetetraacetic acid
EFS	EARLY FLOWERING IN SHORT DAYS
ELF6	EARLY FLOWERING 6
ELF7	EARLY FLOWERING 7
ELF8	EARLY FLOWERING 8
EMF1	EMBRYONIC FLOWER 1
EMF2	EMBRYONIC FLOWER 2

ERF4	ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 4
ERF9	ETHYLENE SIGNALING GENES
ERS1	ETHYLENE RESPONSE SENSOR 1
ESD1	EARLY IN SHORD DAYS 1
F1	First filial generation
FD	FLOWERING LOCUS D
FES1	FRIGIDA-ESSENTIAL 1
FH	Freshly harvested
FIE	FERTILIZATION-INDEPENDENT ENDOSPERM
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FLK	FLOWERING LATE KH MOTIF
FLX	FLC EXPRESSOR
FRIc	FRIGIDA complex
FRL1	FRIGIDA-LIKE 1
FT	FLOWERING LOCUS T
FUS3	FUSCA 3
FWA	FLOWERING WAGENINGEN
GA	Gibberellic acid
GAI	GA INSENSITIVE
GCN5	GENERAL CONTROL NON-DEPRESSIBLE 5
GFP	Green fluorescence protein
GID1A	GA INSENSITIVE DWARF 1A

GNAT	GCN5-related acetyltransferases
GUS	β-glucuronidase
H2Bub1	histone H2B monoubiquitylation
H3K27me3	histone H3 lysine 27 trimethylation
H3K4me3	histone H3 lysine 4 trimethylation
H3K9me2	histone H3 lysine 9 dimethylation
H4R3me2s	histone H4 arginine 3 symmetric dimethylation
HA	HUMAN INFLUENZA HEMAGGLUTININ
HATS	HISTONE ACETYLTRANSFERASES
HCS	HOLOCARBOXYLASE SYNTHETASE
HD2	HISTONE DEACETYLASE 2-RELATED PROTEIN FAMILIES
HDA1	HISTONE DEACETYLASE 1
HDACs	HISTONE DEACETYLASES
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
His	Histidine
HLS1	HOOKLESS 1
HP1	HETEROCHROMATIN PROTEIN 1
HRP	Horseradish peroxidase
HSL1	HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2-LIKE1
HUB1	HISTONE MONOUBIQUITINATION 1
IBM1	INCREASED EXPRESSION OF BONSAI METHYLATION 1

JMJs	JmjC domain–containing proteins
K	Lysine
KYP	KRYPTONITE
LD	LUMINIDEPENDENS
LDL1	LSD1-LIKE 1
LEC1	LEAFY COTYLEDON 1
LFY	LEAFY
LHP1	LIKE HETEROCHROMATIN PROTEIN 1
lncRNAs	long non-coding RNAs
LSD1	LYSINE SPECIFIC DEMETHYLASES 1
MEA	MEDEA
MEE27	MATERNAL EFFECT EMBRYO ARREST 27
MET1	DNA METHYLTRANSFERASE 1
miR402	microRNA 402
MRG1	Morf Related Gene 1
MS	Murashige-skoog
MSI1	Musashi RNA-binding protein 1
MSI1	MULTI-COPY SUPPRESSOR OF IRA 1
MSI4	MULTI-SUBUNIT SUPPRESSOR OF IRA 4
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60
NCEDs	Nine-cis-epoxycarotenoiddioxygenases
ORCs	ORIGIN RECOGNITION COMPLEXES
PAC	Paclobutrazol
PARDs	poly-ADP-ribose glycohydrolases

PARPs	poly-ADP-ribose polymerases
PcG	Polycomb group
PHD	plant homeo domain finger
PHE1	PHERES1
PhoRC	Pho repressive complex
PHYB	PHYTOCROME B
PIE1	PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1
PIFs	PHYTOCHROME-INTERACTING FACTORS
PIL5	PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5
PKL	PICKLE
Pol II	RNA polymerase II
PolIV	RNA Polymerase IV
PRC	POLYCOMB REPRESSIVE COMPLEX
PRMTs	PROTEIN ARGININE METHYLTRANSFERASES
PTMs	Post-translational modifications
PVPP	Polyvinylpolypyrrolidone
qPCR	quantitative PCR
R	Arginines of histone, including Arg2
RdDM	RNA-directed DNA methylation
RDM1	RNA-DIRECTED DNA METHYALTION 1
RDR2	RNA-DEPENDENT RNA POLYMERASE2
REF6	RELATIVE OF EARLY FLOWERING 6
RGA	REPRESSOR OF GAI

RGA1	REPRESSOR OF GA1
RGL1	RGA-LIKE1
ROS1	REPRESSOR OF SILENCING 1
RPD3	REDUCED POTASSIUM DEPENDENCE 3
RRM	RNA RECOGNITION MOTIF
SA	Salicylic acid
SAM	Shoot apical meristem
SDG8	SET DOMAIN GROUP 8
SDS	Sodium dodecyl sulfate
SE	Standard error
SHH1	SAWADEE HOMEODOMAIN HOMOLOG 1
Sin3	Swi-independent 3
SIR2	SILENT INFORMATION REGULATOR 2
siRNAs	small interfering RNAs
SMZ	SCHLAFMÜ TZE
SNL	SIN3-LIKE
SNZ	SCHNARCHZAPFEN
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SOM	SOMNUS
SPT	SPATULA
STM	SHOOT MERISTEMLESS
SUF3	SUPPRESSOR OF FRIGIDA 3
SUMO	Small ubiquitin-related modifier

SUVH4	SU(VAR)3-9 HOMOLOG 4
SVP	SHORT VEGETATIVE PHASE
SWN	SWINGER
TEM1	TEMPRANILLO1
TFL2	TERMINAL FLOWER 2
TOE1	TARGET OF EAT1
TPL	TOPLESS
TrxG	Trithorax group
TSS	Transcription start site
UBC1	UBIQUITIN-CONJUGATING ENZYME 1
UBQ	UBIQUITIN
VIL1	VIN3-LIKE 1
VIM	VARIATION IN METHYLATION
VIN3	VERNALIZATION INSENSITIVE 3
VIP6	VERNALIZATION INDEPENDENCE 6
VRN1	VERNALIZATION 1

Chapter I

General Introduction

1.1 Epigenetic gene regulation

Epigenetics was first defined as a branch of biology, which investigates gene expression and underlying interactions between genes and their products that bring the phenotype (Waddington, 1942). With more and more extensive studies, the term epigenetics is developed and now described as: a branch of biology that searches for heritable changes that are made on a gene locus or a gene-encompassing chromosome without alteration of the DNA sequence (Aaron et al., 2007). Over the past years, numerous efforts have been made in the field of epigenetics to understand biological phenomena that are not explained by genetics. Among diverse regulatory mechanisms of gene expression, epigenetic regulation affects the chromatin structure that subsequently controls transcriptional activity of the target gene. Epigenetic regulation is made possible by DNA methylation, histone modification, non-coding RNAs and the crosstalks among them. These mechanisms will be exclusively described in the first part of this chapter.

1.1.1 DNA methylation

DNA methylation which contributes to gametogenesis, fertilization, and vegetative developments in plant has been considerably studied. Recently, crosstalks with histone modifications are revealed to be also important. Genome-wide DNA methylation levels are regulated by combined activities of DNA methyltransferases (DNMTs) and DNA glycosylases, which may establish, maintain, and/or remove methyl groups from the cytosine bases. In both plants and animals, 5-methyl-cytosine generally results in gene silencing and heterochromatin formation (Li and

Zhang, 2014; Zhao and Garcia, 2014; Furner and Matzke, 2011; Meyer, 2011). Plant DNA methylation may occur in context of CG, CHG, or CHH, where H refers to A, T, or C. It has been identified that DNA methylation is implemented through several pathways.

The RNA-directed DNA methylation (RdDM) pathway is mainly involved in *de novo* DNA methylation on previously unmethylated cytosine residues. In the RdDM pathway, 24-nucleotide (nt) small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) act as major regulatory factors (Wierzbicki et al., 2008; Law et al., 2010). The 24-nt siRNAs, which are generated by DICER-LIKE 3 (DCL3), are loaded onto ARGONAUTE 4 (AGO4) and the siRNA-AGO4 complex then recruits the DNA methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), to the target loci (Law et al., 2010). The generation of siRNA-guide is promoted by the plant-specific RNA polymerase, Polymerase IV (PolIV) (Onodera et al., 2005; Zhang et al., 2007; Herret et al., 2005). The PolIV complex was identified to include CLASSY1 (CLSY1), RNA-DIRECTED DNA METHYLATION 4 (RDM4), SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), and RNA-DEPENDENT RNA POLYMERASE2 (RDR2) (Law et al., 2011). LncRNAs, long nascent transcripts generated from RdDM target loci, can also physically interact with AGO4 and lead DNA methylation factors to the target loci through its complementary sequence (Wierzbicki et al., 2009). The biogenesis of lncRNAs, which is independent of siRNA biogenesis, is facilitated by another plant-specific RNA polymerase, PolV (Wierzbicki et al., 2008). A putative chromatin-remodeling complex, called the DDR complex, also contributes to the biogenesis of lncRNAs. The DDR complex

is named after its components: SWI2/SNF2-like chromatin-remodeling protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), and RNA-DIRECTED DNA METHYALTION 1 (RDM1) (Kannoet et al., 2004; Law et al., 2010; Gao et al., 2010; Kanno et al., 2008).

Depending on cytosine context, distinct DNA methyltransferases work through different mechanisms to maintain DNA methylation level. CG methylation is mainly sustained by DNA METHYLTRANSFERASE 1 (MET1), the plant ortholog of mammalian Dnmt1, which might also contribute to *de novo* CG methylation. VARIATION IN METHYLATION (VIM) family proteins, including VIM1, VIM2, and VIM3, are additionally discovered to associate in the maintenance of CG methylation (Yao et al., 2012; Stroud et al., 2013). CHG methylation is maintained by a plant-unique cytosine methyltransferase CHROMOMETHYLASE 3 (CMT3), which is guided by dimethylated histone H3 lysine 9 (H3K9me2) (Lindroth et al., 2001; Law and Jacobsen, 2010; Du et al., 2012). Interestingly, CHG methylation and H3K9me2 interdependently function for target gene silencing. Methylated CHG loci are preferentially bound by histone methyltransferases KRYPTONITE (KYP)/SU(VAR)3-9 HOMOLOG 4 (SUVH4). KYP/SUVH4, SUVH5, and SUVH6 catalyze H3K9me2 in *Arabidopsis* (Inagaki et al., 2010). The mutuality between DNA and histone methylation is required for further examination. In addition to CMTs and SUVH, DRM2 also maintains CHG methylation via the RdDM pathway (Stroud et al., 2013). CMT2, a CMT3 homolog, has a role in maintaining CHH methylation, presumably via crosstalk with histone modifications (Zemach et al., 2013).

Together with DNA methyltranseferases, DNA glycosylases control DNA methylation levels by counteracting the other, and vice versa. DNA glycosylases eliminate a methyl group from 5-methylcytosine by base excision (Gehring et al., 2006; Gong et al., 2002). In *Arabidopsis*, REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER-LIKE proteins, namely DML2 and DML3, are identified, and they harbor DNA glycosylase domains. DME is required for demethylation of CG dinucleotides (Xiao et al., 2003) and is thought to be especially important in gametophyte developments (Choi et al., 2002; Schoft et al., 2011). DML2 and DML3, DME paralogs, also play a role in demethylation of CG, CHG, and CHH methylation at specific genomic sites (Ortega et al., 2008). Moreover, ROS3, an RNA-binding protein, associates with ROS1 to cause DNA demethylation in its target sites (Zheng et al., 2008). Therefore, multiple DNA glycosylases play a critical role against DNA methyltransferase activities for various cellular processes *in planta*.

1.1.2 Histone modification

In eukaryotic cells, genomic DNA is compacted into the nucleus as a complex structure called chromatin. Chromatin is comprised of repeated units of nucleosome, which consists of 146 base pair (bp) DNA that is wrapped around a histone octamer. The core-protein complex is composed of pairs of each of the four histone proteins, H2A, H2B, H3, and H4, with protruding amino-tails (Kornberg., 1977; van Holde., 1988; Luger et al., 1997; Kornberg and Lorch, 1999). Post-translational modifications (PTMs) of the histone tails lead to dynamic changes in

chromatin structure, and such modifications may contribute to various epigenetic controls of cellular processes and memory (Luger K et al., 1998; Wolffe and Hayes, 1999; Strahl and Allis, 2000; Berger, 2002; Turner, 2002). General correlations between gene-transcription activity and histone modifications, such as acetylation, methylation, phosphorylation, carbonylation, ubiquitylation, sumoylation, ADP ribosylation, and biotinylation, have been studied for about half a century (Allfrey et al., 1964; Jenuwein et al., 2001; Turner, 2000 and 2002; Strahl and Allis, 2000). Histone acetylation and methylation of specific residues, directly affecting gene transcription rate, are well described (Peterson and Laniel, 2004).

Generally, histone modification influences gene activity through conformational change of chromatin. Acetylation is highly dynamic and reversible. It causes an open structure of chromatin by eliminating the positive charge on lysine. Like acetylation, histone phosphorylation is also highly dynamic and facilitates the access of transcription factors to DNA. Phosphorylations occur on serine, threonine, and tyrosine and add negative charges onto histone tails, causing structural change of chromatin. Phosphorylation is involved in the response of DNA damage and promotes chromosomal condensation and segregation (Xiao et al., 2009; Krishnan et al., 2009). Unlike acetylation or phosphorylation, histone methylation does not affect the charge of histone. This type of histone modification rather induces the structural change of chromatin in more complex manners. Lysine methylation is relatively stable than acetylation or phosphorylation, whereas arginine methylation is more or less temporary (Zee et al., 2010; Huang et al., 2013a). Histone methylation plays a pivotal role in epigenetic regulation via mitotically heritable ways, especially in the control of flowering and seed

germination (Cazzanelli et al., 2009; D'Urso and Brickner, 2014; Jeong et al., 2009; Cho et al., 2012). In the sections of below, histone acetylation and methylation will be described more in details.

Carbonylation, an irreversible modification, takes place on arginine and lysine. Histone carbonylation masks the positive charges of histones, consequently resulting in released form of chromatin and easy access of transcription factors (Wondrak et al., 2000). A reversible modification, ubiquitylation, is achieved through the sequential actions of E1-activating, E2-conjugating, and E3-ligating enzymes (Hershko and Ciechanover, 1998) and is removed by the action of isopeptidases (Wilkinson, 2000). Mono-ubiquitylation of histone H2A lysine 119 (H2AK119ub1) participates in transcriptional repression through subsequent binding of the polycomb repressive complex (PRC) (Hunt et al., 2013). H2B ubiquitylation is related to transcriptional activation by facilitating H3K4 methylation and transcription elongation (Lee et al., 2007; Kim et al., 2009; Zhang, 2003). The role of ubiquitylation in transcription regulation is still remained to be largely elusive. Sumoylation, similar to ubiquitylation, is achieved by the E1, E2, and E3 enzymatic cascades (Seeler et al., 2003). However, it is independent from ubiquitylation because sumoylation is not associated with protein degradation. Small ubiquitin-related modifier (SUMO) polypeptide has been shown to attach onto all four core histones. Moreover, a few studies have shown that sumoylation plays a role in transcriptional repression through the competition with acetylation or ubiquitylation to target substrate lysine. (Johnson 2004; Iniguez-Lluh Ί 2006). However, the molecular mechanism of SUMO-dependent chromatin structural changes is yet to be clear. In addition, other studies have suggested that histone

sumoylation causes the repression of transcriptional activity through the recruitment of HISTONE DEACETYLASE 1 (HDAC1) and HETEROCHROMATIN PROTEIN 1 (HP1) (Shiio and Eisenman 2003).

ADP-ribosylation is observed on glutamate and arginine residues as mono- or poly-ADP ribosylated forms (Hassa et al., 2006). ADP-ribosylation is also a reversible modification. ADP-ribosylation are implemented by the ADP-ribosyltransferases (ARTs) and the poly-ADP-ribose polymerases (PARPs) for mono- and poly-ADP-ribosylation, respectively (Messner and Hottiger 2011). These processes can be reversed by ADP-ribosyl hydrolases (ARHs) and poly-ADP-ribose glycohydrolases (PARDs) (Koch-Nolte et al., 2008). Mono-ADP-ribosylation participates in the regulation of cell–cell and cell–matrix interactions (Corda and DiGirolamo 2002; Hassa et al., 2006), whereas poly-ADP-ribosylation is involved in the various cellular processes such as cell differentiation, DNA damage detection and repair, and regulation of transcription via chromatin modification (Masutani et al., 2005; Hassa et al., 2006). Histone biotinylation, a covalent attachment of biotin to the ε-amino group of lysine residues, is a reversible process (Kothapalli et al., 2005). Biotinylation is achieved via two biotinyl ligases: biotinidase (BTD) (Brenner, 2002) and holocarboxylase synthetase (HCS) (Narang et al., 2004). Interestingly, it has been suggested that the alternative splicing variants of BTD might act to catalyze the debiotinylation of histones (Ballard et al., 2002; Zempleni, 2005). Biotinylation participates in gene silencing, cellular responses to DNA damage, genome stability, mitotic condensation of chromatin, and cell proliferation (Kothapalli and Zempleni 2005; Rodriguez-Melendez and Zempleni 2003; Filenko et al., 2011). Biotinylation has been shown

to influence other histone modifications such as acetylation, phosphorylation, and methylation in synergistic or antagonistic ways or vice versa (Camporeale et al., 2004; Kothapalli et al., 2005).

1.1.2.1 Histone acetylation

Histone acetylation, one of the major histone modifications, occurs on all four core histones (Sterner and Berger, 2000; Roth et al., 2001) and neutralizes the positive charge on the histone proteins. Thereby, affinity of the histones for surrounding DNA is reduced and, consequently, the accessibility of chromatin for transcription regulators is increased (Kuo and Allis, 1998). Histone acetylation is mainly catalyzed at K9, K14, K18, and K23 of histone H3 and K8, K12, K16. and K20 residues of histone H4 (Fuchs et al., 2006). Histone acetylation is dynamic and reversible through the action of histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, or HDACs) (Kuo et al., 1998; Brownell and Allis, 1996; Roth et al., 2001)

1.1.2.1.1 Histone acetyltransferases (HATs)

HATs are classified into two categories, Type A and Type B, depending on their subcellular distributions (Brownell and Allis, 1996; Roth et al., 2001). Type B HATs, which are mostly cytoplasmic proteins, catalyze histone acetylation particularly at lysine 5 and 12 of histone H4 (Verreault et al., 1998; Parthun et al., 1996). A type B HAT, which was first characterized in maize, functions as a

heterodimeric complex acting on histone H4 and selectively acetylates lysine 5 and 12 (Eberharter et al., 1996; Kolle et al., 1998; Lusser et al., 1999). On the other hand, the type A HATs mainly catalyze acetylation of nuclear histones and influence gene transcription through changing chromatin assembly (Carrozza et al., 2003). The type A HATs, including GCN5-related acetyltransferases (GNAT), MOZ, Ybf2/Sas3, Sas2 and Tip60 (MYST), CBP/p300, and the TFIID subunit TAF250, are found in eukaryotes (Sterner et al., 2000; Pandey et al., 2002; Carrozza et al., 2003). In *Arabidopsis*, HAT-encoding genes are also grouped as the classified families: four GNATs (HAG1-HAG3, MMC1), two MYSTs (HAM1 and HAM2), five CBPs (HAC1, HAC2, HAC4, HAC5, and HAC12), and two TAFII250s (HAF1 and HAF2) (Pandey et al., 2002; Perrella et al., 2010).

Several studies have suggested that HATs have various roles in plant responses to stress and developmental changes (Bertrand et al., 2003; Long et al., 2006). In yeast, General Control Non-depressible 5 (GCN5) protein coordinates with Alteration/Deficiency in Activation 1 (ADA1) and ADA3 as a subunit of protein complexes to stimulate transcriptional activation (Grant et al., 1997; Balasubramanian et al., 2002). AtGCN5, *Arabidopsis* GCN5-type HAT, also interacts with *Arabidopsis* homologs of the yeast transcriptional adaptor proteins, ADA2a and ADA2b (Stockinger et al., 2001; Mao et al., 2006). It is reported that AtGCN5 and ADA2 interact with transcription factors such as C-REPEAT/DRE BINDING FACTOR 1 (CBF1) in cold acclimation (Vlachonasios et al., 2003).

1.1.2.1.2 Histone deacetylases (HDACs)

HDACs can remove acetyl groups from histone and non-histone proteins. In plant, HDAC proteins can be grouped into three main families, which are RPD3/HDA1 (Reduced Potassium Dependence 3/Histone Deacetylase 1), SIR2 (Silent Information Regulator 2), and HD2 (Histone Deacetylase 2)-related protein families, base on the sequence homology to yeast HDACs (Pandey et al., 2002).

Accumulating data indicate that altered expression of HDACs affect plant growth, development, and responses to abiotic and biotic stress. Based on sequence similarity, the RPD3/HDA1 superfamily is classified into three classes. Class I proteins include HDA6, HDA7, HDA9, and HDA19 which are the best characterized HDACs in *Arabidopsis*. (Hollender and Liu, 2008; Alinsug et al., 2009). HDA2, HDA5, HDA15, and HDA18 are classified as Class II, and HDA8, HDA10, HDA14, and HDA17 belong to Class III family (Pandey et al., 2002; Hollender and Liu, 2008).

HDA6 interacts with a DNA methyltransferase, MET1, in the maintenance of DNA methylation (Liu et al., 2012; To et al., 2011; Aufsatz et al., 2002). *Arabidopsis* HDA6 participates in control of flowering as a histone deacetylase (Wu et al., 2008). Flowering regulation proteins, such as FLD, a LSD1 (Lysine Specific Demethylase 1)-type histone demethylase (Jiang et al., 2007), FVE/ MULTI-SUBUNIT SUPPRESSOR OF IRA 4 (MSI4), and MSI5, are known to co-work with HDA6 (Gu et al., 2011). Especially, the physical association between HDA6 and FLD shows a crosstalk between histone deacetylation and demethylation (Yu et al., 2011). Another well-known member of HDACs is HDA19, which is the closet homolog of HDA6. HDA19 is reported as an important factor for proper vegetative development (Tian et al., 2005; Zhou et al., 2005; Long

et al., 2006). Recent studies have shown that HDA19 interacts with the HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2-LIKE1 (HSL1) and is enriched at target loci to directly repress seed maturation gene expressions during germination (Zhou et al., 2013). It was also clarified that HDA19 is involved in pathogen defense mechanism through salicylic acid (SA) signaling pathways (Choi et al., 2012). Additionally, both HDA6 and HDA19 redundantly regulate the expression of embryogenesis related genes, including *LEAFY COTYLEDON 1* (*LEC1*), *FUSCA 3* (*FUS3*), and *ABA INSENSITIVE 3* (*ABI3*) (Tanaka et al., 2008).

HDA7 was reported to have roles in female gametophyte development and embryogenesis (Cigliano et al., 2013). HDA9 has been reported to function in flowering time regulation through the repression of *AGL19* transcription (Kim et al., 2013; Kang et al., 2015). HDA18 has been suggested to affect cellular patternings in root epidermis (Xu et al., 2005; Liu et al., 2013a). HDA14 was reported to associate with iateed tomis (Tran et al., 2012). HDA15 forms a repression complex with PHYTOCHROME INTERACTING FACTOR 3 (PIF3), a transcription factor that is involved in photomorphogenesis, and regulates light-responsive genes in photosynthesis and chlorophyll biosynthesis (Liu et al., 2013b).

Arabidopsis has two SIR2-encoding genes which are *SRT1* and *SRT2*. SRT2 is involved in mitochondrial energy metabolism and metabolite transport (Koenig et al., 2014). SRT2 has a negative role in plant basal defense through suppression of SA biosynthesis (Wang et al., 2010). In plants, however, the functions of the SIR2 family (Sirtuins) HDACs are not fully understood.

The HD2 protein family is plant-specific. This group has four members:

HD2A/HDT1, HD2B/HDT2, HD2C/HDT3, and HD2D/HDT4. (Pandey et al., 2002). HD2A functions in seed development, and flowering and postembryonic establishment of nucleolar dominance (Wu et al., 2000; Zhou et al., 2004; Pontes et al., 2007). HD2A and HD2B control miR165/166 distribution and the formation of adaxial01baxial leaf polarity by independent association with ASYMMETRIC LEAVES1 (AS1) and AS2 (Ueno et al., 2007). In addition, the contribution of HDACs in plant responses to abiotic stresses has been revealed (Luo et al., 2012a; Yuan et al., 2013). HD2C functionally associates with HDA6 to repress the expression of *ABSCISIC ACID-INSENSITIVE 1* (*ABI1*), *ABI2*, and *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 4* (*ERF4*) (Luo et al., 2012a) and regulates rRNA gene expression through histone modifications (Kim et al., 2014). The expression of HD2-encoding gene itself is repressed by NaCl and ABA (Sridha and Wu, 2006; Luo et al., 2012b).

1.1.2.2 Histone methylation

Histone lysine residues can be methylated, and methylations are classified as mono-, di-, or tri-methylation depending on the number of added methyl groups. Different types of histone methylations, for instance histone H3 lysine 4 mono-/di-/tri-methylation (H3K4me1, H3K4me2, and H3K4me3), histone H3 lysine 27 trimethylation (H3K27me3), and histone H3 lysine 9 dimethylation (H3K9me2), have distinctive functions for epigenetic regulation of transcription.

1.1.2.2.1 H3K4 mono/ di/ trimethylation

H3K4me3 leads to gene transcription as an active mark while the correlations between gene transcription and other methylation marks on H3K4 residue, such as H3K4me1 and H3K4me2, are yet unclear (Zhang X et al., 2009). All of the H3K4 marks exist in euchromatin but not in heterochromatin which are mostly transposons and repetitive DNA sequences. While H3K4me1 is generally enriched in the gene body, H3K4me2 and H3K4me3 are enriched in the promoter region of the gene (Zhang X et al., 2009). The H3K4 methylation is highly conserved in other organisms (Liu et al., 2005; Li et al., 2008; Mikkelsen et al., 2007; Barski et al., 2007; Pokholok et al., 2005). A yeast H3K4 methyltransferase, called SET1, has a conserved SET domain and forms the COMPLEX PROTEINS ASSOCIATED WITH SET 1 (COMPASS) complex that regulates monomethylation, dimethylation, and trimethylation of H3K4 (Eissenberg et al. 2010). In *Drosophila*, homologs of yeast SET1, Trithorax group (TrxG) proteins and Absent, Small, or Homeotic Disc 1 (Ash1), mediate H3K4 methylation. All of TrxG proteins and Ash1 contain a SET domain (Byrd et al., 2003; Schulze et al., 2007).

In *Arabidopsis*, there are five ARABIDOPSIS TRITHORAX PROTEINS (ATXs), seven ARABIDOPSIS TRITHORAX RELATED PROTEINS (ATXRs), and seven ASH1 homologs of which four ASH1 HOMOLOG (ASHH) proteins and three ASH1-RELATED (ASHR) proteins are included (Baumbusch et al., 2001). ASHH2, also named as EARLY FLOWERING IN SHORT DAYS (EFS) or SET DOMAIN GROUP 8 (SDG8), is well studied as histone methyltransferase for both

H3K4 and H3K36 (Ko et al., 2010). ATX1 and ATX2 are reported to have divergent roles in the transcription regulation through H3K4me3 and H3K4me2 enrichments, respectively (Saleh et al., 2008; Alvarez-Venegas et al., 2005). ATXR3, also known as SDG2, mainly acts as a methyltransferase for H3K4me3 (Guo et al., 2010; Berr et al., 2010).

1.1.2.2.2 H3K27 tri-methylation

In *Arabidopsis*, H3K27me3 contribute to epigenetic silencing of several developmental genes such as *FLOWERINGLOCUS C* (*FLC*), *AGAMOUS* (*AG*), *SHOOT MERISTEMLESS* (*STM*), *MEDEA* (*MEA*), and *PHERES1* (*PHE1*) (Pien et al., 2007; Hennig et al., 2009). Distribution of H3K27me3 within *Arabidopsis* chromatin is restricted to short regions which only cover a single gene in contrast to animals (Zhang et al., 2007).

Polycomb group (PcG) proteins mediate tri-methylation of H3K27 in all organisms. PcGs form several complexes, including Polycomb Repressive Complex 1 (PRC1), PRC2, and Pho repressive complex (PhoRC) (Muller et al., 2009). PRC1 recognizes and supports H3K27me3 enrichment (Schwartz et al., 2007). Although any components of PRC1 are not encoded in *Arabidopsis*, Several PRC1-like activities have been reported to recognize H3K27me3 and contribute to the gene repression. In *Arabidopsis*, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)/TERMINAL FLOWER 2 (TFL2) (Zhang et al., 2007; Turck et al., 2007), VERNALIZATION 1 (VRN1) (Mylne et al., 2006), EMBRYONIC FLOWER 1 (EMF1) (Calonje et al., 2008), and AtRING1a and AtRING1b (Xu et al., 2008;

Sanchez-Pulido et al., 2008) are reported as analogues of PRC1 complex components. PRC2 has a key subunit called Enhancer of zeste (E(z)), a specific H3K27me3 methyltransferase. In *Arabidopsis*, three E(z) homologs have been reported; CURLY LEAF (CLF), MEA, and SWINGER (SWN) (Pien et al., 2007; Hennig et al., 2009).

1.1.2.2.3 H3K9 dimethylation

In general, heterochromatin formation depends on trimethylation of H3K9 and interaction of methylated H3K9 with Heterochromatin Protein 1 (HP1) which has a chromo-domain (Lachner et al., 2001; Nakayama et al., 2001; Bannister et al., 2001; Jacobs et al., 2001). In *Arabidopsis*, however, H3K9me3 is typically enriched in euchromatin (Turck et al., 2007). Instead, dimethylation of H3K9 mostly occurs in pericentromeric heterochromatin, transposons, and in small portions of euchromatic repeats (Fuchs et al., 2006). H3K9me2 closely correlates with CHG methylated regions (Bernatavichute et al., 2008).

Su(var)3-9 homologs have been shown in many organisms to play roles in heterochromatin formation and gene silencing (Nakayama et al., 2001; Peters et al., 2001). The Su(var)3-9 family proteins are identified as histone lysine methyltransferases (Rea et al., 2000; Schulze et al., 2007). The SUVH proteins are identified as Su(var)3-9 homologs in *Arabidopsis*. KRYPTONITE (KYP), also known as SUVH4, contributes to heterochromatin formation catalyzing H3K9me1 and H3K9me2 enrichments (Jackson et al., 2004; Malagnac et al., 2002). SUVH5 and SUVH6 are reported to methylate H3K9 and act as histone lysine

methyltransferases (Jackson et al., 2004; Ebbs et al., 2005 and 2006). Although epigenetic regulation mechanisms are shared in plant and other organisms, H3K9 methylation in plant is still obscure. For example, although methylated H3K9 is recognized by HP1, *Arabidopsis* LHP1 (a homolog of HP1) does not affect H3K9 methylation but recognizes H3K27me3 instead (Zhang et al., 2007).

1.1.2.2.4 Arginine methylation

Arginines of histone, including Arg2 (R2), Arg8 (R8), Arg17 (R17), Arg26 (R26) of H3, and Arg3 (R3) of H4, are easily methylated by protein arginine methyltransferases (PRMTs) (Bedford et al., 2005). PRMTs are classified as either type I or type II enzymes. Type I PRMTs catalyze asymmetric arginine dimethylation whereas type II PRMTs enrich symmetric arginine dimethylation (Bedford et al., 2009). In *Arabidopsis*, 9 PRMTs have been identified (Niu et al., 2007). As type I methyltransferases, AtPRMT1a, AtPRMT1b, and AtPRMT10, catalyze histone H4R3 asymmetric di-methylation (Niu et al., 2007; Yan et al., 2007). In addition, AtPRMT4a and AtPRMT4b catalyze asymmetric di-methylation of histone H3 R2, R17, and R26 *in vitro* and H3R17me2a *in vivo*. AtPRMT4a and AtPRMT4b are functionally redundant in the regulation of *FLC*-dependent flowering (Niu et al., 2008). AtPRMT5/SKB1 acts as a type II methyltransferase which dimethylates H4R3 symmetrically (Yan et al., 207; Pei et al., 2007; Schmitz et al., 2008; Wang et al., 2007) Taken together, AtPRMT4a, AtPRMT4b, AtPRMT5/SKB1, and AtPRMT10 are involved in regulation of flowering time, even though they show distinctive enzymatic activities from each other (Niu et al.,

2007; Niu et al., 2008; Pei et al., 2007; Schmitz et al., 2008; Wang et al., 2007).

1.1.2.2.5 Readers of histone methylation

Currently, three superfamilies are classified according to their conserved reader-module types. There are chromo-domain or chomo-like-domain-containing superfamily, plant homeo domain finger (PHD) superfamily, and proteins containing WD40 repeats of WDR5 (Ruthenburg et al., 2007; Taverna et al., 2007). In *Arabidopsis*, a few proteins have been shown to recognize methylated histone as readers. LHP1/TFL2, homologue of the Drosophila heterochromatin protein 1 (HP1) which recognizes H3K9me3, can also bind to PRC2-mediated H3K27me3 modification through its chromo-domain (Exner et al., 2009; Gaudin et al., 2001; Turck et al., 2007; Zhang et al., 2007). The binding of LHP1/TFL2 to H3K27me3 is required to maintain silencing of *FLC* (Mylne et al., 2006) and *FT* (Kotake et al., 2003; Farrona et al., 2008; Turck et al., 2007). The origin recognition complexes (ORCs), ORC1a and ORC1b, contain PHD domains and interact with H3K4me3 (de la Paz Sanchez et al., 2009). Additionally, AtING and AL, PHD proteins, are identified as readers that recognize H3K4me2 and H3K4me3 *in vitro* (Lee et al., 2009). The *Arabidopsis* WDR5a is a homolog of human WDR5 which reads H3K4me2. WDR5a is enriched at the *FLC* locus and is suggested to recognize H3K4 methylation in the presence of FRI (Jiang et al., 2009).

1.1.2.2.6 Erasers of histone methylation

Histone demethylase plays a crucial role as an eraser in histone methylation mechanism. Recently, two classes of enzymes are demonstrated as histone demethylases, which are LYSINE SPECIFIC DEMETHYLASES 1 (LSD1) and JUMONJI C (JmjC) domain–containing proteins. LSD1 and JmjC domain-containing proteins are capable of removing methyl groups from methylated lysine residues through distinct mechanisms of amine oxidation and hydroxylation, respectively. (Shi et al., 2004; Tsukada et al., 2006). LSD1 specifically demethylates mono- and di-methylated but not tri-methylated H3K4 (Shi et al., 2004). In *Arabidopsis*, four LSD1 homologs, FLOWERING LOCUS D (FLD), LSD1-LIKE 1 (LDL1), LDL2, and LDL3, are identified. FLD, LDL1, and LDL2 participate in *FLC* repression as H3K4 demethylases (He et al., 2003; Liu et al., 2007; Spedaletti et al., 2008). LDL1 and LDL2 are also involved in the regulation of *FLOWERING WAGENINGEN* (*FWA*) in vegetative tissues (Jiang et al., 2007).

Unlike LSD1, JmjC domain-containing proteins have demethylase activities towards all of the mono-, di- and tri-methylated lysines of histones (Klose et al., 2006). In *Arabidopsis*, 21 JmjC domain–containing proteins (JMJs) are classified into five subfamilies, including the KDM3/JHDM2, KDM4/JHDM3, KDM5/JARID1, JMJD6, and JmjC domain–only groups. (Hong et al., 2009; Lu et al., 2008). KDM4/JHDM3 group proteins catalyze demethylation of di- and trimethylated histone H3K9 and H3K36 in mammals (Klose et al., 2007). The homolog of KDM4/JHDM3 group proteins, EARLY FLOWERING 6 (ELF6/JMJ11) and RELATIVE OF EARLY FLOWERING 6 (REF6/JMJ12), are functionally characterized in flowering pathways (Lu et al., 2008; Noh et al., 2004). ELF6 and REF6 play divergent roles in the regulation of photoperiodic flowering

and *FLC*, respectively (Noh et al., 2004). More recently, a study suggested that ELF6 and REF6 are involved in the brassinosteroid signaling, being able to catalyze H3K9 demethylation (Yu et al., 2008). As a homolog of human KDM3/JHDM2, INCREASED EXPRESSION OF BONSAI METHYLATION 1 (IBM1/JMJ25) contributes to the repression of the cytosine methylation mechanism, possibly through demethylation of H3K9me1 and me2 (Lu et al., 2008; Miura et al., 2009; Sazeet al., 2008). However, biochemical properties of JMJs as H3K9 demethylase are still remained to be elucidated. The MATERNAL EFFECT EMBRYO ARREST 27 (MEE27/JMJ15), one of KDM5/JARID1 group protein, is reported to be involved in female gametophyte development as a H3K4 demethylase (Pagnussat et al., 2005; Lu et al., 2008). Another KDM5/JARID1 group protein *Arabidopsis thaliana* Jumonji4 (AtJmj4/JMJ14) also shows a demethlyase activity on H3K4me3, H3K4me2, and H3K4me1 and has a role in flowering regulation (Jeong et al., 2009; Yang et al., 2010; Lu et al., 2010). In spite of the efforts to discover powerful histone demethylases, there are still unsolved questions, such as how demethylases functionally regulate developmental processes in plants. Therefore, additional researches on histone demethylation are needed at biochemical and genetic levels.

1.2 Flowering

Flowering, a transition from vegetative to reproductive phases, is important in ensuring the reproductive success in plant life. Determining the proper time for floral transition is controlled by the expression of various flowering-regulatory

genes that are sensitive to internal signals and environmental cues, such as photoperiod and temperature. When a plant acquires potency to flower by endogenous signals, such as hormones, to achieve the appropriate developmental stage, external signals are then incorporated for flowering. In *Arabidopsis thaliana*, it has been shown that various molecular pathways, including photoperiod, vernalization, autonomous, and many others, integrate internal and external cues. Moreover, recent studies have demonstrated that a number of chromatin modifiers contribute to floral transition by regulating key flowering regulatory genes, such as *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T* (*FT*).

1.2.1 FLC, a central floral repressor

Determination of summer-annual accession (which flowers rapidly without prolonged period of cold exposure) or winter-annual accession (which requires vernalization prior to flowering) depends on the expression level of *FLC* (Sheldon et al., 2000; Johanson et al., 2000). A winter-annual possesses dominant *FRI* allele that activates *FLC* expression, whereas a summer-annual has a non-functional *fri* allele (Michaels and Amasino, 2001). FLC, a MADS-box transcription factor, inhibits floral transition by repressing downstream floral integrators *FT*, *FLOWERING LOCUS D* (*FD*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Michaels and Amasino, 1999; Sheldon et al., 1999; Searle et al., 2006). Previous studies have demonstrated that *FLC* is also regulated by chromatin modifications such as H3K4me3, H3K27me3, and others. (He et al.,

2004; Pien et al., 2008; Schuettengruber et al., 2007; Sheldon et al., 2006; Wood et al., 2006; Jiang et al., 2008).

1.2.1.1 Chromatin-mediated activation of *FLC*

H3K4me3 is associated with active gene expression. In *Arabidopsis*, accumulation of H3K4me3 within *FLC* chromatin leads to active *FLC* expression. (He et al., 2004; Pien et al., 2008). Former studies have demonstrated that homologs of yeast and human PAF1 complex, called PAF1-like complex in *Arabidopsis*, recruits MLL/COMPASS to activate *FLC* expression (Zhang and van Nocker, 2002; Zhang et al., 2003; He et al., 2004; Oh et al., 2004). PAF1-like complex components include EARLY FLOWERING 7 (ELF7), the homolog of yeast Paf1, EARLY FLOWERING 8 (ELF8)/VERNALIZATION INDEPENDENCE 6 (VIP6), the homolog of the yeast Ctr9, VIP3, the homolog of human hSki8, VIP4, the homolog of the yeast Leo1, and VIP5, the homolog of yeast Rtf1 (Zhang and van Nocker, 2002; Zhang et al., 2003; He et al., 2004; Oh et al., 2004). It is well-known that PAF1-like complex functions with COMPASS-like H3K4 methyltransferases, ARABIDOPSIS TRITHORAXs (ATXs), to activate *FLC* through the accumulation of H3K4m3 on near transcription start site (TSS) region of the *FLC* locus (Pien et al., 2008; Tamada et al., 2009; Berr et al., 2010; Yun et al., 2012; Guo et al., 2010).

In addition to H3K4me3, H3K36me2 and H3K36me3 are also required for *FLC* regulation. Several studies have reported that EARLY FLOWERING IN SHORT DAYS (EFS)/SET DOMAIN GROUP 8 (SDG8) catalyzes H3K36 methylation within *FLC* chromatin and is also involved in H3K4me3 enrichment

especially with functional FRI protein (Pien et al., 2008; Xu et al., 2008; Ko et al., 2010).

Moreover, histone H2B monoubiquitylation (H2Bub1) acts as an activation mak in *FLC* expression (Liu et al., 2007b; Cao et al., 2008; Gu et al., 2009; Xu et al., 2009a). In yeast, H2B-monoubiquitylating complex is composed of Rad6, an E2 ubiquitin-conjugating enzyme, and Bre1, an E3 ubiquitin ligase, and modifies H2B of target chromatin (Wood et al., 2003). This leads to transcription activation and elongation of the target gene (Pavri et al., 2006; Shilatifard, 2006). In *Arabidopsis*, ubiquitin-conjugating enzyme 1 (UBC1), UBC2, and UBC3, which are homologue of Rad6 (Sullivan et al., 1994; Kraft et al., 2005), and HISTONE MONOUBIQUITINATION 1 (HUB1) and HUB2, which are homologs of Bre1 (Fleury et al., 2007; Liu et al., 2007b), are identified as E2 and E3 ubiquitin ligases, respectively. *Arabidopsis* H2B-monoubiquitylating complex catalyzes H2Bub1 on *FLC* locus and induces either H3K4me3 accumulation or a movement of RNA polymerase II (Pol II) towards the gene body (Cao et al., 2008; Gu et al., 2009).

A histone variant H2A.Z deposition is also associated with *FLC* activation through the *Arabidopsis* SWR1 complex (AtSWR1c) (Deal et al., 2007; Choi et al., 2007). PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1) (Noh and Amasino, 2003), ACITN-RELATED PROTEIN 4 (ARP4) (Kandasamy et al., 2005), SUPPRESSOR OF FRIGIDA 3 (SUF3)/ACITN-RELATED PROTEIN 6 (ARP6)/EARLY IN SHORD DAYS 1 (ESD1) (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), and SWC6 (Choi et al., 2007), are identified as the homologous components of the yeast SWR1c in *Arabidopsis*. Several studies have suggested that AtSWR1c accumulates H2A.Z at the *FLC* locus and mediates *FLC*

transcription (Choi et al., 2007; Zilberman et al., 2008).

Besides chromatin modifiers, FRIGIDA complex (FRIc) activates *FLC* expression as well. Specific regulators, FRIGIDA-LIKE 1 (FRL1), FRIGIDA-ESSENTIAL 1 (FES1), SUPPRESSOR OF FRIGIDA 4 (SUF4), and FLC EXPRESSOR (FLX), form the transcription activator complex FRIc (Schmitz et al., 2005; Kimet al., 2006; Kim and Michaels, 2004 and 2006, Andersson et al., 2008; Choi et al., 2011). Through interacting with FRI, the complex recruits not only general transcription factors but also chromatin modifiers, such as EFS, SWR1c, and COMPASS, on *FLC* chromatin and thus leads to *FLC* activation. (Ko et al., 2010; Jiang et al., 2009; Choi et al., 2011).

1.2.1.2 Chromatin-mediated repression of *FLC*

1.2.1.2.1 The autonomous pathway

In summer-annual accession with a non-functional *fri* allele, autonomous genes, for instance *FCA*, *FPA*, *FLOWERING LATE KH MOTIF* (*FLK*), *LUMINIDEPENDENS* (*LD*), *FY*, *FVE*, and *FLOWERING LOCUS D* (*FLD*), repress *FLC* expression. Autonomous-pathway genes are also involved in various epigenetic mechanisms such as RNA-mediated silencing, demethlaytion of H3K4me2, and deacethylation of H3 acetylation (H3Ac) to repress *FLC* expression.

Several studies have suggested that RNA recognition motif (RRM) type RNA-binding proteins facilitate chromatin structural change and gene silencing (Baurle and Dean, 2008; Baurle et al., 2007). FCA and FPA, putative RNA-binding

proteins, contain RRM domains and they are involved in RNA-mediated *FLC* chromatin silencing (Liu et al., 2010). FY, a homolog of the yeast polyadenylation factor Pfs2p, interacts with FCA and acts in the 3'-end processing of *FLC* transcript (Simpson et al., 2003). FLD, an H3K4 demethylase, is necessary for the FCA- and FPA-mediated *FLC* repression (Liu et al., 2007; Baurle and Dean, 2008). In addition to FLD, FVE also plays a role in *FLC* silencing in cooperation with FPA (Veley et al., 2008). RNA metabolism and processing mechanisms cause repressive modification of chromatin and*FLC* repression (Baurle et al., 2007; Liu et al., 2010).

Moreover, histone deacetylation or demethylation of active marks, such as H3Ac, H3K4me3, H3K36me2, and H3K36me3, are associated with gene repression in general. *Arabidopsis* FVE and FLD are homologs of the musashi RNA-binding protein 1 (MSI1) and the lysine-specific histone demethylase (LSD1), respectively (Ausin et al., 2004; Sanda and Amasino, 1996; He et al., 2003; Metzger et al., 2005) Recently, the histone deacetylation complex, FVE-FLD-HDA6 complex, wasis identified, and it results in *FLC* repression (Ausin et al., 2004). Additionally, FLD has an effect on demethylation of H3K4me1 and H3K4me2 at the *FLC* locus. (He et al., 2003; Jiang et al., 2007; Liu et al., 2007a)

1.2.1.2.2 Histone arginine methylation

Furthermore, protein arginine methyltransferases (PRMTs), catalyzing histone arginine methylation, also play an essential role in *FLC* repression. In *Arabidopsis*, a type I PRMT, AtPRMT10, catalyzes histone H4 arginine 3 asymmetric dimethylation (H4R3me2a) and represses *FLC* expression (Niu et al., 2007).

AtPRMT5, a type II PRMT, contributes to histone H4 arginine 3 symmetric dimethylation (H4R3me2s) within *FLC* chromatin (Pei et al., 2007; Wang et al., 2007). AtPRMT5 has an important function in the establishment and maintenance of vernalization-induced *FLC* silencing in functional *FRI* background. In *atprmt5* mutants of FRI background, both H3K9 and H3K27 methylation levels at the *FLC* locus do not increase by vernalization (Robert et al., 2007). In addition, two type II PRMTs, AtPRMT4a and AtPRMT4b, mediate H3R17me2a and redundantly repress *FLC* expression (Niu et al., 2008).

1.2.1.2.3 Vernalization

In winter-annual plants, vernalization, a prolonged cold exposure, is required to ensure competence to flower. Vernalization mediates epigenetic changes, such as accumulation of H3K27me3 and H3K9me3, within *FLC* chromatin and results in *FLC* silencing. Once silenced by vernalization, repressed *FLC* expression and its compacted chromatin state are mitotically maintained even when the growth condition turns back warm (Kim et al., 2009; Dennis et al., 2007; Angel et al., 2011). A complete silencing of *FLC* during vernalization requires PcG or PcG-related components and long non-coding RNAs (lncRNAs) (Wood et al., 2006; Dennis et al., 2007; Angel et al., 2011; Helliwell et al., 2011; Heo and Sung, 2011).

VERNALIZATION INSENSITIVE 3 (VIN3) (Sung and Amasino, 2004), a plant homeodomain (PHD) protein, is induced by vernalization and forms a complex with PRC2 components, CURLY LEAF (CLF)/SWINGER (SWN), FERTILIZATION INDEPENDENT ENDOSPERM (FIE), MULTI-COPY

SUPPRESSOR OF IRA 1 (MSI1), VERNALIZATION 2 (VRN2), and VRN5/VIN3-LIKE 1 (VIL1) (Kim et al., 2009; De et al., 2008; Wood et al., 2006). The PHD-PRC2 complex deposits H3K27me3 around the first exon of *FLC*, and H3K27me3 subsequently spreads over the entire *FLC* locus (De et al., 2008; Angel et al., 2011; Finnegan and Dennis, 2007). The enriched H3K27me3 then recruits a component of PRC1, LHP1, and a putative PRC1 component, VERNALIZATION 1 (VRN1), to the *FLC* locus. These two repressor complexes are required for the maintenance of stable *FLC* silencing after the cold (Sung et al., 2006; Zheng and Chen, 2011; Turck et al., 2007)

1.2.1.2.4 lncRNAs *COOLAIR* and *COLDAIR*

Recent studies have reported that some RNA transcripts also participate in *FLC* repression. Cold induces two types of long non-coding RNAs (lncRNAs), cold induced long antisense intragenic RNA (COOLAIR) and COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), that function in the vernalization-mediated *FLC* silencing (Swiezewski et al., 2009; Heo and Sung., 2011). While COOLAIR produces polyadenylated antisense *FLC* transcripts, COLDAIR generates unpolyadenylated sense *FLC* transcripts from the first intron of *FLC*. Moreover, *COLDAIR* physically interacts with CLF, recruits the PHD–PRC2 complex to *FLC* chromatin, and thereby represses *FLC* expression (Heo and Sung, 2011). Whereas the presence of COLDAIR is crucial for *FLC* silencing, it has been revealed that *COOLAIR* is not critical in *FLC* silencing, i.e., removal of *COOLAIR* did not affect *FLC* expression level during vernalization (Helliwell et al., 2011).

1.2.2 FT, a key floral inducer

In addition to FLC, FT plays a key role as florigen in promotion of floral transition. *FT* is directly repressed by FLC, whereas CONSTANS (CO) activates *FT* under long day conditions (Searle et al., 2006; Helliwell et al., 2006; Li et al., 2008). FT protein moves from leaves, through phloem, to the shoot apical meristem (SAM), where FT forms a protein complex with a bZIP transcription factor, FLOWERING LOCUS D (FD) (Abe et al., 2005; Wigge et al., 2005). The FT-FD complex subsequently activates floral-meristem identity genes, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*). Functions of such genes are associated with floral meristem differentiation during flowering initiation (Wigge et al., 2005; Abe et al., 2005).

1.2.2.1 Chromatin-mediated *FT* regulation

Recent studies have revealed that *FT* is partially regulated by epigenetic mechanisms. The concurrent actions and crosstalks between the active mark H3K4me3 and the repressive mark H3K27me3 have been reported in detail (Jiang et al., 2008; Yang et al., 2010). Several chromatin-remodeling factors, such as LHP1/TFL2, PRC2, EARLY FLOWERING 6 (ELF6), AtJMJ4/JMJ14, andHDACs are also involved in the chromatin-mediated *FT* regulation.

H3K27me3 is a typical repressive mark, which is mediated by PcG proteins. It has been revealed that H3K27me3 is extensively enriched within *FT* chromatinincluding several kilobases upstream and downstream of the coding

region (Adrian et al., 2010).CLF, a component of PRC2, directly associates with *FT* chromatin and deposits H3K27me3 (Jiang et al., 2008; Farrona et al., 2011; Lopez-Vernaza et al., 2012). Additionally, other PRC2 components, for instance SWINGER (SWN), EMBRYONIC FLOWER 2 (EMF2), and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), are also involved inH3K27me3 to repress *FT* expression (Jiang et al., 2008; Farrona et al., 2011). H3K27me3 mark is recognized by PRC1. Components of plant PRC1, such as LHP1/TFL2 and EMF1, are also required for *FT* repression. LHP1 binds to *FT* chromatin and suppresses *FT* transcription via enriched H3K27me3 (Kotake et al., 2003; Farrona et al., 2008; Turck et al., 2007). Together with these findings, it is suggested that PRC1 and PRC2 cooperate in the repression of *FT* to build and maintain H3K27me3 (Jiang et al., 2008; Farrona et al., 2011; Turck et al., 2007; Moon et al., 2003; Bratzel et al., 2010). Recently, it has been reported that REF6 antagonizes H3K27me3-methyltransferase activity in the epigenetic regulation of *FT* (Lu et al., 2011).

On the other hand, H3K4me3 also participates in epigenetic regulation of *FT*. Lack of H3K27me3 owing to loss of PRC2 results in elevated level of H3K4me3 at *FT*. PRC2-dependent H3K27me3 suppresses H3K4me3 deposition (Jiang et al., 2008). Moreover, several members of the Jmj-family proteins were revealed to act as H3K4 demethylases at the *FT* locus in *Arabidopsis*. Two *Arabidopsis* Jmj-family proteins, AtJmj4 and ELF6, were identified as repressors of *FT* via demethylation of H3K4 (Jeong et al., 2009). They directly associate with *FT* locus and demethylate H3K4me2 and H3K4me3 within *FT* chromatin. (Yang et al., 2010; Jeong et al., 2009; Lu et al., 2010). The increase of H3K4me3 due to loss of *AtJmj4* results in the reduction of H3K27me3 (Yang et al., 2010). Thus, *FT*

expression *FT* is regulated by relative levels of H3K27me3 and H3K4me3.

In addition to the regulatory mechanism of bivalent H3K27me3 and H3K4me3 marks at the *FT* locus, other chromatin-mediated mechanisms, such as H3K36me3, H3Ac, and H2A.Z-related regulation, have also been revealed to influence *FT* expression. Recent studies have reported that *FT* expression is also affected by H3K36 methylation. The *Arabidopsis* Morf Related Gene 1 (MRG1) and MRG2 proteins have been identified as H3K36 methylation readers (Xu et al., 2014; Bu et al., 2014). MRG1 and 2 proteins have important roles in the CO-dependent *FT* activation under the photoperiodic flowering pathway. MRG2 directly binds to the *FT* locus and contributes to the activation of *FT* in H3K36me3-dependent manner (Xu et al., 2014; Bu et al., 2014).

Current findings also suggest that HDACs remove acetyl marks on histones at *FT* specifically at dusk. Two plant-unique functional relatives of the yeast SAP30, SAP30 FUNCTION-RELATED 1 (AFR1) and AFR2, take a part of HDAC complexes, AFR1-HDAC or AFR2-HDAC, to modulate the acetylation level of *FT* chromatin (Gu et al., 2013). AFR1/AFR2-HDAC complex binds to the *FT* promoter region and catalyzes histone deacetylation of *FT* chromatin, which requires CO activity at the end of long days (16 hr light/8 hr dark) (Gu et al., 2013). Moreover, the MADS-domain transcription factor AGAMOUS-LIKE 18 (AGL18) interacts with AFR1 and AFR2 and recruits the AFR1/AFR2-HDAC to *FT* chromatin. AGL18-AFR-HDAC acts as a repressive *FT* regulator to moderate *FT* expression.

Additionally, the SWR1 complex is participated in the *FT* regulation

mechanism through H2A.Z deposition at *FT*. (Kumar and Wigge, 2010). *FT* is regulated by H2A.Z-containing nucleosomes under the thermo-sensory pathway (Blazquezet et al., 2003). A rise of ambient temperature results in the eviction of the H2A.Z from *FT* nucleosome and causes increased *FT* transcription (Kumar and Wigge, 2010). EARLY BOLTING IN SHORT DAYS (EBS) is also involved in *FT*-chromatin remodeling mechanism which leads to *FT* repression (Castillejo and Pela; 2008; Gómez-Mena et al., 2001; Piñeiro et al., 2003).

1.3 Seed germination

Seed germination is an important process in higher plants, and it is controlled by a combination of internal and environmental factors. Environmental conditions, such as light, temperature, nutrient, and moisture, affect seed germination. Seed dormancy, one of core internal factors, prevents germination of viable seeds even if seeds are exposed to favorable external conditions. Phytohormones, including gibberellic acid (GA), abscisic acid (ABA), ethylene, brassinosteroid (BR), and auxin, which elicit response from both external and internal signals, can also influence seed germination (Holdsworth et al., 2008; Nambara et al., 2010). Among them, GA and ABA play central but antagonistic roles in the regulation of seed germination. Their molecular mechanisms have been extensively studied at physiological and molecular levels for many years (Koorneef and Karssen., 1994). GA- and ABA-mediated seed germination will be further discussed in detail. Additionally, interactions and crosstalks between different hormones have been studied in recent years (Wolters and Jurgens, 2009; Gazzarrini and McCourt, 2003).

1.3.1 Gibberellins (GA)

GA plays a central role in plant developmental processes including seed germination, leaf expansion, stem elongation, and flowering (Schwechheimer and Willige, 2009). In *Arabidopsis*, severe GA-deficient mutants, such as *ga1-3* and *ga2-1*, are shown to have a defective phenotype in seed germination (Koornneef and van der Veen, 1980). GA biosynthetic inhibitors, paclobutrazol (PAC) and uniconazole, also prohibit seed germination (Nambara et al., 1991; Jacobsen and Olszewski, 1993). GA is necessary for embryo growth and radicle protrusion in weakening tissues that surround the embryo such as endosperm and testa. (Groot and Karssen, 1987; Silverstone et al., 1997; Telfer et al., 1997). Two *Arabidopsis* GA biosynthetic genes, *GA3ox1* and *GA3ox2*, are mainly expressed during imbibition in embryo axis. They encode GA3 oxidases that catalyze the conversion of inactive GA into active form during seed germination. (Yamauchi et al., 2004; Mitchum et al., 2006).

GA recognition is also important. Interaction between GA receptors and DELLA-domain proteins affects the germination potential of seeds. GA receptors, including GA INSENSITIVE DWARF 1A (GID1A), GID1B, and GID1C, function as key components of GA signaling. Loss of all three *GID1* causes germination failure (Griffiths et al., 2006; Willige et al., 2007; Nakajima et al., 2006). Five members of DELLA-domain proteins, GA INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, act as negative regulators in GA responses (Sun and Gubler, 2004). Especially, RGL2 plays a

major role in the regulation of seed germination. Loss of RGL2 function causes insensitivity to PAC and restores germination ability in *ga1* mutant background. (Lee et al., 2002; Tyler et al., 2004). Other DELLA proteins only have a minor function in germination (Koornneef et al., 1985; Cao et al., 2005).

1.3.2 Abscisic acid (ABA)

ABA is a key hormone in plant stress-responses, development, seed dormancy, and seed germination (Zhu, 2002; Sharp, 2002; Nambara et al., 2010; Finkelstein et al., 2002). ABA facilitates processes that link environmental changes to plant responses. Against drought and salt stresses, plant responses are controlled by the regulation of ABA synthesis and ABA-mediated signaling. (Schroeder et al., 2001; Cutler et al., 2010; Weiner et al., 2010). In addition to stress responses, several developmental processes, including seed maturation and seed germination, require ABA- dependent regulation (Finkelstein et al., 2002). A decrease of endogenous ABA level is needed for seed germination. Treatment of exogenous ABA during seed germination leads to failure of endosperm weakening and rupture (Muller et al., 2006; Karin et al., 2011) A higher level of endogenous ABA contents in imbibed seeds results in the inhibition of seed germination (Nambara et al., 2010). In addition, ABA is involved in the maintenance and reinforcement of seed dormancy (Nambara et al., 2010). Nine-cis-epoxycarotenoiddioxygenases (NCEDs) and ABA 8'-hydroxylases (CYP707As) function in ABA biosynthesis and degradation, respectively (Seo et al., 2006; Toh et al., 2008). *CYP707A2* is highly expressed in the radicle during seed imbibition and affects seed germination

(Okamoto et al., 2006). Several *ABA-INSENSITIVE* (*ABI*) genes are involved in the ABA signaling pathway. Mutations of *ABI3*, *ABI4*, and *ABI5* genes showed ABA-insensitive phenotypes during seed germination and early seedling development (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000b; Soderman et al., 2000). ABI5 transcription factor, which is positively regulated by ABI3, plays an important role in ABA signaling (Finkelstein and Lynch, 2000b; Finkelstein et al., 2002).

1.3.3 Ethylene

Ethylene positively acts on seed germination. In other words, it is antagonistic to ABA but synergistic to GA in *Arabidopsis*. (Kepczynski and Kepczynska, 1997; Beaudoin et al., 2000; Ghassemian et al., 2000) ACC OXIDASE (ACO), a key enzyme in ethylene production, is involved in endosperm rupture to oppose the ABA-mediated inhibition of the rupture. (Kucera et al., 2005; Matilla and Matilla-Va´zquez, 2008; Linkies et al., 2009). ACO activity is repressed by ABA in the radicle, while ABA level is not affected by ethylene. Thus, ethylene counteracts to ABA-mediated inhibition of seed germination via interference of ABA signaling (Linkies et al., 2009). Ethylene synthesis and response are activated by GA. It is reported that *ACO* expression is increased by exogenous GA4 in imbibed *Arabidopsis* seeds (Ogawa et al., 2003). Ethylene-inducible genes, *HOOKLESS 1* (*HLS1*) (Lehman et al., 1996) and *ETHYLENE RESPONSE SENSOR 1* (*ERS1*) (Hua et al., 1998), are up-regulated by GA4 (Ogawa et al., 2003).

1.3.4 Light signaling

As an environmental cue, light is one of the most important factors in plant growth and development, especially in seed germination. The perception of light signals by photoreceptors is crucial in the light signaling pathway (Jiao et al., 2007). Phytochromes (phyA and phyB) or crytochromes (cry1 and cry2) can perceive far-red/red light or blue light, respectively. PHYTOCHROME-INTERACTING FACTORS (PIFs), a group of basic helix-loop-helix (bHLH) transcription factors, function as signaling integrators of light and hormones (Leivar et al., 2008; Castillon et al., 2007). The light-dependent plant developmental process involves many hormonal pathways (Neff et al., 2006). PIF3 and PIF4 participate in photomorphogenesis of seedling and another member, PIF3-LIKE 5 (PIL5)/PIF1, regulates seed germination (Nemhauser et al., 2008; Alabadı´ et al., 2009).

As mentioned above, PIF1 is involved in phytochrome-mediated seed germination and affects GA and ABA signaling also. PIF1 regulates GA responses through direct targeting of *DELLA* genes. GAI and RGA functionally repress GA signaling (Oh et al., 2007). In dark, PIF1 self-activates its transcription and leads to inhibition of GA-induced seed germination. Moreover, PIF1 indirectly affects ABA biosynthesis and *ABI3* expression (Piskurewicz et al., 2009). In short, PIF1 acts as a key regulator in dark to repress seed germination through ABA- and GA-mediated metabolism and signaling pathways (Oh et al., 2006 and 2007). PIF1-downstream proteins also play important roles in seed germination. SOMNUS (SOM), a nuclear-localized CCCH-type zinc-finger protein, participates in the inhibition of seed germination by maintaining balance between GA and ABA levels.

Loss-of-function of *SOM* shows a germination phenotype in dark without any light signals (Kim et al., 2008). DOF AFFECTING GERMINATION 1 (DAG1), a DNA-binding One Zinc Finger (DOF) transcription factor, directly binds to *GA3ox1* and negatively regulates *GA3ox1* transcription (Gabriele et al., 2010).

1.3.5 Epigenetic aspects of seed germination.

Seeds of higher plants undergo several phase transitions, such as seed germination and flowering. Seed maturation and germination are largely regulated through epigenetic mechanisms. For example, DNA methylation-related genes are needed for proper embryo developments and seed viability (Xiao et al., 2006). Reduced nuclei with highly condensed chromatin is established during seed maturation and the reduced nuclear size is restored through germination process (van Zanten et al., 2011). A few studies have demonstrated that DNA methylation is involved in the regulation of seed germination. MicroRNA 402 (miR402) targets *DNA GLYCOSYLASE DEMETER-LIKE PROTEIN 3* (*DML3*) (Sunkar and Zhu, 2004). Under high salt or cold stresses, miR402 constitutively promotes seed germination, suggesting that maintenance of 5-mC level at the *DML3* locus is important in the inhibition of seed germination (Kim et al., 2010).

The DELLA protein RGL2, which plays a key role in GA signaling, also promotes ABA biosynthesis and ABI5 activity (Piskurewicz et al., 2008). Contrarily, other type of DELLA proteins, GAI and RGA, participate in the repression of seed germination in dark (Cao et al., 2005). SPATULA (SPT) and PIF1, two bHLH transcription factors, are reported to repress seed germination by

affecting GA signaling (Penfield et al., 2005; Oh et al., 2004, 2006 and 2007). SPT is also involved in the establishment of dormancy by the repression of *GA3 oxidases*. PIF1 represses not only *GA3 oxidases* but also GA responses via stimulating RGA and GAI (Oh et al., 2007). Active transcription marks (H3K4me3, H3K36me3, and H3K9Ac) are involved in the regulation of DELLA proteins such as RGL2, GAI, and RGA, and germination-related transcription factors, including SPT and PIF1 (Roudier et al., 2011; Charron et al., 2009). In addition, the repressive mark H3K27me3 is also involved in the transcriptional regulation of *GAI*, *RGA*, and *PIF1* (Zhang et al., 2007a; Charron et al., 2009). Other histone repressive marks, H4R3me2 and H3R2me2, are also reported to regulate the transcription of GA anabolic genes, *GA3ox1* and *GA3ox2*. *Arabidopsis* histone arginine demethylases, JMJ20 and JMJ22, directly target *GA3ox1* and *GA3ox2* and enhance seed germination by eliminating H4R3me2s at the *GA3ox1* and *GA3ox2* loci (Cho et al., 2012).

Repression of embryonic properties is required for germination. PICKLE (PKL), the CHD3-class SWI/SNF chromatin-remodeling factor, is needed for the repression of embryonic properties, which is regulated by seed-maturation regulators such as LECs, FUS3, and ABI3 (Ogas et al., 1997 and 1999; Rider et al., 2003). The expression level of *PKL* is low in dry seed but is increased during imbibition (Henderson et al., 2004; Li et al., 2005). In *pkl* mutant seeds, H3K27me3 level is lower compared to wild type at the *LEC1* and *LEC2* loci (Zhang et al., 2008). Moreover, it is recently reported that DNA methylation is also involved in PKL-mediated *LEC1* silencing during germination (Zhang et al., 2012a). Unfavorable environmental conditions such as osmotic stress cause a

growth arrest at the early phase of seed germination. ABI3 and ABI5 are key factors in stress-response mechanisms. In ABA-dependent manner, PKL plays a negative role in an ABA signaling during seed germination (Perruc et al., 2007). Repressive marks, H3K9me and H3K27me, are reduced in *pkl* mutant at the *ABI3* and *ABI5* loci, and the reduction of repressive marks is induced by the treatment of exogenous ABA (Perruc et al., 2007). Therefore, PKL acts as a negative regulator in ABA-dependent seed germination via enrichment of repressive histone marks at the *ABI3* and *ABI5* loci (Perruc et al., 2007; Zhang et al., 2012a).

In addition to PKL, HDA6 and HDA19 redundantly act to repress embryonic properties during early seed germination (Tanaka et al., 2008). *hda6 hda19* double mutant was shown to have various germination-related phenotypes such as growth arrest, failure to proceed cotyledon greening and expansion, and a perpetuation of embryo-like phenotypes after germination (Tanaka et al., 2008). HDA6 is partially redundant with PKL and acts in the DICER-LIKE 1 (DCL1)-mediated *LEC2* and *FUS3* repression mechanism (Willmann et al., 2011). According to recent studies, LHP1, a component of PRC1, is possibly involved in the repression of *LEC1*, *LEC2*, *FUS*, and *ABI3* (Zhang et al., 2007a and 2007b; Charron et al., 2009). FIE, a component of PRC2, is suggested to participate in the regulation mechanisms of seed dormancy and germination. *fie* mutants exhibit germination-defective phenotypes and reinforced seed dormancy (Bouyer et al., 2011). EARLY FLOWERING IN SHORT DAYS (EFS), a putative histone methyltransferase, was reported as a negative regulator of seed germination: *efs* mutant shows an early seed-germination phenotype (Bassel et al., 2011).

However, so far little is known about epigenetic regulatory mechanisms of

seed germination. As described above, efforts to prove that epigenetic regulators play a critical role in seed germination is in progress. Therefore, epigenetic regulation has been considered a promising mechanism to illuminate the veiled area of seed germination.

Chapter II

Repression of *FLOWERING LOCUS T* Chromatin by Functionally Redundant Histone H3 Lysine 4 Demethylases in *Arabidopsis*

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2.1 Abstract

FLOWERING LOCUS T (FT) plays a key role as a mobile floral induction signal during the floral transition. Therefore, precise control of *FT* expression is critical for the reproductive success of flowering plants. Coexistence of bivalent histone H3 lysine 27 trimethylation (H3K27me3) and H3K4me3 marks at *FT* locus and the role of H3K27me3 as a strong *FT* repression mechanism in *Arabidopsis* have been reported. However, the role of an active mark, H3K4me3, in *FT* regulation has not been addressed, nor have the components affecting this mark been identified. Mutations in *Arabidopsis thaliana Jumonji4* (*AtJmj4*) and *EARLY FLOWERING6* (*ELF6*), two of the *Arabidopsis* genes encoding Jmj family proteins, caused an *FT*-dependent additive early flowering correlated with increased expression of *FT* mRNA and increased H3K4me3 levels within *FT* chromatin. Purified recombinant AtJmj4 protein possessed specific demethylase activity for mono-, di-, and trimethylated H3K4. Tagged AtJmj4 and ELF6 proteins associated directly with the *FT* transcription initiation region, a region where the H3K4me3 levels were increased most significantly in the mutants. Thus, our study demonstrates the roles of AtJmj4 and ELF6 as H3K4 demethylases directly repressing *FT* chromatin and preventing precocious flowering in *Arabidopsis*.

2.2 Introduction

Flowering, a critical developmental transition in plants, is controlled by both environmental cues and internal developmental signals. The photoperiod, one of the major environmental cues for flowering, exerts profound effects on flowering in numerous plant species including Arabidopsis. The photoperiodic signal is generated mainly in the leaves by light and circadian-clock signaling and relayed through the photoperiod pathway. *GIGANTEA* (*GI*) (Park et al., 1999; Fowler et al., 1999) and *CONSTANS* (*CO*) (Putterill et al., 1995) act as upstream activators of *FLOWERING LOCUS T* (*FT*) (Kobayashi et al., 1999; Kardailsky et al., 1999) in the photoperiod pathway. On the other hand, *FT* mRNA expression is repressed by *FLOWERING LOCUS C* (*FLC*) (Park et al., 1999; Fowler et al., 1999), and this repression is mediated possibly by a protein complex between FLC and SHORT VEGETATIVE PHASE (Li et al., 2008). Thus, *FT* acts not only as a component in the photoperiod pathway but also as a floral integrator that combines the photoperiodic floral activation signal and the *FLC*-mediated floral repression signal. FT protein, as a graft-transmissible signal, is translocated from the vascular tissue of leaves to the shoot apex (Corbesier et al., 2007), where it interacts with FD and stimulates the floral transition (Abe et al., 2005; Wigge et al., 2005).

Recent studies have shown that *FT* expression is affected by histone modifications. *FT* locus was shown to be enriched with trimethylated histone H3 lysine 27 (H3K27me3) (Turck et al., 2007; Zhang et al., 2007), and loss of putative polycomb repressive complex 2 (PRC2) components results in decreased H3K27me3 within *FT* chromatin, which in turn increases *FT* expression (Jiang et al., 2008). Furthermore, lack of LIKE-HETEROCHROMATIN PROTEIN1

(LHP1), which can bind to H3K27me3 and silence the chromatin (Turck et al., 2007; Zhang et al., 2007), also causes increased *FT* expression (Kotake et al., 2003; Takada S and Goto K, 2003). Therefore, *FT* transcription is repressed by H3K27me3 and its effecter protein.

Methylation at histone residues had been considered an irreversible epigenetic modification for a long period of time. However, more recently at least two classes of enzymes have been shown to be capable of removing methyl groups from either histone lysine or arginine (R) residues. Human Lysine-Specific Demethylase1 (LSD1), a nuclear amine oxidase, specifically demethylates mono- and dimethylated but not trimethylated H3K4 (Shi Y et al., 2004). After the discovery of LSD1, a human Jumonji (Jmj) C domain-containing protein, JHDM1A, was first shown to be able to remove methyl groups from H3K36 (Tsukada et al., 2006). Soon after the identification of JHDM1A, a number of JmjC domain-containing proteins have been demonstrated to be H3K4, H3K9, H3K27, H3K36, H3R2, and H4R3 demethylases (Christensen et al., 2007; Chang et al., 2007; Klose RJ and Zhang Y, 2007). Unlike LSD1, JmjC domain-containing proteins are capable of demethylating all of the mono-, di- and trimethylated lysines of histones (Klose et al., 2006). Thus, JmjC family proteins are considered the major histone demethylases in eukaryotic cells.

Arabidopsis has twenty-one genes encoding JmjC family proteins (*Arabidopsis thaliana Jumonji* (*AtJmj*) *1~21*) (Hong et al., 2009). To date, three of these genes have been functionally characterized. *EARLY FLOWERING6* (*ELF6*; *AtJmj1*) and *RELATIVE OF EARLY FLOWERING6* (*REF6*; *AtJmj2*) were shown to be involved in photoperiodic flowering and *FLC* regulation, respectively (Noh et

al., 2004). INCREASED EXPRESSION OF BONSAI METHYLATION 1 (IBM1; AtJmj15), represses genic cytosine methylation, possibly through demethylation of H3K9me (Saze et al., 2008). In this report, we show that ELF6 and another Arabidopsis JmjC family protein (AtJmj4) directly repress *FT* expression via demethylation of H3K4me. Thus, our study demonstrates the presence of an H3K4me demethylation-mediated mechanism in addition to the previously characterized H3K27 methylation-mediated mechanism in the chromatin repression of a key flowering time regulator, *FT*.

2.3 Materials and Methods

2.3.1 Plant materials and growth

atjmj4 T-DNA insertion lines in the Col background were obtained from the SALK collection (http://signal.salk.edu/; *atjmj4-1*, SALK_135712; *atjmj4-2*, SALK_136058). The following mutants are in the Col background and were described previously: *elf6-4* (Noh et al., 2004), *vin3-5* (Mylne et al., 2006), *flc-3* (Michaels et al., 1999), *gi-2* (Park et al., 1999), *co-101* (Takada S and Goto K, 2003), *ft-10* (Yoo et al., 2005), *lhp1-4* (Larsson et al., 1998), *fld-3* (He et al., 2003), *fve-4* (Ausin et al., 2004), *FRI* (Lee et al., 1994). All plants were grown under 100 $\mu E\ m^{-2}\ s^{-1}$ cool white fluorescent light at 22℃.

2.3.2 T-DNA flanking sequence analysis

The T-DNA borders of *atjmj4-1* and *atjmj4-2* alleles were defined by sequencing PCR products obtained using a T-DNA border primer (SALKLB1; Table 2.1) and gene-specific primers. For *atjmj4-1*, AtJmj4-1-R and AtJmj4-1-F primer pair (Table 2.1) was used to detect wt allele while AtJmj4-1-R and SALKLB1 primer pair was used to detect *atjmj4-1* allele. For *atjmj4-2*, AtJmj4-2-F and AtJmj4-2-R primer pair (Table 2.1) was used to detect wt allele while AtJmj4-2-F and SALKLB1 primer pair was used to detect *atjmj4-2* allele.

2.3.3 RT-PCR and qPCR analyses

Total RNA was isolated from seedlings using TRI Reagent (Molecular Research Center, INC.) according to the manufacturer's instructions. RT was performed with M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's

instructions using 3 µg of total RNA. PCR was performed on first strand DNA with i-Taq DNA polymerase (iNtRON Biotechnology). Primers used for RT-PCR and qPCR analyses are listed in Table S2. qPCR was performed in 96-well blocks with an Applied Biosystems 7300 real-time PCR system using the SYBR Green I master mix (Bio-Rad) in a volume of 20 µl. The reactions were performed in triplicate for each run. The comparative $_{\Delta\Delta}$CT method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (Ct) was automatically determined for each reaction by the system set with default parameters.

2.3.4 GUS and GFP assays

The *FT$_{pro}$::GUS* (Yoo et al., 2005) and the *ELF6::GUS* (Noh et al., 2004) were described previously. For the construction of the *AtJmj4::GUS* translational fusion construct, a 6-kb genomic DNA fragment of *AtJmj4* containing 1.5-kb 5' upstream region and the entire coding region was generated by PCR amplification using AtJmj4GUS-F and AtJmj4GUS-R as primers (Table 2.3). After restriction digestion with *Sal*I-*Sma*I, the PCR product was ligated into pPZP211-GUS (Noh et al., 2003) at *Sal*I-*Sma*I sites. The final construct was introduced into wt Col plants by the floral dip method (Clough et al., 1998) through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 50 µg ml^{-1} kanamycin. Histochemical GUS staining was performed as described (Schomburg et al., 2001).

AtJmj4 cDNA obtained from Col RNA through RT-PCR using AtJmj4OE-F and AtJmj4OE-R as primers (Table 2.3) was used for the construction of the

CaMV35S$_{pro}$*::AtJmj4::GFP*. The cDNA was cloned into the *Sal*I site of p35SsGFP/pGEM which has the open reading frame of *sGFP* (Niwa et al., 1999) behind the Cauliflower Mosaic Virus 35S promoter (*CaMV35S*$_{pro}$). Mesophyll protoplasts were isolated from rosette leaves of Col plants grown for 4 weeks in LD as described (Yoo et al., 2007). The LHP1::RFP (Choi et al., 2005) was included as a nuclear protein control. Protoplasts were co-transformed with the *CaMV35S*$_{pro}$*::AtJmj4::GFP* and the *LHP1::RFP* constructs, each with 10 μg of plasmid DNA prepared with Nucleo Bond Xtra Midi Kit (Macherey-Nagel). After 16 h incubation at 22℃ in dark, protoplasts were observed with LSM 510 confocal microscope (Zeiss). The GFP and RFP fusion proteins were excited at 488 nm and 543 nm, respectively. The autofluorescence of chlorophylls, GFP, and RFP were analyzed with LP650, BP500-530IR, and BP565-615IR filters, respectively. The merged image was obtained using the LSM Image Browser (Zeiss).

2.3.5 AtJmj4::FLAG

The *AtJmj4::FLAG* construct is consisted of a 0.8 kb 5' upstream region of *AtJmj4* (*AtJmj4*$_{pro}$), the sequence for 3xFLAG tags, and the full coding sequence of *AtJmj4* cDNA. *AtJmj4* cDNA was obtained from Col RNA through RT-PCR using AtJmj4OE-F and AtJmj4OE-R1 as primers (Table 2.3), digested with *Sal*I, and cloned into the *Sal*I site of a construct containing *3xFLAG* behind the *CaMV35S*$_{pro}$ in pPZP211 vector. The *CaMV35S*$_{pro}$ was replaced with the *AtJmj4*$_{pro}$ obtained from Col genomic DNA through PCR using Atjmj4FLAG-F and Atjmj4FLAG-R as primers (Table 2.3) at *Pst*I site. Then the *AtJmj4*$_{pro}$*::3xFLAG::AtJmj4 cDNA* was PCR-amplified using AtJmj4FLAG-F1 and Atjmj4FLAG-R1 as primers (Table

2.3). After restriction digestion with *Nhe*I, the PCR fragment was ligated into the *Sma*I-*Xba*I sites of the binary vector pPZP221B (Kang et al., 2001). The final construct was introduced into *atjmj4-1* mutants by the floral dip method through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 25 μg ml^{-1} glufosinate ammonium.

Protein samples were extracted using 2xloading buffer from wt Col and transgenic plants harboring the *AtJmj4::FLAG* construct, and their concentrations were determined by Protein Assay (Bio-Rad). 3.75 μg of protein samples were size-fractionated on a 7% SDS-PAGE gel, transferred to Pure Nitrocellulose (GE Water & Process Technologies), and blocked with 10% skim milk power in TTBS (0.1% tween 20, 20 mM Tris-HCl pH7.4, 150 mM NaCl). AtJmj4::FLAG protein was detected using Anti-FLAG M2-Peroxidase (HRP) antibody (Sigma), ECL Western Blotting Detection Kit (GE Healthcare), and JP/LAS-3000 Luminescent Image Analyzer (Fujifilm).

2.3.6 AtJmj4 protein expression and purification

For the expression of AtJmj4 protein in insect cells, the full length *AtJmj4* coding region was PCR amplified from a cDNA clone using JMJ4_pENTR_For and JMJ4_pENTR_Rev as primers (Table 2.3) and ligated into the Klenow-filled *Eco*RI site of pFastBac HT A vector (Invitrogen). The resulting *AtJmj4::pFastBac HT A* construct with amino-terminal 6xHis tag was used to transform DH10Bac *E. coli* competent cells (Invitrogen), and the recombinant baculovirus DNA was selected and used for the infection of *sf9* cells following the Bac-to-Bac system instructions (Invitrogen). Cells positive for the recombinant-protein expression as

tested by western blot with anti-His antibody (Santa Cruz) was used to infect cells to produce *6His-AtJmj4* baculovirus stocks. Viral stocks were stored at 4 ℃. For protein expression, 2.5 ml of viral stock was used to infect approximately 2×10^6 adherent *sf*9 cells in 400 ml of sf-900 II SFM serum free medium (Gibco) and cultured at 27℃ for 48 h. Then, cells were harvested and washed with PBS and frozen at -80℃ until further purification. Frozen cells were thawed on ice and resuspended with 20 ml equilibration buffer (80 mM Na_2HP0_4, 20 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 100 μM PMSF, 10% glycerol). Cells were disrupted by sonication, and the lysate was clarified by centrifugation at 10,000x g for 20 min at 4℃. The supernatant was applied into a Ni-NTA-Agarose (Qiagen) chromatography column and washed with 10x column volume of equilibration buffer. Protein was eluted from the column with 3x bed volume of elution buffer (80 mM Na_2HP0_4, 20 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 100 μM PMSF, 10% glycerol). Purified recombinant 6His-AtJmj4 protein was dialyzed against dialysis buffer (40 mM HEPES-KOH pH 7.9, 50 mM KCl, 10% glycerol, 1 mM DTT, 0.2 mM PMSF) overnight at 4℃. Next-day, dialyzed 6His-AtJmj4 proteins was quantified and stored at -20℃.

2.3.7 Histone demethylase assay

In vitro histone demethylation assay was performed as previously described (Whetstine et al., 2006) with minor modifications. Briefly, two or four μg of purified 6His-AtJmj4 protein was incubated with 4 μg of calf thymus histones type II-A (Sigma) in the DeMTase reaction buffer 1 (20 mM Tris-HCl pH 7.3, 150 mM

NaCl, 50 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6(H_2O)$, 1 mM α-ketoglutarate, 2 mM ascorbic acid) for 5 h at 37℃. Histone modifications were detected by western blot analysis with antibodies as follows: anti-H3K4me1 (Upstate 07-436), anti-H3K4me2 (Upstate 07-030), anti-H3K4me3, (Abcam ab8580), anti-H3K9me3 (Upstate 07-442), anti-H3K36me2 (Upstate 07-369), anti-H3K27me3 (Upstate 07-449), and anti-H3 (Abcam ab1791-100).

2.3.8 ChIP assay

ChIP was performed as described by Han et al. (Han et al., 2007) using 55- to 60-d-old plants grown in SD. Briefly, leaves were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin was isolated and sonicated into ~0.5 to 1 kb fragments. Specific antibody against GUS (Invitrogen A5790), FLAG (Sigma A8592-0.2MG), H3K4me3 (Upstate 07-473), or H3K27me3 (Upstate 07-449) was added to the chromatin solution, which had been precleared with salmon sperm DNA/Protein A agarose beads (Upstate 16-157). After subsequent incubation with salmon sperm DNA/Protein A agarose beads, immunocomplexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immunocomplexes were removed by incubation with proteinase K, followed by phenol/chloroform extraction. DNA was recovered by ethanol precipitation. The amount of immunoprecipitated *FT*, *CO*, and *Actin1* chromatins was determined by PCR with primer pairs in Table S4.

2.4 Results

2.4.1 Mutations in *AtJmj4* cause early flowering

To address the biological roles of Arabidopsis genes encoding JmjC domain-containing proteins, we obtained T-DNA insertion lines of these genes from the SALK T-DNA collection and tested their phenotypes. Two independent homozygous T-DNA insertion mutants of *Arabidopsis thaliana Jumonji4* (*AtJmj4* or *At4g20400*) (Hong et al., 2009) showed an early flowering phenotype both in long days (LD; 16 h light/8 h dark) and short days (SD; 8 h light/16 h dark; **Figures 2.1A-C**). The early flowering phenotype was not due to an accelerated leaf initiation rate (**Figure 2.8**) but resulted from accelerated transition of the shoot apical meristem (SAM) from the vegetative to the reproductive phase as characterized by a lower number of rosette and cauline leaves at the onset of flowering (**Figure 2.1C**). No other phenotypic traits were noticed in *atjmj4* mutants. Plants heterozygous for the T-DNA insertions displayed a wild-type (wt) flowering time (data not shown), indicating that *atjmj4-1* and *atjmj4-2* are recessive mutations. Because both alleles displayed similar early flowering behaviors, *atjmj4-1* was chosen to carry out all the rest of genetic and molecular analyses.

2.4.2 Early flowering of *atjmj4* is due to increased expression of *FT*

Because AtJmj4 might have a role in histone modification and affect the transcription of its target gene(s), we tested the mRNA expression of key flowering time regulators, namely *GI, CO, FT, SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Samach et al., 2000; Lee et al., 2000), and *FLC*, in *atjmj4-1* to understand its early flowering phenotype (**Figure 2.2**). In LD, the mRNA levels of

GI and *CO* were not affected by the *atjmj4-1* mutation. However, the mRNA expressions of the floral integrators, *FT* and *SOC1*, were up-regulated in *atjmj4-1* and that of *FLC* was slightly reduced (**Figure 2.2A**). To further confirm these observations, we monitored the expression of these genes in SD (**Figure 2.2B**). *FT* mRNA expression showed a stronger up-regulation by the *atjmj4-1* mutation in SD than in LD, but the expression of *SOC1* mRNA was not affected significantly. Because *CO* expression was not increased by the *atjmj4-1* mutation in SD, the up-regulation of *FT* might be caused by de-repression instead of induction. The mRNA level of *FLC* was slightly reduced in *atjmj4-1* mutants compared to in wt as was in LD.

Then we compared the mRNA expressions of *FLC*, *FT*, and *SOC1* in wt versus *atjmj4-1* at different developmental stages both in LD and SD (**Figures 2.2C** and **2.2D**). Notably, *FT* mRNA levels were higher in *atjmj4-1* than in wt throughout the developmental stages tested. *FT* promoter showed an increased activity both in LD and SD when the construct containing the *FT* promoter fused with *β-glucuronidase* (*FT::GUS*) (Takada S and Goto K, 2003) was introduced from wt Col into *atjmj4-1* homozygous mutants (**Figure 2.2E**). *FT::GUS* expression which was detected in the marginal minor veins of wt leaves were also observed in the central minor veins of *atjmj4-1* mutant leaves in LD. In SD, *FT::GUS* expression was robust in the major veins of *atjmj4-1* mutant leaves, although its expression was weak in the same wt leaf tissue. Thus, the *atjmj4* mutation leads to increased expression of *FT* mRNA through enhanced activity of *FT* promoter. However, the specific expression of *FT* in leaf veins was not affected by the *atjmj4* mutation.

Since the *atjmj4* mutation also caused a slight reduction in *FLC* mRNA levels (**Figures 2.2A-D**), we tested if the decrease in *FLC* expression is the major cause for the increased expression of *FT* in the mutants. For this, we first compared *FT* mRNA levels between in an *flc* null mutant (*flc-3*) (Michaels et al., 2001) and in *atjmj4-1* (**Figure 2.9**). Although the expression levels of *GI*, *CO*, and *SOC1* mRNAs were similar in the two genotypes, *FT* mRNA level was clearly higher in *atjmj4-1*. Furthermore, when we compared the flowering times between *atjmj4-1* single and *atjmj4-1 flc-3* double mutants both in LD and SD, the double mutants flowered earlier than the single mutants in both photoperiodic conditions (**Figures 2.3A** and **2.3B**). *atjmj4* showed normal response to vernalization as wt, while a double mutant between *atjmj4* and the vernalization unresponsive *vernalization insensitive3* (*vin3*) (Sung et al., 2004) did not (**Figure 2.10**). Further, *atjmj4 vin3* flowered earlier than *vin3* without or with vernalization (**Figure 2.10**) which acts largely through *FLC* (Sheldon et al., 1999; Michaels et al., 1999). Taken together, these data indicate that the *atjmj4* mutation causes an early flowering independently of *FLC* expression.

The idea for the *FLC*-independent activity of AtJmj4 was reinforced by our studies on genetic interactions between *atjmj4* and several autonomous-pathway mutants (Koornneef et al., 1991) (**Figure 2.3C**). Double mutants between *atjmj4* and *flowering locus d* (*fld*) (He et al., 2003) or *fve* (Ausin et al., 2004) represented intermediate flowering time of each single mutant. When a functional *FRIGIDA* (*FRI*) (Lee et al., 1994; Koornneef et al., 1994) allele was introduced into *atjmj4*, *atjmj4-1 FRI* flowered also at intermediate time between *atjmj4-1* and *FRI*. These

results indicate that AtJmj4 controls flowering process mainly through an *FLC*-independent pathway.

To test interactions between *AtJmj4* and genes acting in the photoperiod pathway, we made double mutants, namely *atjmj4-1 gi-2*, *atjmj4-1 co-101*, *atjmj4-1 ft-10*. The early-flowering phenotype of *atjmj4-1* was attenuated by LD-specific late flowering phenotypes of *gi-2* and *co-101* (**Figures 2.3D** and **2.3E**). However, the early flowering of *atjmj4-1* was fully suppressed by the *ft-10* mutation (**Figure 2.3F**). Therefore, AtJmj4 might affect *FT* expression independently of GI and CO. Consistent with this hypothesis, *FT* mRNA level in *atjmj4-1 gi-2* or *atjmj4-1 co-101* double mutants was higher compared to that in *gi-2* or *co-101* single mutants, respectively (**Figure 2.11**). LHP1 directly represses *FT* chromatin (Turck et al., 2007; Zhang et al., 2007) such that *FT* is strongly de-repressed in *lhp1* mutants (Kotake et al., 2003). Consistent with the repressive roles of AtJmj4 and LHP1 in *FT* expression, *atjmj4-1 lhp1-4* double mutants flowered at similar times with the severe early flowering mutant *lhp1-4* in both LD and SD (**Figures 2.3G** and **2.3H**).

Because all the data above indicated that AtJmj4 acts as an *FT* repressor, we were tempted to test if AtJmj4 affects *FT* expression indirectly through controlling the expression of *FT* regulators. To address this, we compared the mRNA levels of known *FT* regulators, namely *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3* (Jung et al., 2007; Aukerman et al., 2003), *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) (Jung et al., 2007; Schmid et al., 2003), *CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX1* (*CIB1*) (Liu et al., 2008), *TEMPRANILLO1* (*TEM1*), *TEM2* (Castillejo et al., 2008), *SHORT VEGETATIVE PHASE* (*SVP*) (Li et al., 2008; Lee et al., 2007; Hartmann et al., 2000), *AGAMOUS-like15* (*AGL15*),

and *AGL18* (Adamczyk et al., 2007), in between wt and *atjmj4* mutants, but none showed detectable differences (**Figure 2.12**).

2.4.3 AtJm4 is a nuclear protein preferentially expressed in vascular tissues and shoot/root apices

When we compared the mRNA expression of *AtJmj4* between in SD- and in LD-grown seedlings at a similar developmental stage, we could observe higher expression of *AtJmj4* mRNA in SD-grown seedlings, although the expression of *FT* mRNA was clearly higher in LD-grown seedlings (**Figure 2.4A**). This observation was consistent with the higher *AtJmj4* promoter activity in SD than in LD as studied using an *AtJmj4 promoter::GUS* fusion construct (Hong et al., 2009). Because the results might mean a preferential repressive role of AtJmj4 in *FT* expression in SD, we studied further to see if the expression level of AtJmj4 protein is also higher in SD than in LD. For this, we made an *AtJmj4::FLAG* fusion construct that contains an *AtJmj4* promoter fragment, 3 copies of FLAG tags, and the *AtJmj4* cDNA with the entire coding sequence. The construct fully rescued the early flowering phenotype of *atjmj4-1* when was introduced into the mutants (**Figure 2.4B**). When we measured the expression levels of the AtJmj4::FLAG fusion protein in transgenic plants at the same developmental stage with the one used to study the expression of *AtJmj4* mRNA (**Figure 2.4A**), however, there was no difference in the expression level between in SD- and LD-grown seedlings (**Figure 2.4C**). Thus, AtJmj4 expression might be further controlled at posttranscriptional level(s), although its promoter activity *per se* is regulated by a

day-length signal, and AtJmj4 protein exerts its repressive role for*FT* in both LD and SD.

Since AtJmj4 protein expression is distinct from its mRNA expression, we studied the spatial expression pattern of AtJmj4 protein using a construct harboring the entire genomic region of *AtJmj4* including 1.5 kb promoter in frame with *GUS* (*AtJmj4::GUS*). In seedlings, AtJmj4::GUS expression pattern was similar in SD and LD (**Figures 2.4D** and **2.4E**). The GUS activity was detected in most organs, but strong activity was observed in the shoot apex (**Figure 2.4J**), primary root tip (**Figure 2.4H**), trichomes of young leaves (**Figure 2.4F**), and leaf vascular tissues (**Figure 2.4G**). In floral organs, strong GUS activity was detected in anther filaments and styles (**Figure 2.4I**). Importantly, the *AtJmj4::GUS* expression domain showed an overlap with the *FT* expression domain (Takada S and Goto K, 2003) in leaves.

The subcellular localization of AtJmj4 protein was evaluated by protoplast transfection assay using a fusion protein between AtJmj4 and green fluorescence protein (GFP; AtJmj4::GFP) expressed by the *Cauliflower Mosaic Virus 35S* (*CaMV35*) promoter. The fusion protein between LHP1 and red fluorescence protein (RFP; LHP1::RFP), which was known to be localized into the nucleus (Choi et al., 2005), was co-expressed with the AtJmj4::GFP. Both RFP and GFP signals were detected only in the nucleus (**Figure 2.4K**). This result is in agreement with the possible role of AtJmj4 as a chromatin and/or transcriptional regulator.

2.4.4 AtJmj4 and ELF6 play redundant roles in *FT* repression as H3K4-specific demethylases

In our previous study, we reported that *ELF6* (*At5g04240*), a gene encoding an Arabidopsis Jmj-domian protein, acts as a repressor in the photoperiodic flowering pathway (Noh et al., 2004). Therefore, we were tempted to study the relationship between *AtJmj4* and *ELF6* in the regulation of photoperiodic flowering. For this, *elf6-4 atjmj4-1* double mutant was generated and assayed for flowering time. The double-mutant plants flowered earlier than the either single-mutant as well as the wt Col plants both in LD and SD (**Figures 2.5A-C**). Since both *ELF6* and *AtJmj4* have repressive roles in the photoperiod pathway, then we tested the mRNA expression of genes acting in the photoperiod pathway, namely *GI*, *CO* and *FT*, using RNAs isolated from SD-grown 56-d old plants. mRNA expressions of *GI* and *CO* were similar among the wt, the *elf6-4* and *atjmj4-1* single mutants, and the *elf6-4 atjmj4* double mutants at ZT4 and ZT11 (**Figure 2.5D**). However, *FT* mRNA level was increased in the *elf6-4* and the *atjmj4-1* single mutants by at least 3 folds compared to that in the wt, and this increase was more significant in the *elf6-4 atjmj4-1* double mutants (**Figures 2.5D** and **2.5E**). Therefore, the data for *FT* mRNA expression as well as the flowering time data (**Figures 2.5A-C**) indicate that *ELF6* and *AtJmj4* have redundant repressive roles in photoperiodic flowering through negatively regulating *FT* mRNA expression.

Our unpublished phylogenetic analysis on the JmjC domains of ELF6, AtJmj4, and human Jmj proteins showed that the JmjC domains of ELF6 and AtJmj4 are clustered along with the JmjC domains of human JARID1 family which is known to specifically demethylate H3K4me3 and H3K4me2 (Christensen et al., 2007;

Klose RJ and Zhang Y, 2007). Hence, we studied if the level of H3K4me within *FT* chromatin is affected by *elf6* and *atjmj4* mutations through chromatin immunoprecipitation (ChIP) assay. Sets of primers covering different regions of *FT* locus were used for the ChIP assay (**Figure 2.6A**). H3K4me3 levels were increased in G, I, and EX1 regions of *FT* locus by the *elf6-4* and *atjmj4-1* mutations, and the increase was more significant when both the mutations were combined (**Figures 2.6B-D**). However, H3K4me3 levels in regions F and N were not affected significantly by these mutations. Level of another histone methylation, H3K27me3, which was reported to be enriched within *FT* chromatin (Turck et al., 2007; Zhang et al., 2007), was slightly reduced by the *elf6-4* but not by the *atjmj4-1* mutation in some of the *FT* regions tested (**Figure 2.6C**). These results indicate that ELF6 and AtJmj4 repress *FT* mRNA expression by negatively affecting the methylation of H3K4 but not H3K27 within *FT* chromatin.

To test if ELF6 and AtJmj4 are active histone demethylases, we tried to express the full-length ELF6 and AtJmj4 proteins in several expression systems. Although we could not express the full-length ELF6 in any systems employed, we could express the full-length AtJmj4 in insect *sf9* cells as an amino-terminal 6 histidine-tagged protein (6His-AtJmj4) with a molecular mass of 130 kilo-daltons. 6His-AtJmj4 was purified near homogeneity (**Figure 2.6E**) and subjected for *in vitro* histone demethylase activity assay. 6His-AtJmj4-mediated histone demethylase activity was analyzed by decreased signals in western blots with antibodies specific to methylated histone H3 residues (**Figure 2.6E**). Incubation of the recombinant 6His-AtJmj4 protein with histone substrates in the demethylase assays resulted in reduced levels of H3K4me1, H3K4me2, H3K4me3, but not of H3K9me3,

H3K36me2, and H3K27me3 (**Figure 2.6E**). The levels of H3K4me3 and H3K4me2 were deceased more than the level of H3K4me1. These results indicate that AtJmj4 is an intrinsic H3K4-specific demethylase which has higher activity for H3K4me3 and H3K4me2 than H3K4me1.

The results in **Figure 2.5** and **Figure 2.6** suggested that AtJmj4 and ELF6 might directly target *FT* chromatin and repress the transcription activity of *FT* by reducing the methylation level of H3K4. To test if *FT* chromatin is directly targeted by ELF6 and AtJmj4 proteins, we preformed ChIP assays using transgenic plants expressing functional *ELF6::GUS* (Noh et al., 2004) and *AtJmj4::FLAG* (**Figures 2.4B** and **2.4C**) as demonstrated by the complementation of the *elf6-4* and *atjmj4-1* mutant phenotypes, respectively. PCR was then carried out using primers amplifying various regions of *FT* locus (**Figure 2.7A**). ELF6::GUS showed binding with broad regions of the *FT* locus around the transcription start site with strongest binding with region I (**Figures 2.7B**). However, ELF6::GUS did not show binding with region N which is a part of the first intron of *FT* and the transcription initiation region of *CO*. AtJmj4::FLAG showed a similar binding pattern with ELF6::GUS to *FT* chromatin (**Figures 2.7C**). It showed strong bindings with regions I to EX1 like ELF6::GUS, but its binding was not detected with regions F and G unlike ELF6::GUS. AtJmj4::FLAG binding to region N and the *CO* region was not detected as for the case of ELF6::GUS. In summary, both ELF6::GUS and AtJmj4::FLAG can associate directly and specifically with the transcription initiation region of *FT* locus where the H3K4me3 levels showed the largest increase in *elf6, atjmj4,* and *elf6 atjmj4* mutants. Thus, ELF6 and AtJmj4 proteins directly target *FT* chromatin and regulate flowering time via

demethylation of H3K4me.

2.5 Figures and Tables

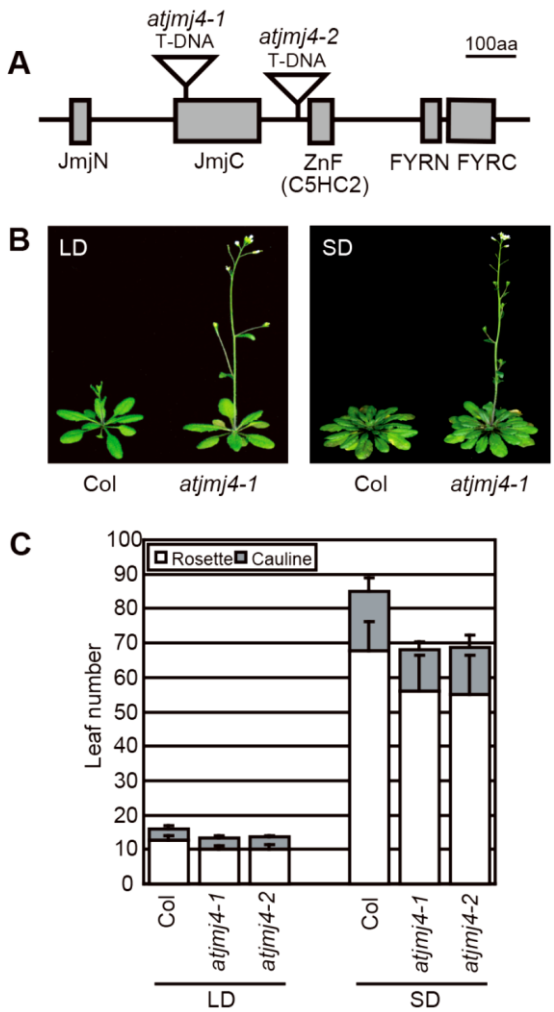

Figure 2.1 Early flowering of *atjmj4* mutants.

A) Domain organization of AtJmj4. Domains were predicted by SMART (http://smart.embl-heidelberg.de/). Lines indicate interdomain regions. T-DNA insertion sites on the genomic sequence of *AtJmj4* in *atjmj4-1* and *atjmj4-2* are marked on the corresponding positions of their translated protein products. **B)** Early flowering phenotype of *atjmj4-1* mutant plants grown in either LD or SD. **C)**

Flowering time of *atjmj4* mutants. Wt Col and *atjmj4* mutant plants were grown in either LD or SD and their flowering times were determined as the number of primary rosette and cauline leaves formed at bolting. At least 12 individuals were scored for each genotype. Error bars represent sd.

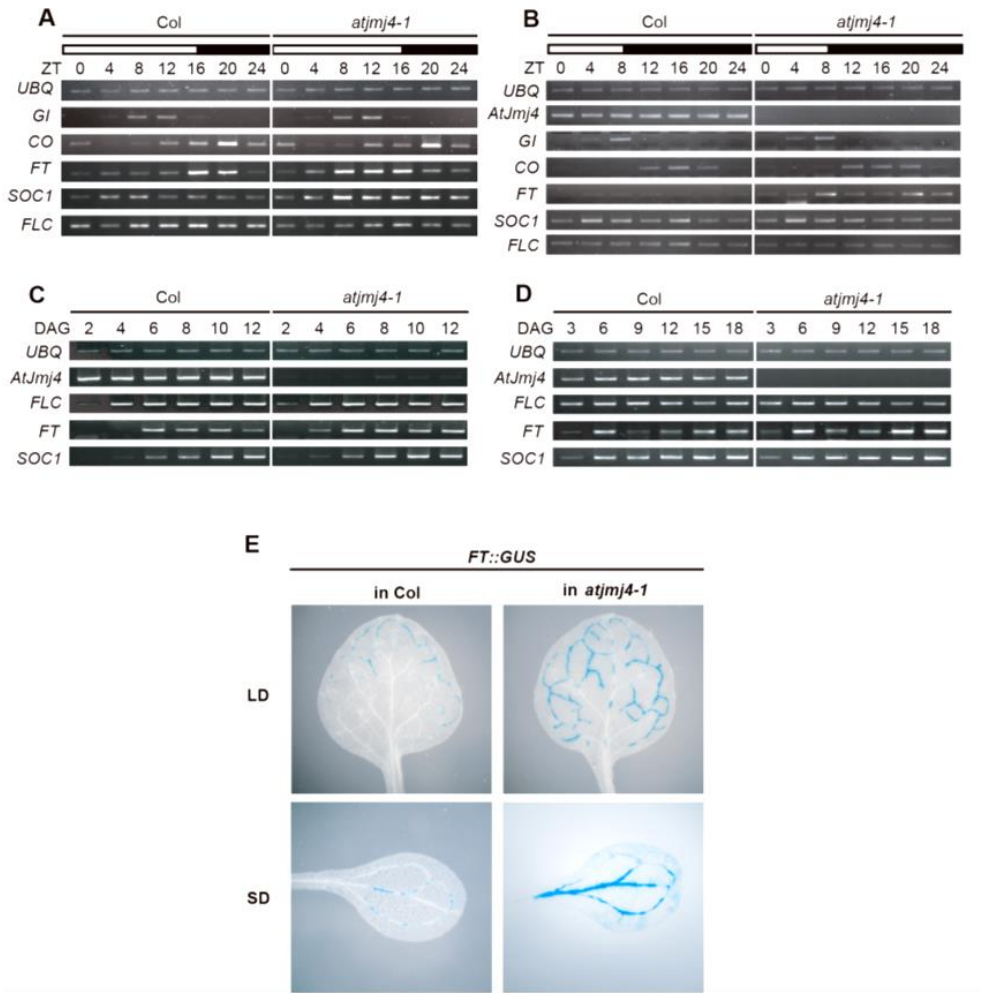

Figure 2.2 Increased expression of *FT* in *atjmj4* mutants.

A and B) Expression of flowering genes in *atjmj4-1* mutants. Col and *atjmj4-1* plants were grown in LD (A) for 10 d or in SD (B) for 15 d, harvested every 4 hours (h) at indicated zeitgeber (ZT; h after light-on) for one d, and used for RT-PCR analyses. *Ubiquitin* (*UBQ*) was included as an expression control. Identical results were obtained from two independent experiments, and one of them is shown. White and black bars represent light and dark periods, respectively. **C and D)**

Temporal expression of flowering genes in *atjmj4-1* mutants. Col and *atjmj4-1* plants were grown for up to 12 days after germination (DAG) in LD (C) or 18 DAG in SD (D). Plants were harvested during the growth period at ZT14 (LD) or ZT8 (SD) of designated DAG and used for RT-PCR analyses. **E)** Histochemical GUS staining of transgenic plants harboring *FT::GUS* fusion construct in Col or *atjmj4-1* plants. Plants were grown for 16 d either in LD or SD before GUS staining.

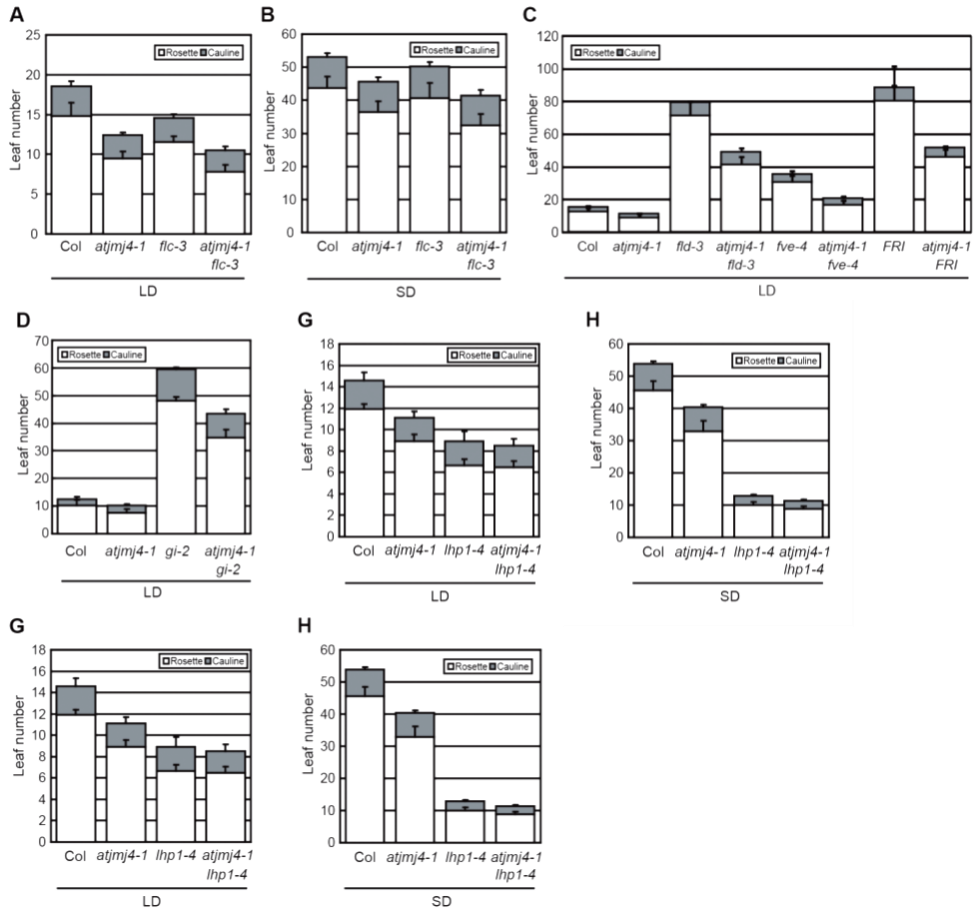

Figure 2.3 Genetic interaction between *Atjmj4* and other flowering genes.

A and B) Flowering time of *atjmj4-1 flc-3* double mutants. **C)** Genetic interaction between *AtJmj4* and *FLC* regulators. **D to F)** Genetic interaction between *AtJmj4* and photoperiod-pathway genes. **G and H)** Genetic interaction between *AtJmj4* and *LHP1*. Flowering times were determined in LD (A and C to G) or SD (B and H). At least 12 individuals were scored for each genotype (A to H). Error bars represent sd (A to H).

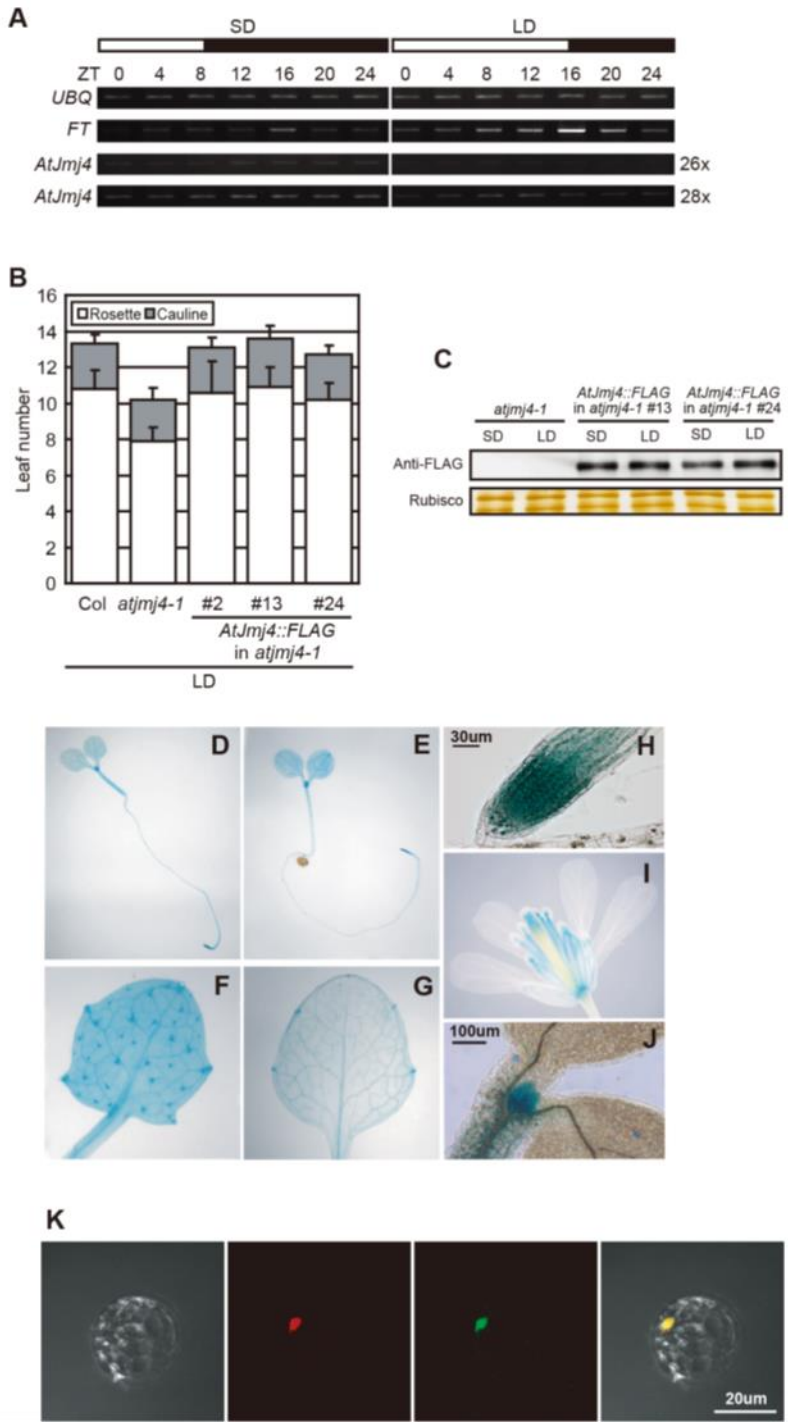

Figure 2.4. *AtJmj4* expression.

A) mRNA expression of *AtJmj4* in SD and LD. Wt Col plants were grown in SD for 12 d or in LD for 8 d and used for RT-PCR analyses. *UBQ* was used as an expression control. Number of PCR cycles used for *AtJmj4* is indicated on the right. **B)** Genomic complementation of *atjmj4-1*. Three independent transgenic lines of *atjmj4-1* containing *AtJmj4::FLAG* (see text for details) were grown in LD and their flowering times were determined as the number of rosette and cauline leaves formed at bolting. At least 12 individuals were scored for each genotype. Error bars represent sd. **C)** Expression of the AtJmj4::FLAG fusion protein in SD and LD. Plants of *atjmj4-1* and two of the complementation lines shown in (B) were grown for 12 d in SD or 8 d in LD, harvested at ZT12, and used for Western blot analyses. Upper panel: Western blot with anti-FLAG antibody. Lower panel: Silver stained gel image of rubisco subunits. **D to J)** Histochemical GUS staining of transgenic Col plants harboring *AtJmj4::GUS*. Plants grown in LD (D and I) or SD (E, F, G, H, and J) were used for GUS staining. (D) In 4 d-old seedling. (E) In 6-d old seedling. (F) In trichomes. (G) In leaf. (H) In root tip. (I) In floral organs. (J) In shoot apex. **K)** Subcellular localization of AtJmj4 in Arabidopsis mesophyll protoplast. From left to right; bright-field image, LHP1::RFP fusion protein, AtJmj4::GFP fusion protein, merged image of the left three images.

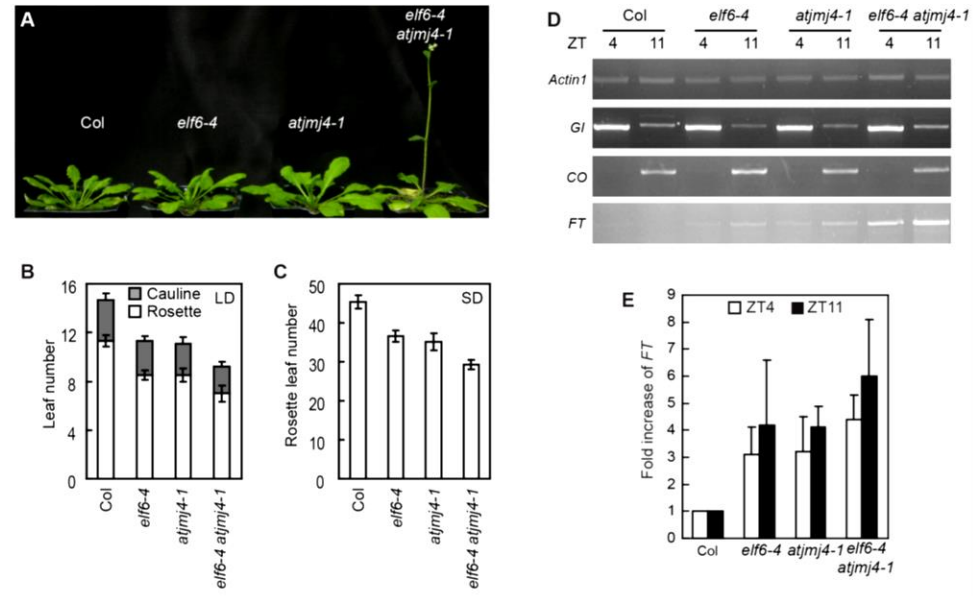

Figure 2.5 Additive effect of *elf6* and *atjmj4* mutations on *FT*-dependent early flowering.

A) Early flowering phenotype of *elf6-4 atjmj4-1* double mutant. All plants were grown in SD for 63 d before taken picture. **B and C)** Flowering time of wt Col, *elf6-4*, *atjmj4-1*, and *elf6-4 atjmj4-1* double mutants in LD and SD as determined by number of leaves formed at bolting. At least 15 individuals were scored for each genotype. Error bars represent sd. **D)** Expression of flowering genes in *elf6-4 atjmj4* double mutants. Plants of each genotype were grown in SD for 57 d and harvested at ZT4 or ZT11 for RT-PCR analyses. *Actin1* was included as an expression control. Identical results were obtained from two independent experiments and one of them is shown. **E)** qPCR analysis of *FT* expression. The same RNAs used in (D) were evaluated. The wt Col levels were set to 1 after normalization by *Actin1* for qPCR analysis. Error bars represent sd.

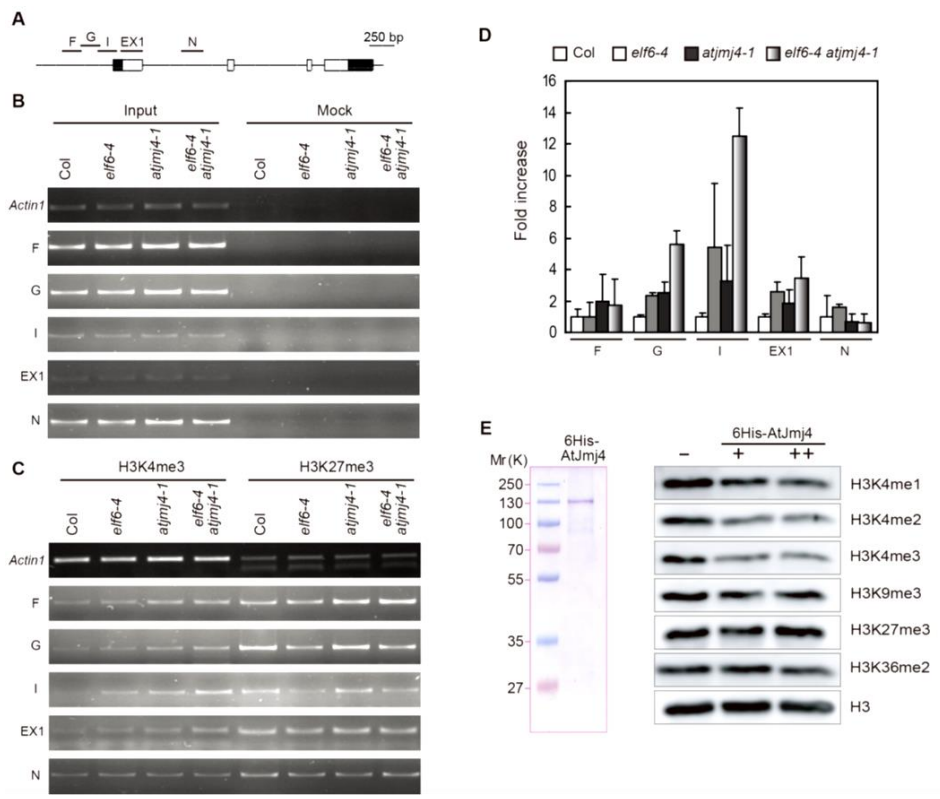

Figure 2.6 Increased trimethylation of H3K4 at *FT* locus by *elf6* and *atjmj4* mutations.

A) Schematic of *FT* locus showing regions (F, G, I, EX1, and N) amplified by the primers used for ChIP analysis. The front and the rear black boxes indicate 5' and 3' UTRs, respectively. White boxes indicate exons, while lines indicate introns and intergenic regions. **B and C)** ChIP assay of *FT* chromatin with antibody against H3K4me3 or H3K27me3. Plants of each genotype were grown in SD for 57 d and harvested for ChIP assay. 'Input' indicates chromatins before immunoprecipitation. 'Mock' refers to control samples lacking antibody. *Actin1* was used as an internal control. **D)** qPCR analysis of the ChIP assay for H3K4me3 described in (B and C).

The wt Col levels were set to 1 after normalization by input. Error bars represent sd. **E)** Coomassie-blue stained 6His-AtJmj4 protein purified from *sf9* cells (left), and *in vitro* histone demethylation activity assay using the purified protein (right). Assays were performed without (-) or with either two (+) or four (++) µg of purified 6His-AtJmj4 protein. Mr (K), molecular mass in kilo-daltons.

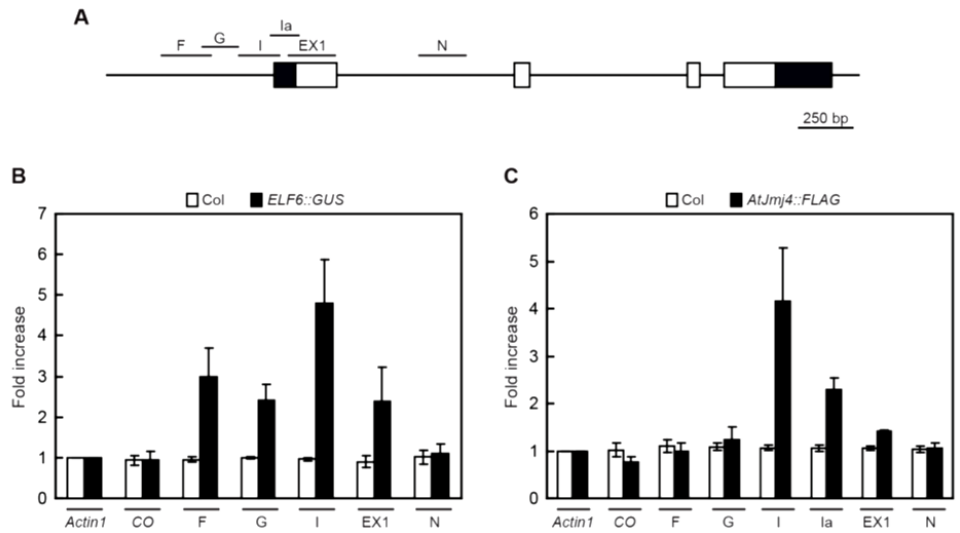

Figure 2.7 Direct association of ELF6 and AtJmj4 with *FT* chromatin.

A) *FT* regions tested for ChIP assay. Schematic is as described in **Figure 6A** except for the region Ia, which was added in assays in (C). **B)** ELF6 binding to *FT* chromatin. LD grown 16 d-old wt Col and *ELF6::GUS*–containing transgenic *elf6-4* plants [22] were harvested and used for ChIP assay using GUS-specific antibody. Amount of immunoprecipitated chromatin was measured by qPCR (B and C). *Actin1* and *CO* were used as internal controls, and the level of *Actin1* in each sample was set to 1 for normalization (B and C). Error bars represent se of three independent biological replicates (B and C). **C)** AtJmj4 binding to *FT* chromatin. LD grown 16 d-old wt Col and *AtJmj4::FLAG*–containing *atjmj4-1* plants were harvested and used for ChIP assay using FLAG-specific antibody.

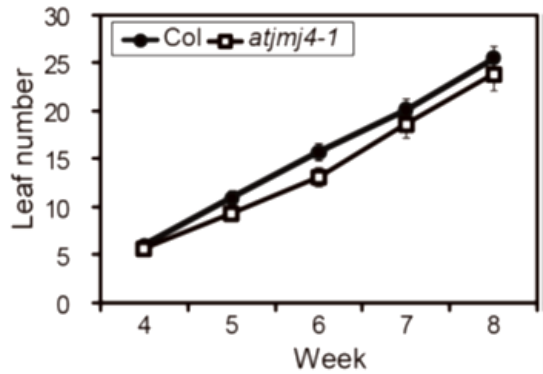

Figure 2.8 Leaf initiation rate of *atjmj4-1* mutants.

Wt Col (black circles) and *atjmj4-1* mutant plants (white squares) were grown in SD and their leaf numbers were scored every week from four weeks after planting. At least 10 individuals were scored for each genotype. Error bars represent sd.

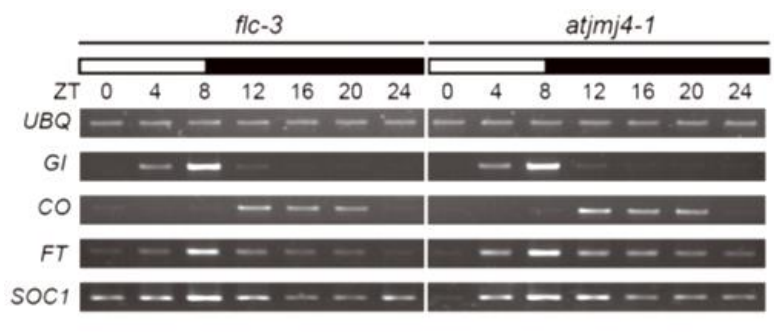

Figure 2.9 *FLC*-independent function of *AtJmj4*.

Expression of flowering genes in *flc-3* and *atjmj4-1* mutant plants grown in SD for 12 d as determined by RT-PCR analysis. *UBQ* was used as an expression control.

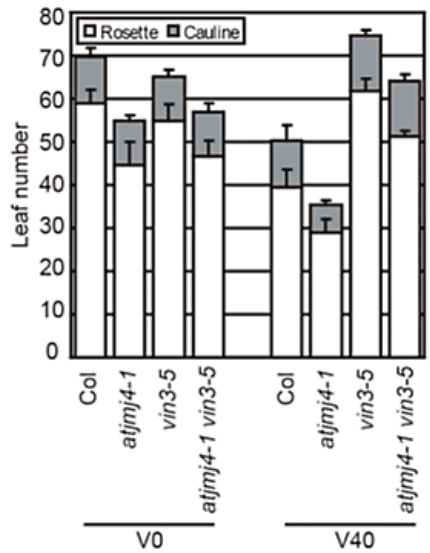

Figure 2.10 Vernalization response of *atjmj4* mutants.

Plants of each genotype were treated with vernalization for 40 days (d) as described previously [56]. Flowering time was scored as leaf number for plants either without (V0) or after (V40) vernalization treatment. At least 12 individuals were scored for each genotype. Error bars represent sd.

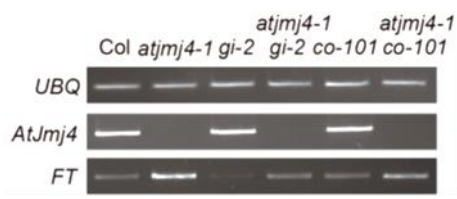

Figure 2.11 *CO*- and *GI*-independent increase of *FT* expression in *atjmj4*.

Plants of each genotype were grown in LD for 14 d and harvested at ZT8 for RT-PCR analyses. *UBQ* was used as an expression control.

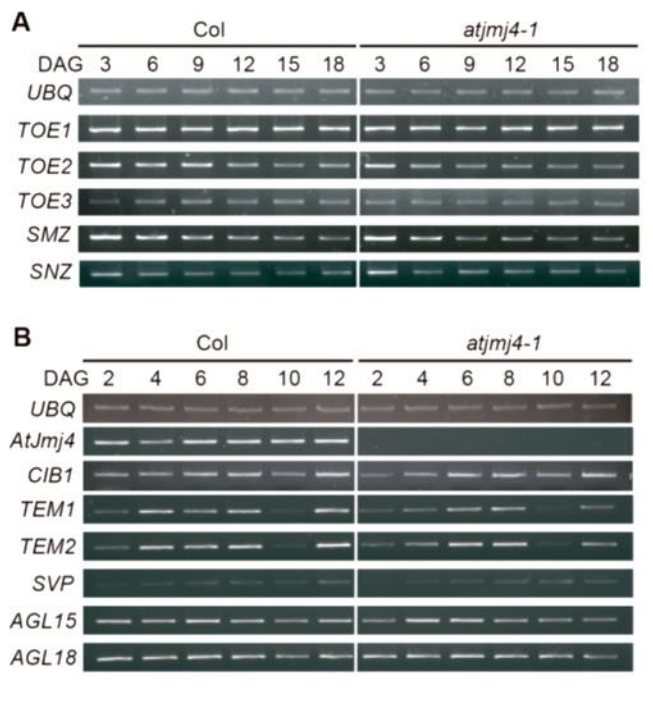

Figure 2.12 Expression of *FT* regulators in *atjmj4*.

A and B) Temporal expression of *FT* regulators in *atjmj4-1*. Col and *atjmj4-1* plants were grown in SD (**A**) or in LD (**B**) until indicated DAG and harvested at ZT14 (LD) or ZT8 (SD) for RT-PCR analyses. *UBQ* was used as an expression control.

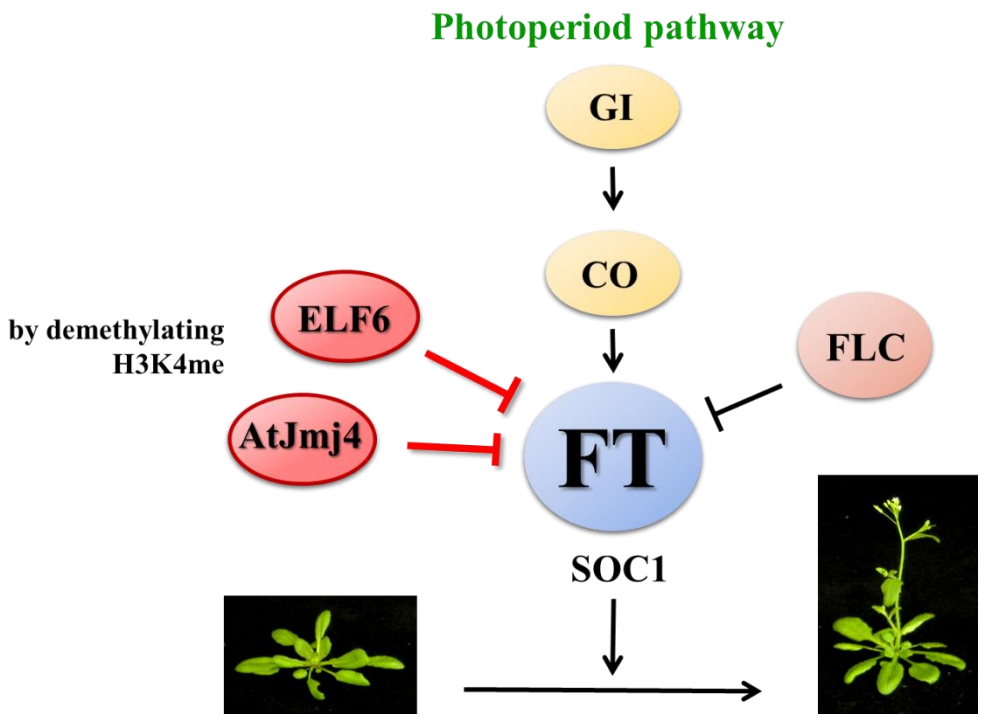

Figure 2.13 Proposed model for the role of AtJmj4 and ELF6 in pathways regulating flowering time in Arabidopsis

ELF6 and AtJmj4 have redundant repressive roles in flowering through negatively regulating *FT* expression via demethylation of H3K4me.

Table 2.1. Oligonucleotides used for T-DNA flanking sequence analysis

Name	Sequence
SALKLB1	5’-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3’
AtJmj4-1-F	5’-GAGAAGTTGCGCTCTAAAGCAGAATC-3’
AtJmj4-1-R	5’-TGGATCACCTGTGTGTAAGTAGTTCATGG -3’
AtJmj4-2-F	5’-AATATGTATCTCACTCTGCACC-3’
AtJmj4-2-R	5’-AAATCACCCTCCATCTTTCGC-3’

Table 2.2. Oligonucleotides used for RT-PCR analysis

Gene	Name	Sequence
Ubiquitin	UBQ-F	5’-GATCTTTGCCGGAAAACAATTGGAGGATG GT-3’
	UBQ-R	5’-CGACTTGTCATTAGAAAGAAAGAGATAAC AGG-3’
AtJmj4	AtJmj4-F	5’-GCTTGACCCAACAAACCTAACC-3’
	AtJmj4-R	5’-TCTCACCACACAGAAGTCCATGC-3’
GI	GI-F	5’-GTTGTCCTTC AGGCTGAAAG-3’
	GI-R	5’-TGTGGAGAGC AAGCTGTGAG-3’
CO	CO-F	5’-AAACTCTTTCAGCTCCATGACCACTACT-3’
	CO-R	5’-CCATGGATGAAATGTATGCGTTATGGTTA-3’
FT	FT-F	5’-GCTACAACTGGAACAACCTTTGGCAAT-3’
	FT-R	5’-TATAGGCATCATCACCGTTCGTTACTC-3’
SOC1	SOC1-F	5’-TGAGGCATACTAAGGATCGAGTCAG-3’
	SOC1-R	5’-GCGTCTCTACTTCAGAACTTGGGC-3’
FLC	FLC-F	5’-TTCTCCAAACGTCGCAACGGTCTC-3’
	FLC-R	5’-GATTTGTCCAGCAGGTGACATCTC-3’
TOE1	TOE1-F	5’-ACTCAGTACGGTGGTGACTC-3’
	TOE1-R	5’-CGAGGATCCATAAGGAAGAGG-3’
TOE2	TOE2-F	5’-CACTTTCTATCGGAGGACAG-3’
	TOE2-R	5’-CTTCCACATACGGAATTGTT-3’
TOE3	TOE3-F	5’-GTTACGTTTTACCGACGAAC-3’
	TOE3-R	5’-TGCTTGCAATATCAGACTTG-3’
SMZ	SMZ-F	5’-AATGGTGAAGAAGAGCAGAA-3’
	SMZ-R	5’-CTTTCCGATGATGATGAAAT-3’
SNZ	SNZ-F	5’-TTTGGAATCCTTAAACGAAA-3’
	SNZ-R	5’-TATCTCATTGCATTTTGCTG-3’
CIB1	CIB1-F	5’-GCATAGCAGAACGAGTTAGAAGAG-3’
	CIB1-R	5’-ATCAGAACTGGTATTCACTTGCTG-3’
TEM1	TEM1-F	5’-GCGTGTTGTTTCGGTATCACTA-3’
	TEM1-R	5’-ATTCAGAGAACGGCGTCGA-3’

TEM2	TEM2-F	5’-TTCCTCAGCCTAACGGAAGAT-3’
	TEM2-R	5’-TCCTTGACGAATCGACTCCAT-3’
SVP	SVP-F	5’-CGCTCTCATCATCTTCTCTTCCAC-3’
	SVP-R	5’-GCTCGTTCTCTTCCGTTAGTTGC-3’
AGL15	AGL15-F	5’-TTATCTAGATGGGTCGTGGAAAAATCGAG-3’
	AGL15-R	5’-TTAGCGGCCGCAGAGAACCTTTGTCTTTTG GCTTC-3’
AGL18	AGL18-F	5’-ATGGGGAGAGGAAGGATTGAGATTAAGA A-3’
	AGL18-R	5’-TCAATCAGAAGCCACTTGACTCCCAGAGT-3’

Table 2.3. Oligonucleotides used for constructs

Name	Sequence
AtJmJ4GUS-F	5’-gtcgacGTCTCCTCTCTATCGCCATTCTTG-3’
AtJmJ4GUS-R	5’-CTTcccgggAAAGGACTTATCTCCATC-3’
AtJmJ4OE-F	5’-CAgtcgacATGGATCAGCTTGCATCTC-3’
AtJmJ4OE-R	5’-TGCgtcgacAAGGACTTATCTCCATC-3’
AtJmJ4OE-R1	5’-TGCgtcgacTTAAGGACTTATCTCCATC-3’
AtJmj4FLAG-F	5’-TTctgcagGTCTTCGTCTCCTCTCTATCGC-3’
AtJmj4FLAG-R	5’-GGGctgcagCATTTACAGTGAGATTAAGTTC-3’
AtJmj4FLAG-F1	5’-GAgctagcTCCTCTCTATCGCCATTCTT-3’
AtJmj4FLAG-R1	5’-GTCTCGAGGAATTCCCAAACATATAGTAGATG-3’
JMJ4_pENTR_For	5’-CACCATGGATCAGCTTGCATCTCTAG-3’
JMJ4_pENTR_Rev	5’-AGGACTTATCTCCATCTTATC-3’

Restriction sites used for cloning are in small letters and underlined**.**

Table 2.4. Oligonucleotides used for ChIP assay

Gene	Region	Name	Sequence
FT	F	FF	5’-ACTTGGCGGTACCCTACTT-3’
		FR	5’-ATATCTCCCACTTGGTAG -3’
	G	GF	5’-GTCGAGAGAGGTATCTTGTTAAAG-3’
		GF	5’-ATCATAGGCATGAACCCTCTACAC-3’
	I	IF	5’-TATGTGTAGAGGGTTCATGCCTATG-3’
		IR	5’-TGGCCATAACCTTTAGAGTG -3’
	Ia	IaF	5’-CCACCTGTTTGTTCAAGATC-3’
		IaR	5’-GAAGGCCTTAGATCCAAGCC-3’
	EX1	EX1F	5’-ATGTCTATAAATATAAGAGACCCTC-3’
		EX1R	5’-CTTCTCCACCAATCTCAACTCTTG-3’
	N	NF	5’-TCCACCAACTTCTTGCATAAGTGA-3’
		NR	5’-CCACAACAGAGATTCATCAATATAT-3’
Actin1		Actin1 F	5’-CGTTTCGCTTTCCTTAGTGTTAGCT-3’
		Actin1 R	5’- AGCGAACGGATCTAGAGACTCACCTTG-3’
CO		CO F	5’-GGCACTCAGGATTCGATCTCC-3’
		CO R	5’-CCGGCATGTGTCACAGGGTCG-3’

2.6 Discussion

Recent studies have shown that the expression of some key flowering genes, such as *FLC* and *FT*, are regulated through chromatin modifications and have also discovered factors involved in the chromatin modification processes (Farrona et al., 2008; Schatlowski et al., 2008). In this study, we showed that AtJmj4 and ELF6 play a role in the repression of *FT* transcription by removing methyl groups from H3K4 at *FT* locus.

FT chromatin contains bivalent marks, thus the active mark (H3K4me3) and the repressive mark (H3K27me3) exist simultaneously (Jiang et al., 2008). Of these, only H3K27me3 has been studied. It plays a critical role in preventing precocious floral transition by establishing and maintaining the repressive *FT* chromatin as default state. The PRC2-like complex comprised of CURLY LEAF, SWINGER, EMBRYONIC FLOWER2, and FERTILIZATION INDEPENDENT ENDOSPERM, are required for H3K27me and the repression of *FT* (Jiang et al., 2008).

Unlike H3K27me3, H3K4me3 has been known to be positively associated with transcriptional activities (Li et al., 2007). H3K4me3 can be recognized by the TFIID complex via the PHD finger of TAF3, which in turn recruits RNA polymerase II, leading to transcription activation (Vermeulen et al., 2007). Therefore, H3K4me3 might also affect the chromatin state and expression of *FT*. In this study, we demonstrate that AtJmj4 is involved in *FT* repression as an H3K4-specific demethylase directly targeting *FT* locus with the following data: 1) The loss of *AtJmj4* function increased *FT* expression via enhanced *FT* promoter activity (**Figure 2.2**); 2) The loss of *AtJmj4* function increased H3K4me3 level in the

transcription initiation region of *FT* (**Figures 2.6C** and **2.6D**); 3) The purified AtJmj4 protein can specifically demethylate H3K4me1, H3K4me2, and H3K4me3 *in vitro* (**Figure 2.6E**); 4) The AtJmj4::FLAG binds to the transcription initiation region of *FT*. Up-regulation of *FT* expression could also be responsible, at least in part, for the increased H3K4me3 level in *atjmj4* at the *FT* locus.

It is notable that the increase of H3K4me3 at *FT* locus caused by the loss *AtJmj4* is not substantial. This suggests that there might be other histone demethylases having redundant roles with AtJmj4 for the demethylation of H3K4 at *FT* locus. ELF6 is turned out to be one of those histone demethylases from our following data: 1) The loss *ELF6* function increased *FT* expression and H3K4me3 level in the transcription initiation region of *FT*, and these increases were more significant when *AtJmj4* and *ELF6* functions were lost together (**Figures 2.5D**, **2.5E**, **2.6C**, and **2.6D**); 2) The ELF6::GUS binds to the promoter and transcription initiation region of *FT*. Previously we reported that both AtJmj4 and ELF6 belong to the same group (Group I) of Arabidopsis Jmj family proteins (Hong et al., 2009). Our unpublished phylogenetic analysis indicates that eight Arabidopsis Jmj family proteins belonging to this group have JmjC domains clustered together with the JmjC domains of human JARID1 family that are H3K4 demethylases (Christensen et al., 2007; Klose RJ and Zhang Y, 2007). Therefore, not only AtJmj4 and ELF6 but also other members of the Arabidopsis Group I Jmj family proteins have a potential to be H3K4 demethylases acting at *FT* locus. However, their genetic and biochemical roles need yet to be addressed in the future study.

According to recent studies, the antagonistic histone marks, H3K27me3 and H3K4me3, are coordinately regulated by protein complexes containing both

histone methyltransferase and histone demethylase (Lee et al., 2007; Pasini et al., 2008). Pasini et al (Pasini et al., 2008) reported that the RBP2 H3K4 demethylase is recruited by the PRC2 to repress the expression of target genes in mouse embryonic stem cells, and the loss of RBP2 increases the expression of the target genes. At this moment, it is not clear if a similar interaction between AtJmj4/ELF6 and the Arabidopsis PRC2 components occurs for the repression of *FT*. However, the report that the level of H3K4me3 within *FT* chromatin is increased in the absence of CURLY LEAF activity (Jiang et al., 2008) suggests such scenario is plausible. In this study, we could not observe a significant reduction of H3K27me3 level within *FT* chromatin in *elf6 atjmj4* double mutants. Thus, H3K4 demethylases might be recruited by PRC2, but the PRC2 recruitment might not be affected by H3K4 demethylases.

The coexistence of bivalent H3K27me3 and H3K4me3 marks at the same locus has been proposed to poise genes for the activation upon appropriate developmental cues (Bernstein et al., 2006; Azuara et al., 2006). Thus, the bivalent chromatin marks within *FT* chromatin might be a strategy for plants to achieve reproductive success by a precise regulation of *FT* expression and flowering time. It might be possible that enriched H3K27me3 favors constitutive *FT* repression, while a proper level of H3K4me3 provides appropriate accessibility for transcription factors controlled temporally such that *FT* expression can be regulated by changing developmental or environmental cues. Interestingly, the region I of *FT* locus, in which both AtJmj4::FLAG and ELF6::GUS showed strongest binding (**Figures 2.7A-C**) and the largest increase of H3K4me3 by the *elf6* and *atjmj4* mutations was observed (**Figures 2.6A**, **2.6C**, and **2.6D**), contains binding sites for *FT*

transcriptional regulators, namely TEM1/TEM2 (Castillejo et al., 2008) and a CO-containing protein complex (Wenkel et al., 2006). Thus, it would be of interest in the future to test if the binding of these *FT* transcriptional regulators is altered by the activity of AtJmj4 and ELF6.

Chapter III

Arabidopsis SIN3 Homologs Negatively Regulate *ABI* genes in Seed Germination and Early Seedling Establishment

3.1 Abstract

Seed germination accompanies a number of morphological changes representing developmental transitions. During seed germination, expression of a number of genes is transcriptionally and post-transcriptionally modulated. As a major factor that links and converts environmental signals to plant responses, abscisic acid (ABA) has a key role in seed maturation and germination. Especially, precise regulation of *ABA INSENSITIVE* (*ABI*) genes, which assist ABA-signaling, is required for adequate germination. During germination, histone deacetylation-based mechanisms antagonize histone acetyltransferase activity to regulate ABA signaling-related genes on a platform of SIN3-LIKE (SNL) in *Arabidopsis*. In this study, I demonstrate that seed germination and early seedling establishment of *SNL* quadruple mutant (*snl1234*) mimics the germination process under hyperactive ABA-signaling conditions. SNL1, SNL2, SNL3, and SNL4 protein expressions are spatially overlap each other in developing and mature embryos. This is consistent with the idea that SNL proteins have redundant roles during germination. Dormancy released *snl1234* seeds and embryos showed delayed germination, and this phenotype was exaggerated with exogenous ABA treatment. Although, endogenous ABA content was not altered in *snl1234*, different expression patterns of the ABA-signaling genes, *ABI3*, *ABI4*, and *ABI5*, were observed in the mutant during germination and early seedling establishment. Moreover, attuned *ABI* expression in *snl1234* was associated with enriched histone acetylation within *ABI* chromatin. Furthermore, SNL3 directly targets *ABI3* and *ABI5* loci. Taken together, SNLs play an important role during seed germination and early seedling establishment as bedrock for histone deacetylation-based mechanism of *ABI* gene

regulation.

3.2 Introduction

Proper transition from seed germination to early seedling establishment in the life of higher plants is both ecologically and economically important. Seed germination begins with imbibition of after-ripened seed and finishes with radicle protrusion or endosperm rupture (Bewley, 1997; Bewley et al., 2013). Seed germination is also a successive process from seed development to early seedling establishment (Angelovici et al., 2010). If a favorable condition for a plant to begin its life cycle is given, the after-ripened seed germinates and subsequently develops to become a seedling. For the control of seed germination and early seedling development, various environmental factors, such as light, temperature, water and nutrient availability, are incorporated into hormonal regulatory pathways (Holdsworth et al., 2008). At molecular level, seed germination is largely influenced by the regulation of phytohormones, such as gibberellic acid (GA), abscisic acid (ABA), ethylene, and auxin. Moreover, a number of gene-expression changes through transcriptional and post-transcriptional modulations are also required to complete the seed germination (Nakabayashi et al., 2005; Soeda et al., 2005).

The signaling networks that incorporate light signals to hormonal responses are crucial in seed development, dormancy, germination, and post-germinative developmental processes (Bai et al., 2012; Finkelstein et al., 2008; Oh et al., 2012; Rajjou et al., 2012). Especially, the signaling mechanisms of phytohormones, namely GA and ABA, are essential in the regulation of seed dormancy, germination, and early seedling development. Since these phytohormones function antagonistically in the seed germination process, the

metabolism and signaling balance between GA and ABA should be tightly regulated (Koornneef et al., 1982; Razem et al., 2006; Holdsworth et al., 2008). GA breaks dormancy and promotes seed germination, while ABA induces and maintains seed dormancy (Koornneef and Karssen, 1994; White et al., 2000). During germination processes, GA-related germination mechanisms are well-characterized at both physiological and molecular levels, whereas ABA-related mechanisms have mostly been examined in relation only with physiological responses. Therefore, more detailed studies are necessary for the understanding of ABA-related molecular mechanisms acting during seed germination and early seedling establishment.

ABA-mediated inhibition of seed germination occurs through various signaling components, including ABA-INSENSITIVE 3 (ABI3) (Giraudat et al., 1992), ABI4 (Finkelstein et al., 1998; Soderman et al., 2000), and ABI5 (Finkelstein and Lynch, 2000). *ABI3*, *ABI4*, and *ABI5* encode transcription factors which are highly expressed in seeds and repressed in vegetative tissues (Giraudat et al., 1992; Finkelstein et al., 1998, 2002; Finkelstein and Lynch, 2000). It has been demonstrated that mutations in *ABI3*, *ABI4*, and *ABI5* cause ABA-insensitivity in seed and yield viviparies (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Soderman et al., 2000). To more specifically describe, ABI3 encodes a member of B3-domain transcription factor. During seed maturation, it acts in the embryo for the accumulation of storage-proteins or lipids and, more importantly, induces seed dormancy (Stone et al., 2001; Kroj et al., 2003; To et al., 2006). An ERF/AP2-type transcription factor ABI4 was revealed to participate in the regulation of seed maturation-related genes and also in the acquisition seed

dormancy (Liu et al., 2007). A basic leucine-zipper transcription factor ABI5, of which expression is enhanced by ABI3 and ABI4, stimulates the expression of many critical genes that are involved in the arrest of seedling growth after germination (Bossi et al., 2009; Lopez et al., 2002). It is clear that the network of ABA signaling components, namely ABI3, ABI4 and ABI5 as the key regulators, is critical in seed development, especially for ABA-mediated inhibition of germination (Park et al., 2011). However, more comprehensive regulatory mechanisms of such genes are yet to be revealed.

Only recently, some studies have reported that a germination-arrest mechanism is mediated by chromatin-remodeling factors (Perruc et al., 2007; Li et al., 2005). Since then, more and more studies have demonstrated that various epigenetic regulations are entailed for gene-expression changes during seed germination and very early phase of seedling establishment (Liu et al., 2011; Cho et al., 2012). Histone methylation is a renowned epigenetic control for these processes. In *Arabidopsis*, histone arginine demethylases, JUMONJI 20 (JMJ20) and JMJ22, promote seed germination in red light-dependent manner by eliminating histone H4 arginine 3 symmetric dimethylation (H4R3me2s) at the *GA3 oxidase 1* (*GA3ox1*) and *GA3ox2* loci via the PHYB-PIL5-SOM pathway (Rider et al., 2003; Cho et al., 2012). When PHYTOCROME B (PHYB) is inactive, PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5 (PIL5)/PIF1 directly upregulates the expression of *SOMNUS* (*SOM*), a repressor of seed germination, which in turn represses *GA3ox1* expression. In a previous report, SOM was shown to be enriched in the promoter of *JMJ20* or *JMJ22*, and these JMJs directly target *GA3ox1* and *GA3ox2* chromatin (Cho et al., 2012). When seeds are exposed to red-

light, H4R3me2s levels at *GA3ox1* and *GA3ox2* are decreased in wild type (wt) but not in *jmj20 jmj22* double mutants. Moreover, it has been proven that a crosstalk between histone methylation and other epigenetic modifications exists in seed germination. Although how H4R3me2s influences the level of other histone marks is not yet clear, histone H3 lysine 9 trimethylation (H3K9me3) or H3K4me3 levels are increased or decreased, respectively, in *jmj20 jmj22* compared to those in wt. Another example of epigenetic control reported is the role of PICKLE (PKL), a SWI/SNF-type chromatin remodeling factor, in the repression of embryonic trait genes, *LEAFY COTYLEDON* (*LEC1*), *LEC2*, and *FUSCA 3* (*FUS3*), during seed germination and at multiple developmental points (Rider et al., 2003). It was reported that *pkl* mutant seeds fail to develop into seedling phase because seed-associated traits are continually expressed after germination and GA biosynthesis is up-regulated in *pkl* mutant. It was recently informed that repressive role of PKL on those embryonic genes was implemented via H3K27me3 enrichment (Zhang et al., 2008).

Interestingly, among many histone modifier-mutant seeds we tested, a few mutants including non-functional *SIN3-LIKE* (*SNL*) mutants exhibited delayed germination phenotypes. *Arabidopsis* SNLs are the orthologs of yeast Swi-independent 3 (Sin3). The molecular mechanism of Sin3 in other organisms has been widely studied. In 1987, Sin3 of budding yeast was first identified as a negative transcriptional regulator (Sternberg et al., 1987). Since then, a number of researches have ascertained that Sin3 is a transcriptional co-repressor involved in gene-silencing mechanism in concert with a histone deacetylase (HDAC), Reduced potassium dependency 3 (Rpd3) (Kasten et al., 1997; Vidal and Gaber, 1991). It

has also been revealed that Sin3 itself does not possess an intrinsic DNA-binding domain. Instead, it is considered a master scaffold which serves as a platform for the association of HDACs and transcription factors (Zhang et al., 1997).

In *Arabidopsis*, six homologs of yeast Sin3 (SNL1, SNL2, SNL3, SNL4, SNL5, and SNL6) are identified by the analysis of protein sequence similarity. Moreover, a few studies have identified the roles of SNL proteins in various biological processes in plants. It has been revealed that SNL3 induces APETALA2/EREBP-type transcription factor AtERF7, which functions as an important transcriptional repressor in ABA responses upon drought stress (Song et al., 2005). Very recently, it was reported that SNL1 and SNL2 participate in the acquisition of seed dormancy via antagonistic mechanisms on the ethylene- and ABA-mediated pathways (Wang et al., 2013). In *snl1 snl2* double mutants, histone acetylation levels of ethylene synthesis-related genes (*ACO1* and *ACO4*), ethylene signaling genes (*ERF9*, *ERF105*, and *ERF112)*, and ABA hydrolase-encoding genes (*CYP707A1* and *CYP707A2*) were slightly enriched. However, these enrichment levels were less than two-fold, and these observations were made during the acquisition of seed dormancy.

Since former findings have suggested that epigenetic control of phytohormone signalings might be associated with various developmental processes such as plant stress responses and the acquisition of seed dormancy, using *snl1234* quadruple mutant seeds, here we attempted to demonstrate that all or some SNL proteins may function in seed germination and early seedling establishment via phytohormone signaling pathway,. Because both endogenous hormone level and its signaling greatly influence seed germination and early

seedling establishment, we aimed to clarify whether SNL proteins are associated with hormonal metabolism or signaling. Especially, as direct relationship between histone acetylation and seed germination is poorly understood, epigenetic aspects of hormone-related seed germination mechanism have to be more clearly elucidated. Therefore, we intended to focus on the roles of SNLs on ABA-mediated regulation during seed germination and early seedling establishment. In addition, as SNLs may function as a platform for Sin3-HDAC complex repressing target gene expression, the purpose of this study was to identify the role of SNL proteins as co-repressors of their target genes as well as to test a possibility of SNL-mediated role of histone acetylation in seed germination and early seedling establishment.

3.3 Materials and Methods

3.3.1 Plant materials and growth

snl3 and *snl4* T-DNA insertion lines in the Col background were obtained from the SALK collection (http://signal.salk.edu/; *snl3-1*, SALK_920633; *snl3-2*, SALK_028140; *snl4-1*, SALK_042565; *snl4-2*, SALK_053319). The genotype of each mutant allele was defined by gene-specific primers (listed in **Table 3.1**). The following mutants are in the Col background and were described previously: *snl1* and *snl2* (Wang et al., 2013), *abi3* (Michaels & Amasino, 1999). Double, triple and quadruple mutants were obtained by cross. All seeds were sown after After-ripening (3 months later from seed harvested) and were grown under 100 umol/m^2s, long day (16 hr light/ 8 hr dark).

For generation of the *SNL3pro::SNL3:HA* translational fusion construct, a 7.3kb genomic DNA fragment of *SNL3* containing 1.27kb 5' upstream region and the entire coding region was generated by PCR amplification using SNL3-fullength F and SNL3-fullength R primers (listed in **Table 3.2**). The amplified region was cloned into the vector pEarleyGate 301 (Earley et al., 2006). The fusion construct was introduced into *snl1234-1* by the floral dip method (Clough and Bent, 1998) through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 25 μg ml^{-1} glufosinate ammonium. The *HDA19pro::HDA19:FLAG* (Choi et al, 2012) and *HDA9pro:HDA9:HA* (Kang et al, 2015) were described previously.

3.3.2 Germination Assay

For the Germination assays, seeds were selected that were grown as side by side at the same time. Seeds were surface-sterilized and plated on half Murashige-skoog (MS) media without sucrose (0.8% phytoagar, pH 5.7). Then, the seeds were placed in a growth chamber at 22℃ under white light. Germinated seeds, which were completely penetrated the seed coat (protrusion of the radicle) were scored at the indicated times. At least 50-100 seeds were used for each germination assay and three biological replicates were performed for statistical analyses. To determine the effect of GA3 or ABA, Seeds were placed on half MS Media supplemented with variable concentrations of GA3 or ABA.

3.3.3 Gene Expression Analyses

Total RNA was isolated from seed with previously described but modified method. (Ling Meng and Lewis Feldman, 2010). The RNA purified using Qiagen RNeasy Plant mini kit with on-cloumn DNase I digestion step (Qiagen). The 3 ug of total RNA was reverse transcribed using MMLV Reverse Transcriptase) according to the manufacturer's instructions (Fermentas, USA). The qRT-PCR analysis was performed using Rotor-Gene Q (Qiagen) with the SYBR Green Fast qPCR master mix (Kappa Biosystems) in a total volume of 20ul. Quantification of amplified products was performed by generating standard curves using serial dilutions of all cDNA sample to be analyzed. Expression level of each genes were calculated as fold change compared to *UBQ11*. All qRT-PCR results were performed in triplicate reactions of three- biological repeats as means±STD. Primer used for qRT-PCR

analysis are listed in **Table 3.3**.

3.3.4 GUS and GFP assays

For the construction of the *SNL1pro::GUS*, *SNL2pro*::*GUS*, *SNL3pro*::*GUS*, and *SNL4pro*::*GUS* transcriptional constructs, each promoter regions of *SNL1*, *2*, *3*, and *4* were amplified by specific primer sets (listed in **Table 3.2**) and then subcloned in pPZP211 vector (Hajdukiewicz et al. 1994). The final construct were introduced into Col by the floral dip method. For GUS assay, *SNL1pro*::*GUS*, *SNL2pro*::*GUS*, *SNL3pro::GUS*, and *SNL4pro::GUS* transgenic plants were reciprocally crossed with Col. 7days after pollination seeds and completely maturated embryo were used to GUS assay. Seeds were fixed by acetone on the ice for 30 min, kept in the dark wrapped with foil and washed three times with KPO4 buffer. The fixed seeds were dissected by forceps, and the extracted embryos were stained with X-Gluc solution for 2hr 30 min or overnight. The stained embryos were photographed using AxioVison under optical microscope (Carl Zeiss, Germany).

For the *SNL3pro::SNL3:GFP* transgenic line, the *SNL3* genomic locus (including 1.27kb upstream of the start codon) were amplified with the primer fullength-SNL3 F and fullength-SNL3 R (listed in **Table 3.2**). The PCR product was ligated into modified pEarleygate301 that has GFP and His tag from pEarleygate103. The construct was transformed to *snl234 triple mutant* plants following the floral dip method. Subcellular localization of SNL3::GFP was determined by LSM 510 confocal microscope (Zeiss). Embryos of *SNL3pro::SNL3:GFP* are rescued from seed coat by forceps 1hr after dark

imbibition.

3.3.5 Quantification of Endogenous ABA level

Endogenous ABA was extracted with previously described. (Francisco et al. 2000). To quantify the ABA content of *snl1234-1,-2,* and wild-type plants, 100-200 mg of dry or imbibed seeds were homogenized in 10 ml of ABA-extraction buffer (10 mm HCL, 1% PVPP in methanol). Sample was shaken overnight at 4℃, and then a supernatant was collected. Add 150μl of 1M NaOH for neutralization as described (Peña-Cortes et al. 1989). ABA was quantified with a Phytodetek-ABA-kit (AGDIA Inc., IN) using the protocol provided.

3.3.6 Chromatin immunoprecipitiation (ChIP) assay

ChIP was performed as described previously described (Jeong et al. 2009) with modifications. 500mg seeds were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin was isolated by using lysis buffer (containing 50mM HEPES, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 1mM PMSF, and Protease inhibitor with 0.1% SDS) and sonicated into ~0.5 to 1 kb fragments. Specific antibody against H3Ac (Millipore, 06-599), HA (Abcam, ab9110). FLAG (Sigma A8592-0.2MG) was added to the chromatin solution, which had been pre-cleared with salmon sperm DNA/Protein A agarose beads (Upstate 16-157). After subsequent incubation with salmon sperm DNA/Protein A

agarose beads, immune-complexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immune-complexes were removed by incubation with proteinase K, followed by DNA extraction. DNA was recovered by a DNA purification kit (Qiagen). The amount of immune-precipitated chromatins was determined by PCR with primer pairs in **Table 3.4**.

3.4 Result

3.4.1 Mutations in *SNL1, SNL2, SNL3,* and *SNL4* Cause Delayed Seed Germination

As seed germination should require turn-off and turn-on of numerous embryonic and post-embryonic genes, respectively, we hypothesized that seed germination might involve an epigenomic reprogramming. Hence, we have searched for epigenetic mutants defective in seed germination. Six arabidopsis SNL peptide sequences were identified from the *Arabidopsis* genomic DNA sequence database. It is known that SNL genes have sequence similarity to each other and share HDAC-interaction domains (Bowen et al, 2010). A phylogenic tree was generated with human, mouse, and yeast Sin3 peptide sequence with six arabidopsis SNL peptide sequences using ClustalW (Thompson et al., 1994; http://align.genome.jp/). The resultant phylogenetic tree, six SNL proteins were subdivided into three groups: SNL1 (AT3G01320) and SNL2 (AT5G15020); SNL3 (AT1G24190) and SNL4 (AT1G70060); and SNL5 (AT1G59890) and SNL6 (AT1G10450). Because Sin3 homologs of other organisms, yeast, human, and mouse, are not clustered with *Arabidopsis* SNL, it is proposed that these proteins may function in plant-specific biological contexts (**Figure 3.1A**). To address if SNLs have a functional redundancy, we searched for collections of T-DNA insertional lines of the corresponding genes and series of double, triple, and quadruple mutants of the *snl1*, *snl2*, *snl3*, and *snl4* are generated. One or two allelic homozygous T-DNA insertional mutant lines for corresponding *SNL1*, *2*, *3*, or *4* genes are obtained from the SALK collection and T-DNA insertions for each gene were depicted in

Figure3.1B. All combinations of double and triple mutants were generated by using *snl1-1*, *snl2-1*, *snl3-1*, and *snl4-1*. *snl1234-1* quadruple mutant is generated by using *snl1-1*, *snl2-1*, *snl3-1*, and *snl4-1*. Additionally, independent quadruple mutant *snl1234-2* is generated by using *snl1-1*, *snl2-1*, *snl3-2*, and *snl4-2*. The germination test of these double, triple and quadruple mutants was subsequently performed using non-dormant seeds. In the end of embryo maturation, seeds are highly dormant, which are marked by a very high mRNA expression level of *DELAY OF GERMINATION 1*(*DOG1*). Immediately after maturation, freshly harvested (FH) seeds have not yet acquired the competence for seed germination. The measurement of germination ability at FH seed stage is used to indicate the level of seed dormancy. On the other hand, germination-competence is obtained after endurance of a certain period of time to release dormancy. During dry storage, several physiological changes are followed in seeds so that greenish color become brown and seeds hold adequate amounts of moisture and oil contents, and endogenous phytohormone levels. When the seed state is optimized for germination, seeds become non-dormant and ready to germinate upon a signal, which are called after-ripened (AR) seeds. For all the germination tests, non-dormant AR seeds, which now have a germination-competence, are used. In the **Figure 3.1C**, the result has shown that the quadruple mutants *snl1234-1* and *snl1234-2* have a delayed seed germination phenotype compared with the wildtype. In **Figure 3.1E** and **F**, all of double and triple mutants did not show any notable germination phenotype. Differences during germination and early seedling establishment process between wildtype and *snl1234* mutant seeds were depicted in **Figure 3.1D**. *snl1234-1* mutant allele was shown to have a little stronger

phenotype than *snl1234-2*, so we used *snl1234-1* allele for all of followed experiments. As shown in **Figure 3.1E and F**, wildtype and each combination of double and triple mutant seeds were completely germinated within 4 days after sowing. However, *snl1234-1* and *snl1234-2* quadruple mutant seeds were not fully germinated until 10 days after sowing. These results suggest that SNL 1, 2, 3, and 4 have a functional redundancy in seed germination process. In addition, transgenic *snl1234-1* containing the *SNL3pro::SNL3:HA* construct showed a restored germination ability at wildtype level (**Figure 3.1C**). Therefore, it is clear that *Arabidopsis* SNLs have functional redundancy in seed germination processes.

To assess the roles of SNLs in seed germination in more details, we generated *SNLs pro::GUS* transgenic plants which have a SNL1, 2, 3, or 4 transcriptional fusion with β-glucuronidase (GUS) construct. The spatial expression patterns of each SNL protein were examined by histochemical GUS staining (**Figure 3.2A**). Since the seed germination is affected by both the maternal and zygotic-originated tissues, we examined the SNLs expression in distinguished tissues, using seeds and mature embryos of F1 progeny, which were obtained by reciprocal cross between Col and each *SNLs pro::GUS*. In maternal tissue seed coat, expression of SNL2pro::GUS and SNL3pro::GUS were visualized but those of SNL1pro::GUS and SNL4pro::GUS were not shown. For zygotic-originated tissues embryo and endosperm, all of SNLs pro::GUS were clearly observed in embryos under both stages of developmental and mature embryos but none of them were visible in endosperm of developing seed. This indicates that SNL1, 2, 3, and 4 expressions are overlapped in embryo from developmental stage to after progression of mature seed (**Figure 3.2A**). Previously SNLs were reported that they function as a

platform of HDAC-associated complex. Additionally, it was demonstrated that SNL1 colocalizes with HDA19 in the nucleus of tobacco leaf cell by a ratio metric BiFC Assay (Perrella et al., 2013). To confirm the expression patterns of SNLs in the nucleus at embryonic stage, the subcellular localization of SNL3 protein was depicted. The subcellular localization of SNL3 protein was demonstrated by transgenic plants containing the construct that have its own promoter and SNL3 protein fused to green fluorescence protein (GFP; *SNL3pro::SNL3:GFP*) (**Figure 3.2B**). GFP signals were detected in the nucleus of *SNL3pro::SNL3:GFP* transgenic embryo. *snl234-1* is used as a negative control and no GFP signal was detected. Altogether, it is suggested that SNLs may function redundantly in embryo, within the nucleus, and be involved in the regulation of seed germination.

We next investigated whether the *snl1234* mutant embryo can grow without defects and to reach the typical seedling stage (**Figure 3.3**). Embryos were separated from the seed coats and grown. Embryo-germination tests were performed by counting the emerged green cotyledons at 7days after sowing on half MS media with sucrose (**Figure 3.3A**). In comparison with seed germination test, embryo germination test was depicted in **Figure 3.3A** and the percentages of green cotyledons among scattered embryos or seeds were represented in graph as in **Figure 3.3B**. While more than 90% wildtype embryos were developed into seedlings with green cotyledons, less than 80% *snl1234* mutant embryos showed green cotyledons (**Figure 3.3B**). On the other hand, more than 80% wildtype seeds were germinated and expanded green cotyledons, whereas less than 50% *snl1234* mutant seeds showed green cotyledons. This result suggests that seed germination is controlled more acutely than embryos germination and the delayed germination

by *snl1234* is more prominent with the seed coat. This result supports that SNLs are involved in seed germination process, which represents the development of embryo to seedling, better accomplished in the presence of the seed coats. Interestingly, sizes of seeds and embryos are slightly reduced in *snl1234* compared with wildtype and the small seedling phenotype was continuously observed after germination (**Figure 3.3C**).

In addition, it is reported that the regulation of embryo-specific genes, such as *LEC1*, *LEC2*, and *FUS3*, are not only critical for seed developmental stages but also in seedling developmental stages (Dean Rider et al, 2003; To et al, 2006). Ectopic expression of embryo-specific genes during embryo development and de-repression of those gene expressions in subsequent stages cause the failure of seedling growth after germination. Moreover, it is reported that HDACs, candidates of SNL-functional partner, might be involved in the repression embryo-specific genes during germination (Tanaka et al., 2008; Rider et al., 2003; Zhou et al., 2004; Tai et al., 2005). To address such embryo-specific genes' expression level in *snl1234*, quantitative realtime (qRT)-PCR was performed with gene specific primer sets listed in **Table 3.3**. The mRNA levels of each embryo-specific genes were monitored from dry seeds, through 4 days of stratification until 7 days on MS under constant light (**Figure 3.4**). As the result of qRT-PCR analyses, there was neither relevance nor consistency between altered expression patterns of embryo-specific genes and the phenotype of *snl1234*. Expressions of *LEC1* and *FUS* in *snl1234* were more suppressed than those in wildtype. Although expression patterns of *LEC2*, *CRC*, and *RAB18* were altered in *snl1234* at specific time points during stratification until the first day after light treatment but they were not

constantly maintained. Rather, delayed germination of *snl1234* was not caused by alteration of embryo-specific gene expression and expression alteration of embryonic genes may be the result of the delayed germination in *snl1234*.

3.4.2 SNL1, SNL2, SNL3, and SNL4 Are Independent to GA Metabolism, Bioactive GA Levels, or Expression of GA Signaling Mediators

To determine whether the delayed germination phenotype of *snl1234* is caused by the deficiency or the insensitivity of endogenous GA hormone, we performed germination tests with exogenous GA treatment on the media. First, 100μM of GA was added in the media (**Figure 3.5A**). As a result, germination in wildtype was little shifted to earlier dates after sowing. This means 100μM of exogenous GA was sufficient to induce early germination and show seed germination rate in GA-dependent manner. However, *snl1234* seeds still showed delayed germination phenotypes as in the germination test without GA. Therefore, the delayed germination phenotype of *snl1234* was not due to lack of endogenous GA. Moreover, GA signaling is also important in seed germination and it is known that the disruption of GA signaling pathway leads to the failure of seed germination. To test if SNLs are involved in the GA signaling, we determined the GA sensitivity in *snl1234* mutant. GA sensitivity was represented by the germination rate on media containing paclobutrazol (PAC), GA biosynthesis inhibitor, with various GA concentrations indicated in the **Figure 3.5B**. As in the GA-only supplied media, *snl1234* mutant seeds were germinated less efficiently than wildtype in the

condition of 100μM GA supplemented with 80μM of PAC. In addition, the germination rates of *snl1234* mutant seeds were elevated in accordance with the increase of GA concentration. This result suggests that the delayed germination of *snl1234* quadruple mutants is not associated with GA-sensitivity.

Additionally, we measured the expression levels of GA metabolic and signaling genes in different stages of germination process using dried or stratified seeds and seeds under constant light treatment for seven days. First, the expression patterns of *GA20ox2*, *GA3ox1*, and *GA3ox2* are measured as GA biosynthetic genes. GA20ox2 catalyzes a multi-step oxidation to produce immediate precursors of active GA and, GA3ox1 and GA3ox2 convert these GA precursors to their bioactive forms in plant. As in **Figure 3.5C**, the expressional peak of *GA3ox1* in *snl* quadruple mutant is shifted to earlier day, at day 1 after stratification instead of day 3 in wildtype. Without any shifts in time, the peak of *GA3ox2* mRNA level of was higher in *snl1234* than in wildtype during germination. In addition, although *GA20ox2* expression was low during stratification in the mutant, it became almost the same as that in wildtype during germination. On the other hand, to consider negative regulators of GA signaling into our account, we measured mRNA expression patterns of genes that encode DELLA proteins such as *REPRESSOR OF GA1 (RGA1)*, *RGA-LIKE1 (RGL1)* and *RGL2*. Expression levels of the repressive genes were increased both in wildtype and *snl1234* mutant during germination to repress GA responses and integration of GA signaling during the process. However, after germination, expressions of these DELLA protein-encoding genes were decreased. This is consistent in both wildtype and *snl1234* mutant. Overall, these results suggest that GA metabolism and signaling pathway are not associated with

the delayed germination phenotype of *snl1234*. In other words, GA-mediated germination pathway is not affected by mutations of *SNLs*.

3.4.3 SNL1, SNL2, SNL3 and SNL4 Do Not Affect the Endogenous ABA Level

ABA is one of the key hormones in sustaining seed dormancy. In order for seeds to germinate, ABA level must be down-regulated. Thus, biological regulations of ABA synthesis and signaling are also critical in seed germination mechanism (Schroeder et al., 2001; Cutler et al., 2010; Weiner et al., 2010). It is known that the mutations of *SNL1* and *SNL2* cause alteration of ABA contents in dormant seeds (Wang et al., 2013). To test whether endogenous ABA contents are altered by SNL-mutations during seed germination, ABA concentration levels in wildtype and the quadruple mutants were measured in non-dormant dried or imbibed seeds (**Figure 3.6A**). As known in wildtype that endogenous ABA level is decreased by imbibition, the ABA level in *snl1234* was also decreased by imbibition but maintained at the same level of wildtype in both dried and imbibed samples. Unlike in dormant seeds, endogenous ABA contents were not affected by *SNL* mutation, even in the condition where four members of *SNL* subfamily members were disrupted. This result suggests that the delayed germination phenotype of *snl1234* was not caused by endogenous ABA contents. To add more, mRNA expression levels of ABA biosynthetic and metabolic genes were measured during germination (**Figure 3.6B**). *9-cis-EPOXYCAROTENOID DIOXYGENASE6 (NCED6)* and *NCED9* are known to be seed-specifically expressed and represent ABA

biosynthetic genes during seed development. During germination, from dried seeds until the endosperms are ruptured, the transcription level of *NCED6*, encoding the rate-limiting enzyme in ABA biosynthesis, fluctuates and hits a peak at the end of stratification in wildtype seeds. In *snl1234* mutant seeds, varied pattern is similar but with smaller amplitudes and likely a slowed rate of decrease until the germination is completed. The mRNA expression level of *NCED9* also undulates during germination in both wildtype and *snl1234* mutant seeds. The re-repression rate of *NCED9* expression in *snl1234* during germination, after the expressional peak at day1, seemed to be little slowed down than in wildtype. Furthermore, the transcription level of *CYP707A2*, which encodes ABA 8'-hydroxylase an enzyme that degrades ABA, was also measured during germination. Its expression in wildtype seeds peaks at the end of stratification while that in the mutant seeds is highest on the day 1 after stratification. To recapitulate the results, up-and-down patterns of both ABA biosynthetic and degrading genes exist in both wildtype and *snl1234* and it is obvious that the expressional peak of the critical ABA biosynthetic gene, *NCED6*, is concurrently overlapped with that of ABA degrading gene, *CYP707A2*. However, it is interesting that the expressional peak in both mechanism was delayed and the fluctuation of transcriptional level is dulled in *snl1234* than in wildtype. Altogether, this suggest a possibility that, although total endogenous ABA level is sustained in *snl1234* mutant seeds compared with that in wildtype, ABA-related metabolism is somewhat affected in the *snl* quadruple mutant during germination. It suggests that the rate of ABA metabolism and correspondingly the ABA sensitivity in *snl1234* mutant is altered.

3.4.4 SNL1, SNL2, SNL3 and SNL4 Play a Role in ABA Signaling During Germination

To verify and confirm the roles of SNLs in ABA-mediated signaling pathway, we tried to evaluate ABA sensitivity of *snl1234* seeds. Wildtype and *snl1234* seeds were sown on half MS media containing 0, 0.5, 1, 3, or 8 μM of ABA and their germination efficiency was measured. At 48 hours after sowing, wildtype seeds were almost completely germinated on the media containing up to 3 μM of ABA. In *snl1234*, however, the percentage of seed germination was notably decreased on the media containing only 0.5 μM of ABA and the germination was practically inhibited by 1 μM of exogenous ABA (**Figure 3.7A**). To observe the germination dynamics, we measured germination rates at more specific time points under the indicated ABA conditions (**Figure 3.7B**). Under low ABA concentration conditions, which do not affect germination of wildtype seeds, *snl1234* showed a delayed germination pattern. 0.5 μM ABA in the media was sufficient to delay the germination rate of *snl1234* mutant seeds. Therefore, we could infer that *snl* quadruple mutant is hypersensitive to ABA. Obviously, more severe phenotype in *snl1234* with more reduced rate and efficiency in seed germination was exhibited according to the increased ABA treatments. In addition, at 78 hours after sowing, wildtype seeds were fully germinated even with exogenous ABA treatments while *snl1234* seeds were germinated less than 70%, 50%, or 25% on 1 μM, 3 μM, or 8 μM ABA-containing media, respectively. These results support that *snl1234* is hypersensitive to ABA and SNLs are involved in the ABA-mediated signaling pathway during seed germination.

To identify how SNLs are associated in ABA signaling mechanism, we primarily examined mRNA expression of the central ABA signaling component genes, *ABI3, ABI4,* and *ABI5,* during seed germination and seedling establishment. Transcriptional levels of *ABI* genes were determined by qRT-PCR in wildtype and *snl1234* seeds during germination in the same condition as other previous observations were made (**Figure 3.8**). In wildtype, mRNA expression pattern of *ABI3* and *ABI4* dramatically increase after stratification. This pattern is also kept in *snl1234*. Interestingly, on the third day after stratification when all the *ABI* genes become completely re-repressed in wildtype, the expression is still remained and the extent of the decrease is less steep in the mutant throughout the germination process. Although the overall expression pattern of *ABI5* during germination process is little different from other *ABI* gene patterns, uncontrolled expression on day3 in *snl* mutants is also consistent. It is remarkable that induced *ABI* expression levels were tardily decreased in *snl1234* than in wildtype. Representing as ABI-downstream genes, mRNA expression levels of *RD29B* and *SOM*, were also elevated in *snl1234*. Moreover, un-repressed expression of these genes on day3 was observed as well. Again, these results show that ABA hypersensitivity mediated by SNL mutations were most significant on day3 during germination or early seedling establishment.

As shown in Figure 1, *snl* single, double, or triple mutants did not have defects in seed germination and it was implied as that SNLs may have a functional redundancy during seed germination. To confirm whether the redundancy is elaborated within the ABA-signaling, the expression patterns of *ABI* genes were observed from the seeds of various combinations of *snl* double mutants. The

mRNA levels of *ABI* genes from germinating seeds at day2 after the stratification were shown in **Figure 3.9**. Consistent with the germination phenotypes, *ABI* expressions were not induced in any of the *snl* double mutant seeds compared to the *snl* quadruple mutants. Moreover, it was reported that, for seed dormancy, SNL1 and SNL2 function in redundant manner in dormant seeds through the regulation of ABA-ethylene antagonism. These results support the idea that all of four SNLs, SNL 1, 2, 3, and 4, have a functional redundancy in the ABA-signaling regulation mechanism during seed germination and early seedling establishment. Furthermore, this results approve that ABA-mediated regulation mechanism for seed dormancy and seed germination is through separate and distinctive pathways.

To ensure the function of SNLs within ABA signaling pathway, we generated quintuple mutant *snl1234-1 abi3*. Mutation in *ABI3* exhibits aberrant seed development and *abi3* seeds fail to desiccate during seed maturation, resulting in loss of viability upon matured dry seed (Huang et al., 2008). Thus, the germination test involving *abi3* mutant seeds were implemented using FH seeds of Col, *abi3-16*, *snl1234-1* and *snl1234-1 abi3-16* (**Figure 3.10**). Because FH seeds have elevated dormancy, seeds were no to germinate even if the proper condition for germination is given. To partially break the seed dormancy, seeds were stratified for a longer period of time than usual before performing the germination test. Due to the raised dormancy, germination in wildtype was reduced to less than 40 % and that in *snl1234-1* seeds were also reduced but still with the delayed phenotype. As expected, in *abi3-6,* where the dormancy was not acquired at all, showed early germination phenotype. Interestingly, however, *snl1234-1 abi3-16* seeds germinated in equal rate with *abi3-16* single mutant seeds. This result

indicates that *ABI3* mutation masks the effects of *snl1234* on seed germination and supports that SNLs function in seed germination through the ABI genes. It also suggests that *ABI* genes may be the targets of SNL complex during seed germination and early seed establishment.

3.4.5 SNL1, SNL2, SNL3, and SNL4 Affect *ABI* Transcription via Regulation of H3Ac Level

In yeast and mammals, Sin3 is involved in the transcriptional inactivation by interacting with HDACs. (Lai et al., 2001; Grzenda et al., 2009) It proposes that SNLs may affect the transcription of *ABI* genes during germination via regulation of histone acetylation. To evaluate whether the H3Ac levels of *ABI* genes were affected by *snl1234*, we performed chromatin immunoprecipitation (ChIP) assay using specific antibody against acetylated histone H3 (H3Ac) (**Figure 3.11**). Seeds were collected when differential transcript levels were observed between wildtype and *snl1234* mutant in the previous experiments, at 3d during germination. The relative H3Ac levels were determined by quantitative PCR (qPCR) amplifying various regions of *ABI* genes depicted in **Figure 3.11A**. Increased H3Ac levels in *snl1234* relative to wildtype were detected at promoter regions of all *ABI* genes, *ABI3*, *ABI4*, and *ABI5* (**Figure 3.11B and C**). In particular, a strong ABA-signaling factor, *ABI3*, also has an increased acetylation levels on exonic regions as well as the promoter region. This result approves that SNLs repress *ABI* genes by mediating the regulation of histone deacetylation levels.

To authenticate whether *ABI* genes are directly targeted by SNLs, we

performed a binding assay using functionally expressed *proSNL3::SNL3:HA* in *snl1234-1* transgenic line (**Figure 3.12**). Enrichment of SNL3:HA was observed on the 5'UTR and promoter regions of *ABI3* and *ABI5*. Concisely, SNL3 is strongly bound on the 5'UTR and a promoter regions of the *ABI* genes to directly repress their transcription. It is coherent with the fact that the 5'UTR region of *ABI3* is essential for the negative regulation of its transcription expression (Ryu et al, 2014; Zhou et al, 2013). Moreover, similarly with the H3Ac enrichment levels, enhanced SNL3 enrichment was also observed on the exonic regions of *ABI3* locus. These results support that *ABI* transcriptional regulation is mediated by direct involvement of SNLs through modification of histone acetylation mechanism. Alternatively, association of SNL3 was not detected within *ABI4* locus despite of that H3 hyperacetylation was retained on *ABI4* promoter region in *snl1234*. In other words, direct interaction of SNL3 is executed only on *ABI3* and *ABI5* but not on *ABI4* chromatin. This result implies that, although there exists a high redundancy among the SNL proteins, there also exists a target specificity among SNLs. Further study is required to clarify the specificity of each SNL for their target in ABA signaling mechanism during germination.

Then, it was indispensable to find a SNL-complex components since SNLs do not have a DNA-binding domain themselves and it is notorious that metazoan Sin3 forms a complex with Class I HDACs to regulate H3Ac level of its target. Thus, to find an ally, among various HDACs, that is correlated with the germination phenotype of *snl1234*, ChIP analyses were performed (**Figure 3.13**). HDA19 or HDA9 enrichment on *ABI3* loci was displayed using *HDA19pro::HDA19:FLAG* or *HDA9pro:HDA9:HA* transgenic line and a FLAG- or HA-antibody, respectively.

Recently, it was reported that HDA19 form a complex with brassinosteroid (BR)-activated BES1 (BRASSINAZOLE-RESISTANT 2) and WD40 domain-containing TPL (TOPLESS) to mediate an inhibitory action of BR-signaling to repress transcription of *ABI3* and *ABI5* (Ryu et al., 2014). Moreover, another latest study demonstrated that HDA9 negatively influences germination and represses seedling traits in dry seeds (Zanten et al., 2014). Unfortunately, either HDA19 or HDA9, the best characterized HDACs in *Arabidopsis* associated with ABA signaling and germination, does not show prominent enrichments on all examined regions of *ABI3* locus, representing that there is no direct interaction between the tested HDACs and *ABI* genes for SNL-mediated germination and early seedling establishment processes.

With an inquisitiveness to identify a component of SNL-HDAC complex, various candidate *hdac* mutants were examined for *ABI* mRNA expression levels (**Figure 3.14**). Different classes of HDAC mutants were tested. Among the classified HDACs, *had6, hda9* and *hda19* as a Class I RPD3-type, *hda14* as an unclassified of RPD3-type, and *srt2-1* and *srt2-2* as SIR2-type HDAC were used in the experiment using the appropriate ecotype for control. Within abiotic stress or ABA response mechanism, a model was proposed in which HDA6 and HDA19 redundantly function through a binding of ERF4 and ERF7 to their target genes, possibly recruited by AtSin3 (Chen et al., 2010). However, expression of ABA signaling genes are not altered in *hda6* or *hda19* mutants implicating that HDA6 or HDA19 are not recruited by SNLs during seed germination for ABA signaling. Interestingly, it was observed that, in *hda14* mutant seeds, the expression all of *ABI* genes was increased compared with wildtype. Currently, HDA14 is unclassified

within RPD3-type subfamilies in *Arabidopsis*, and only one report was made on *HDA14* (Tran et al., 2012). Therefore, more profound studies are required on HDA14, as a candidate for SNL-partner, to elucidate the distinctive acetylation modulation mechanism in ABA signaling during seed germination and early seedling establishment processes.

3.5 Figures and Tables

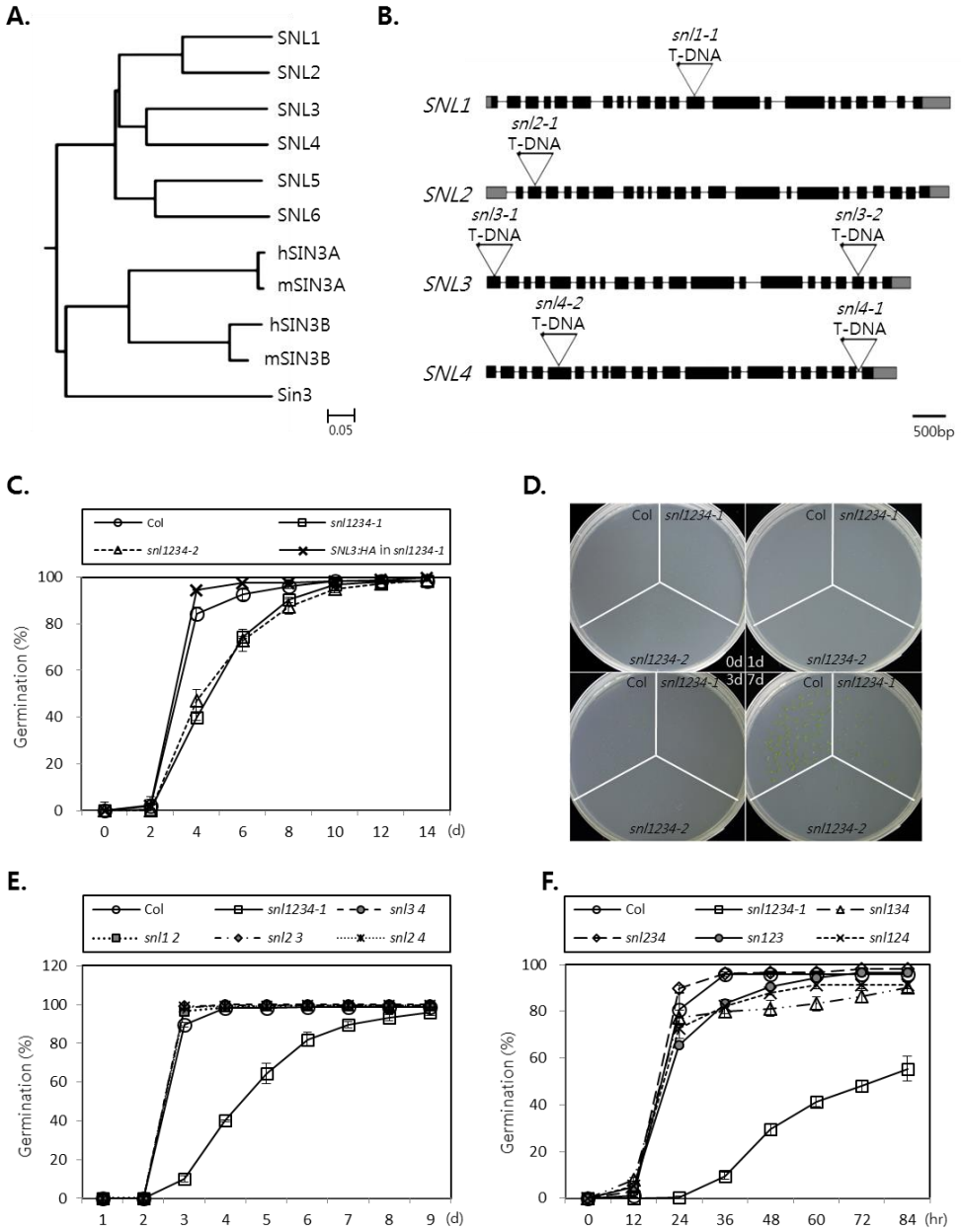

Figure 3.1 Delayed seed germination phenotype of *snl1234* quadruple mutant

(A) Phylogenic analysis of yeast Sin3 and its homolog proteins of Arabidopsis, human, and mouse. Analysis of the six SNL peptide sequences was performed by

ClustalW (Thompson et al., 1994; http://align.genome.jp/). **(B)** Schematic representation of the *SNL1*, *2*, *3*, and *4* gene structures, indicating the positions of the T-DNA insertions. Exons are represented as black boxes, UTR regions as gray boxes and introns as black lines. **(C)** Seed germination of *snl1234* quadruple mutants and a complementation line generated by an introduction of *SNL3pro::SNL3:HA* construct into *snl1234-1*. Germination was scored by radicle protrusion at the indicated time after sowing on half MS media without sucrose. Seed germination assays were performed in three replicates using at least 100 seeds for each genotype and the average value is graphed with ±Standard error (SE). **(D)** Photographs of Col, *snl1234-1*, and *snl12334-2* at 0, 1, 3, and 7 days after sowing on half MS medium without sucrose. **(E and F)** Seed germination of different combinations of double or triple mutants of *SNL1*, *2*, *3*, and *4*. Seed germination was scored at the indicated time on half MS media without sucrose. All seed germination assays were performed in three replicates using more than 100 seeds for each genotype and the average value is shown with ±SE.

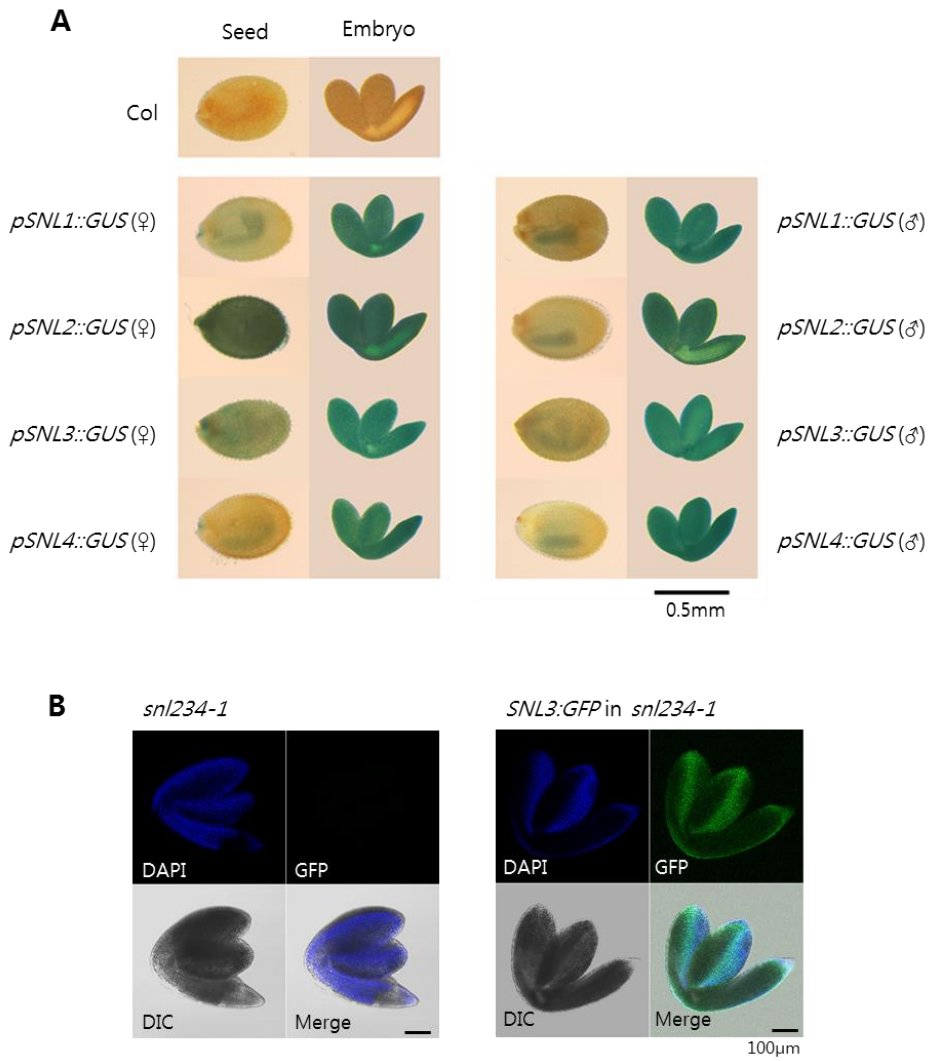

Figure 3.2 Spatial or subcellular expression patterns of the SNL1, 2, 3, and 4 in seeds or embryos

(A) Histochemical GUS staining of F1 whole seeds or embryos. Female (left panel) or male (right panel) plants harboring *SNL1, 2, 3, or 4pro::GUS* constructs was used for reciprocal cross with Col. 7 days after pollination, seeds with seed coats and rescued embryos from mature seeds were used in GUS staining. The bar

represents 0.5mm **(B)** Subcellular localization of SNL3 protein. Transient expression of *SNL3pro::SNL3:GFP* showed localization of SNL3 in the nucleus. Images were obtained by confocal laser scanning microscopy. The bar represents 100µm. Nuclear-specific DAPI staining was also depicted in addition to SNL3:GFP image, using differential interference contrast (DIC) image, the merged image of all three images clearly showed SNL3 localizes in nucleus.

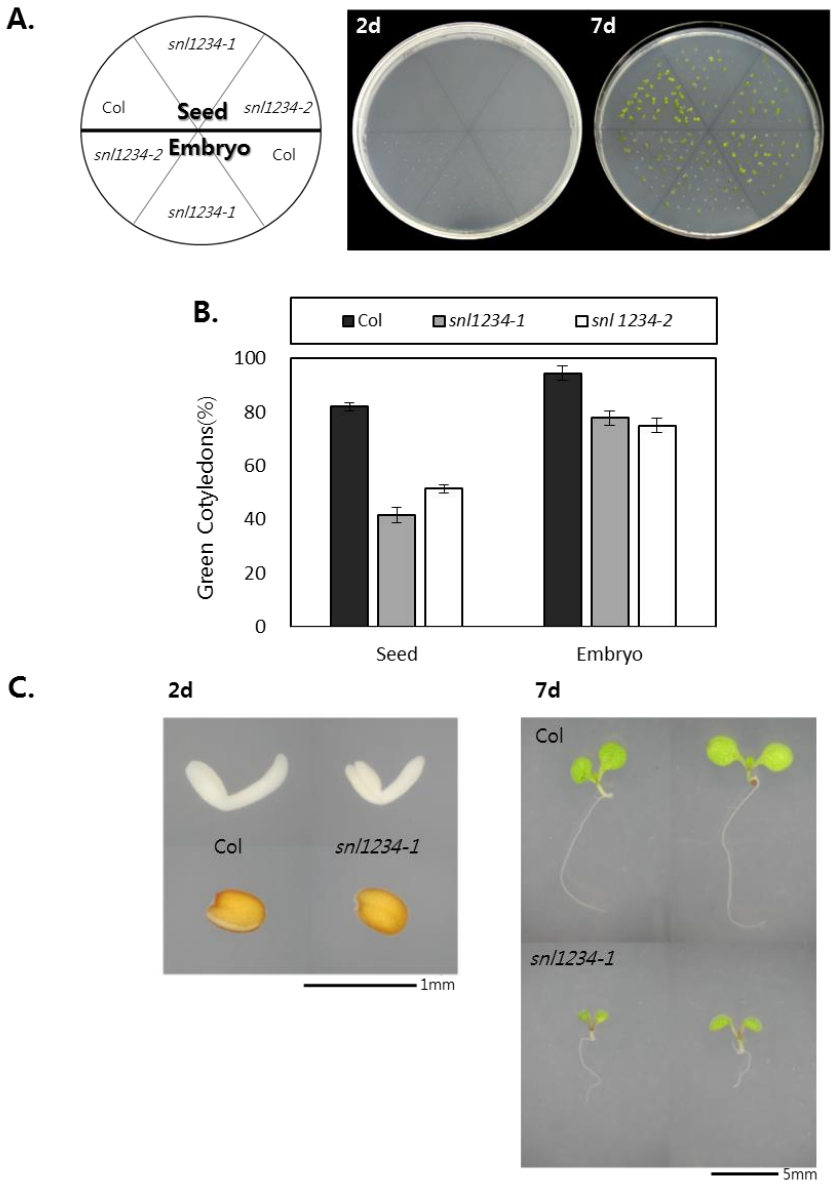

Figure 3.3 Delayed embryo germination of *snl1234* quadruple mutant

(A) Photographs of embryo germination with or without the seed coat at 2 days or 7 days after sowing. Whole seeds and rescued embryos without seed coats from wildtype, *snl1234-1* and *snl1234-2* were germinated and grown on half MS media

without sucrose for 7 days and depicted at the indicated time. **(B)** Embryo germination scored by appearance of green cotyledons at 7 days after sowing. Experiments were performed using embryos with or without seed coats and the scoring was graphed from duplicates of at least 30 embryos or seeds. The average value is shown with ±SE. Embryos were dissected from 20 min of imbibed seeds then sown on half MS medium with 1% sucrose. **(C)** Morphological phenotype of *snl1234* mutant seeds, embryos and seedlings. Seeds and embryos were germinated and grown on half MS media without sucrose for 7 days. Pictures were taken in detail on day 2 or 7 during germination. Left panel illustrates embryos and seeds of wildtype and *snl1234-1* at 2 days. Radicle protrusion is obvious in wildtype seeds. Right panel illustrates seedlings of wildtype and *snl1234-1* at 7 days. Bar represents 1mm (left) or 5mm (right).

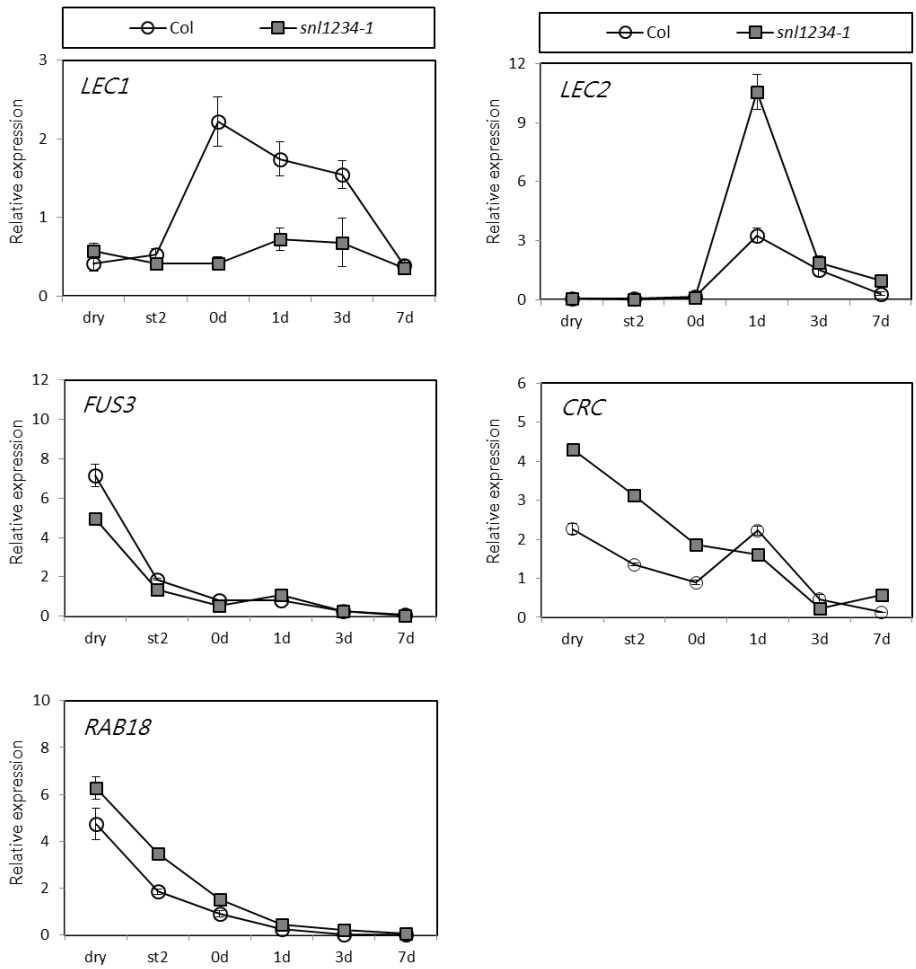

Figure 3.4 Relative transcript levels of embryo-related genes during germination

qRT-PCR analyses of transcript abundances of various embryo-related genes in wildtype and *snl1234* mutant seeds. *LEC1*, *LEC2*, *FUS3*, *CRC*, and *RAB18* were measured. The seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification), and harvested at the indicated time for RNA extraction. In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period

of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents reanscript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were quantified, in relative to UBQ11. Error bars represent ±SE of three independent biological replicates.

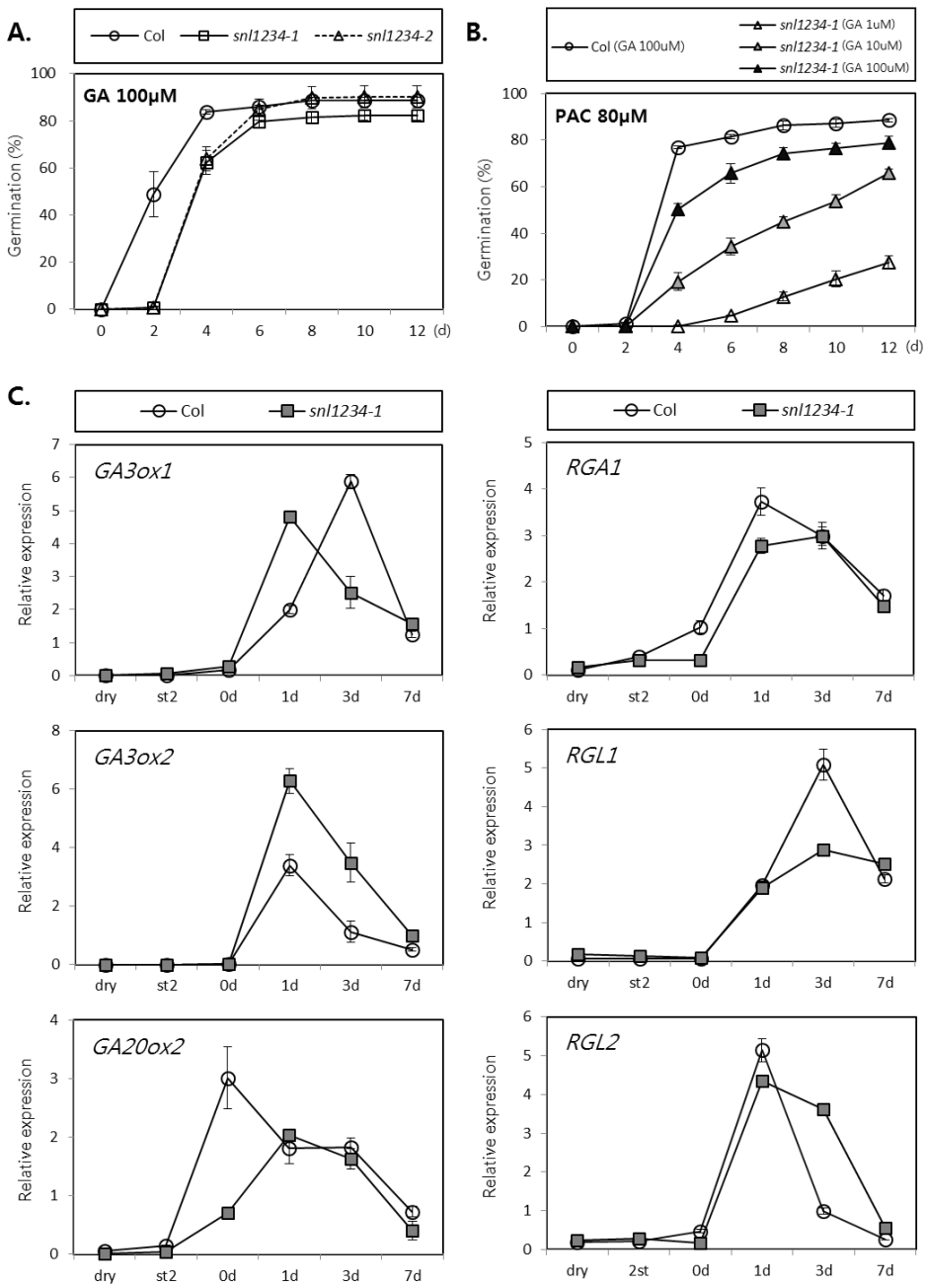

Figure 3.5 Effect of GA in *snl1234* quadruple mutant

(A) Germination of Col, *snl1234-1*, and *snl1234-2* seeds scored upon exogenous

GA. Germination assay was performed on 100μM GA-supplemented media. Each circle, square, or trigngle represents wildtype, *snl1234-1*, or *snl1234-2* mutant seeds, respectively. Germination assays were examined in triplicates with more than 100 seeds each genotype. The average value is shown with ±SE. **(B)** Percentage of germination in wildtype, *snl1234-1*, *snl1234-2* seeds measured in response to GA supplemented with 80μM of PAC. Different concentrations of 1, 10, or 100 μM GA in the media was used to determine GA response in *snl1234*. Open circle represents germination of wildtype seeds under 100 μM GA condition and white, gray, or black triangle is germination of *snl1234-1* mutant seeds on 1, 10, or 100 μM GA supplemented media, respectively. All germination assays were examined in triplicates of more than 100 seeds from each genotype. The average value is shown with ±SE. **(C)** Relative transcript levels of GA metabolic genes (*GA3ox1*, *GA3ox2*, and *GA20ox2*) and GA signaling-related genes (*RGA1*, *RGL1*, and *RGL2*) in Col and *snl1234-1* mutant seeds. Seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification), and then seeds and seedlings were harvested at the indicated time for RNA extraction. On the horizontal axis in the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

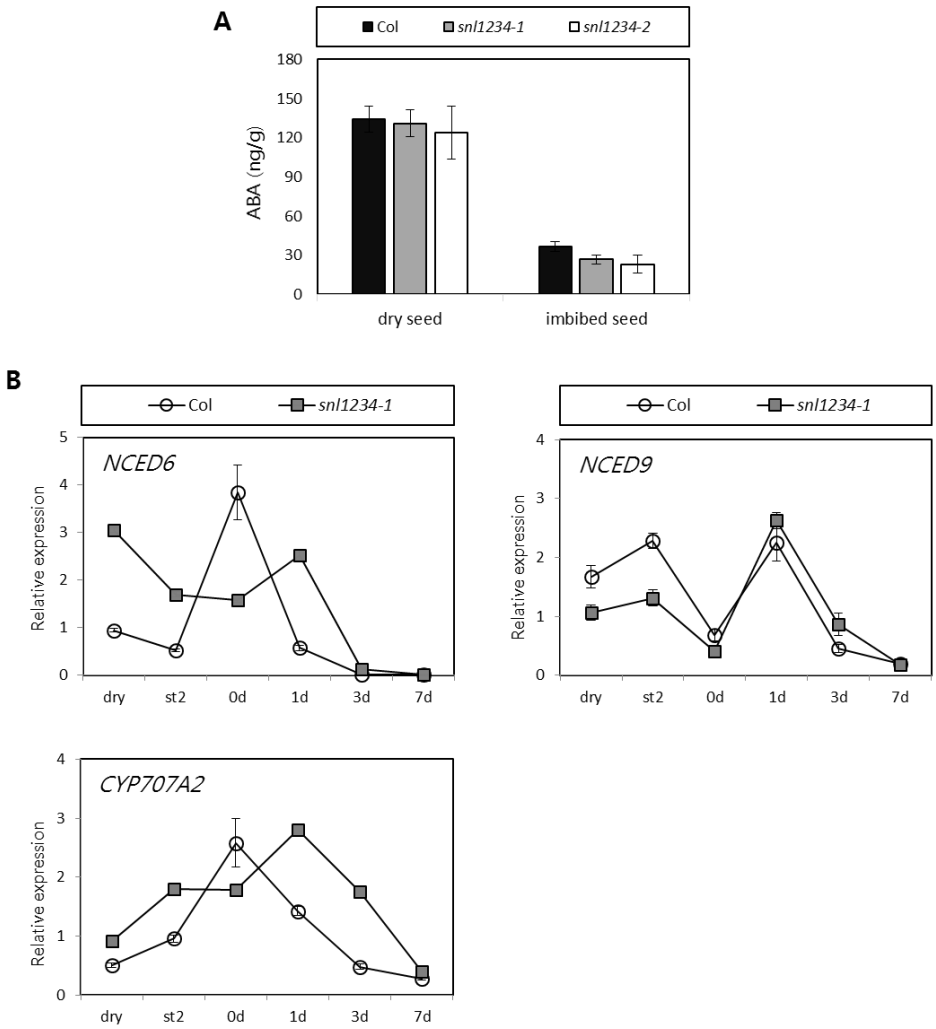

Figure 3.6 Effect of ABA in *snl1234* quadruple mutant

(A) ABA levels in Col, *snl1234-1*, and *snl1234-2* mutant seeds. Endogenous ABA concentration was measured in dried (left) or imbibed (right) seeds from wildtype and *snl1234* mutants. Imbibed seeds were supplied with water and stored at 4℃ for 24h dark condition. Black bars represent ABA concentration in wildtype. Gray or white bars are ABA concentration in *snl1234-1* or *snl1234-2* mutant,

respectively. The data is shown with ±SE of biological duplicates. **(B)** Relative transcript levels of ABA metabolic genes (*NCED6*, *NCED9*, and *CYP707A2*) in Col, *snl1234-1* mutant. All the conditions were the same as in Fig 5C. In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

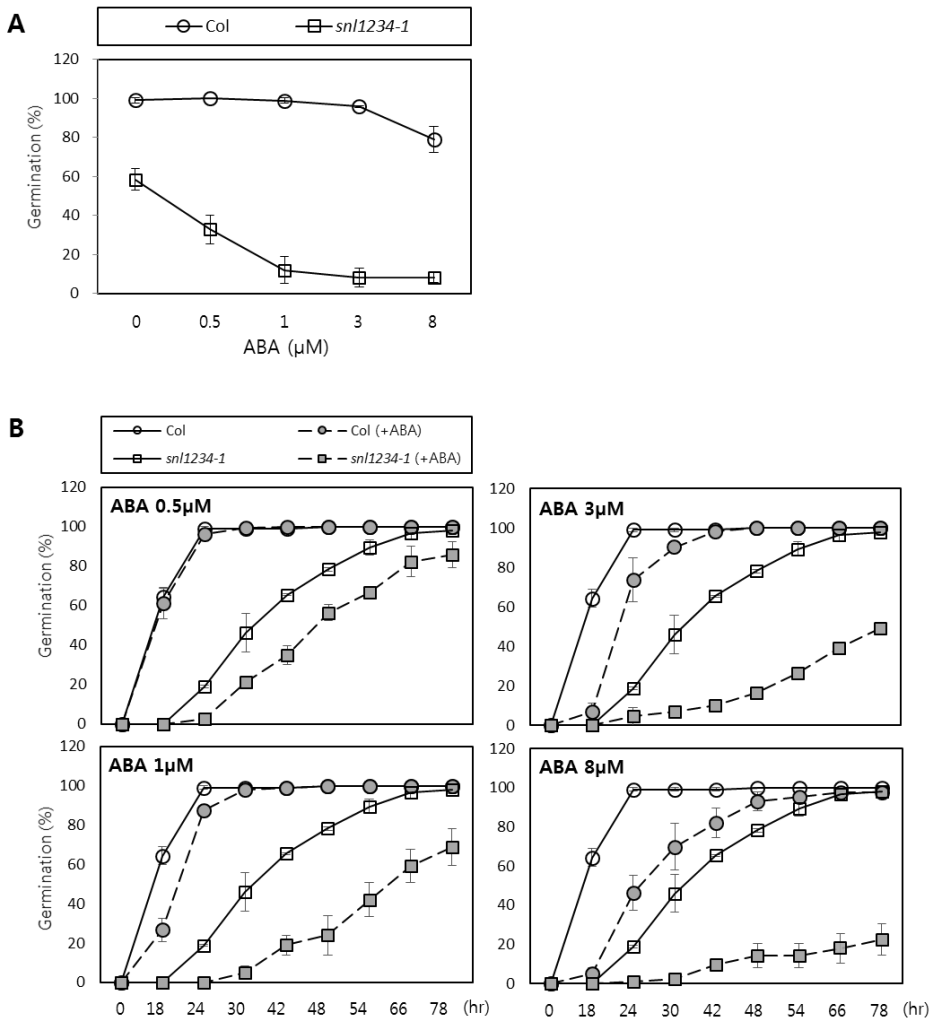

Figure 3.7 ABA hypersensitivity of *snl1234* in seed germination

(A) ABA response of Col and *snl1234-1* seeds. The germination was scored in half MS media containing 0, 0.5, 1, 3, or 8 μM ABA concentrations at 48h after sowing. The circle represents germination of wildtype seeds and the square is that of *snl1234-1* mutant seeds. Germination assay is performed in three replicates using about 100 seeds for each genotype and the average value is shown with ±SE. **(B)** Seed germination of Col and *snl1234-1* mutant in time course under various ABA

concentration conditions. Seed germination was scored at the indicated time under media containing 0, 0.5, 1, 3, or 8 μM ABA. Open circle or square represents germination of wildtype or *snl1234-1* mutant seeds on plain half MS media, respectively, while closed circle or square does that on ABA supplied condition. All germination assays are performed in three replicates using about 100 seeds for each genotype and the average value is shown with ±SE.

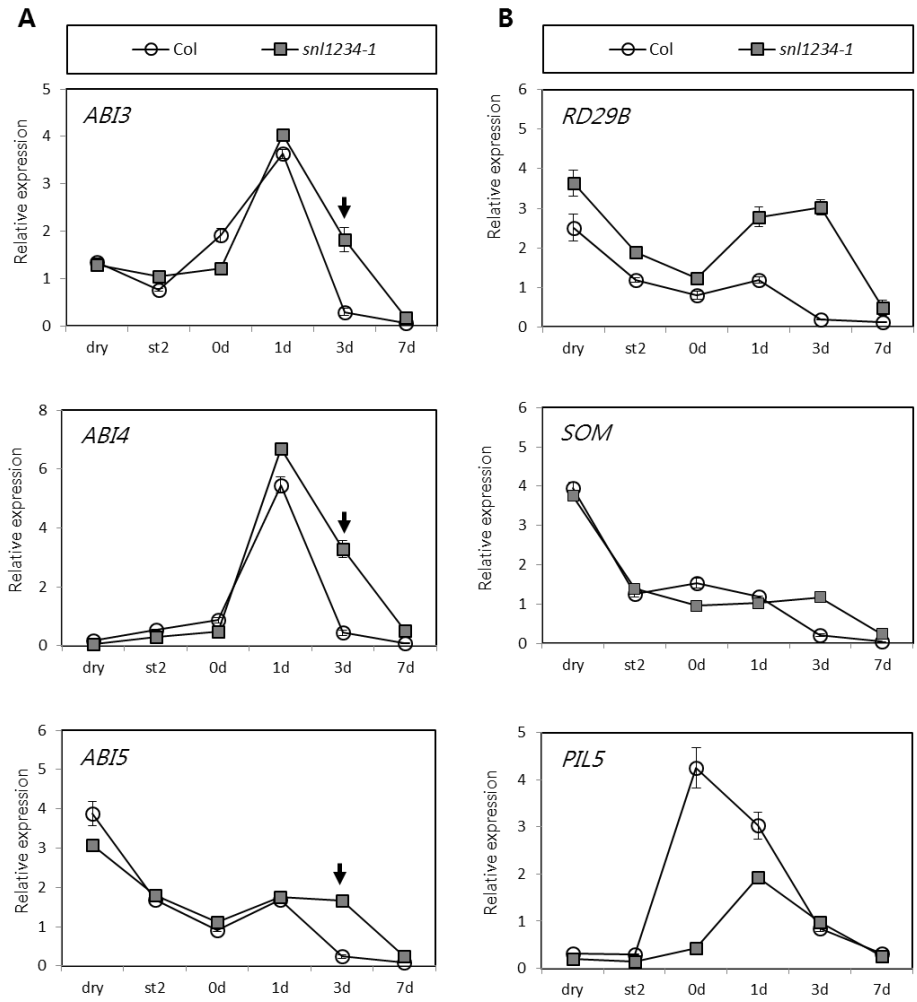

Figure 3.8 Expression of ABA signaling genes in *snl1234* quadruple mutant

(A) Relative expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in Col and *snl1234-1* mutant seeds during germination. Seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification). Then seeds and seedlings were harvested at the indicated time for RNA extraction. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE from three independent biological

replicates. **(B)** RNA expression of *RD29B*, *SOM*, and *PIL5*, that may be altered by *ABI3* during seed germination. All the conditions are the same as (A). In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. *UBQ11* was used as an internal control. Experiments were repeated with three biological sets and the average value was represented with ±SE.

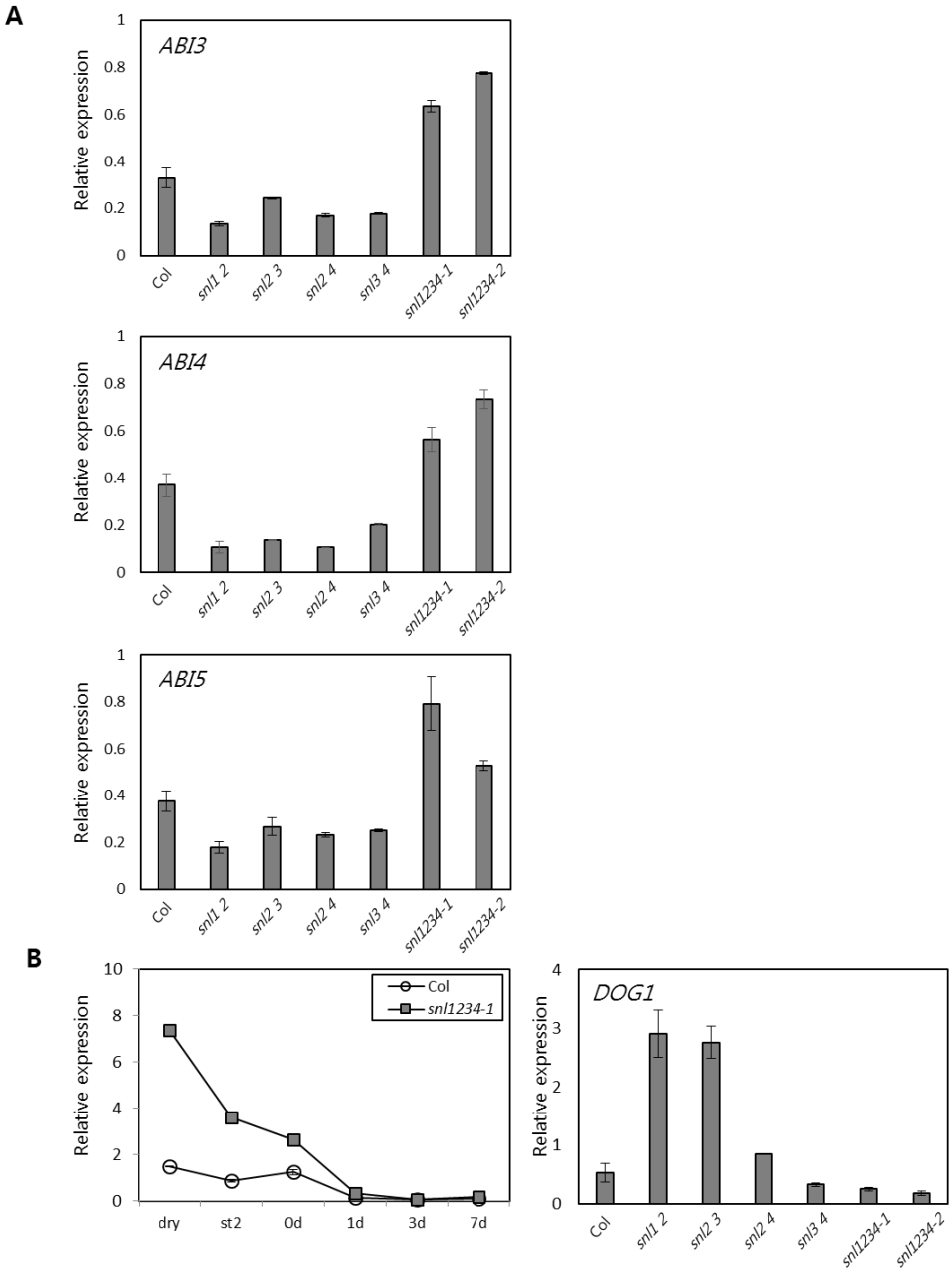

Figure 3.9 Expression of *ABI* genes in *snl* double mutants

(A) Relative expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in Col-0, different combinations of *snl* double and *snl* quadruple mutant seeds. Col, *snl double mutants* (*snl1snl2*, *snl2snl3*, *snl2snl4*, and *snl3snl4*) and *snl* quadruple mutants

(*snl1234-1* and *snl1234-2*) seeds were germinated and grown on half MS media without sucrose for 6 days (2 days under constant light condition after 4 days of stratification). Then germinated seeds were harvested for RNA extraction. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates. **(B)** Relative expression levels of DOG1 in wildtype and *snl1234* mutant seeds was measured during 11 days of germination (left). Relative mRNA of *DOG1* in Col and various *snl* double and *snl1234* quadruple mutant seeds at day 2 under constant light condition after stratification (right). All the conditions were the same as Fig 8A and 9A, respectively. The circle represents transcript levels in wildtype and the dark square does those in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of two independent biological replicates.

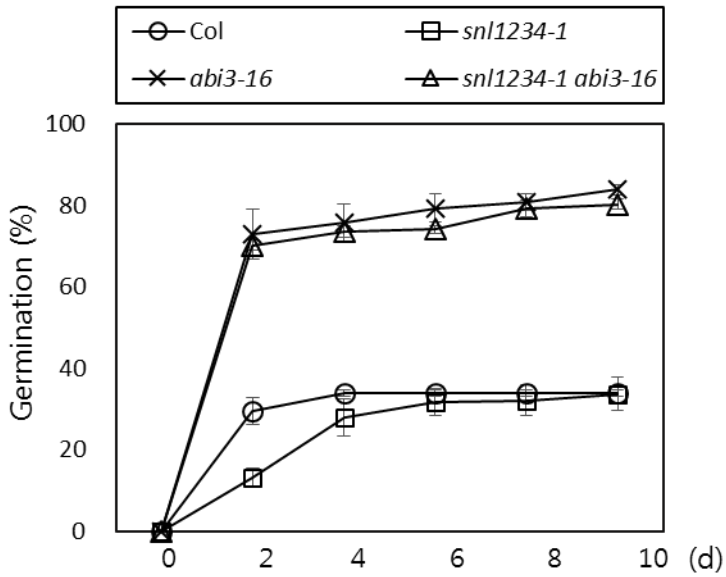

Figure 3.10 Suppression of the delayed germination phenotype of *snl1234* by the *abi3-16* mutation

Seed germination of Col, *abi3-16*, *snl1234-1*, and *snl1234-1 abi3-16* quintuple mutant. Seeds were freshly harvested and stratified for 7 days to break the seed dormancy. Dormancy-released seeds were used for germination assay on half MS medium without sucrose. Germination were scored every 2 days for 10 days. Opened circles represent wildtype seeds; square do *snl1234-1*; Xs do *abi3-16*; and triangles do represents *snl1234-2* mutant seeds. All germination assays were performed in triplicates using at least 100 seeds for each genotype and the average value is shown with ±SE.

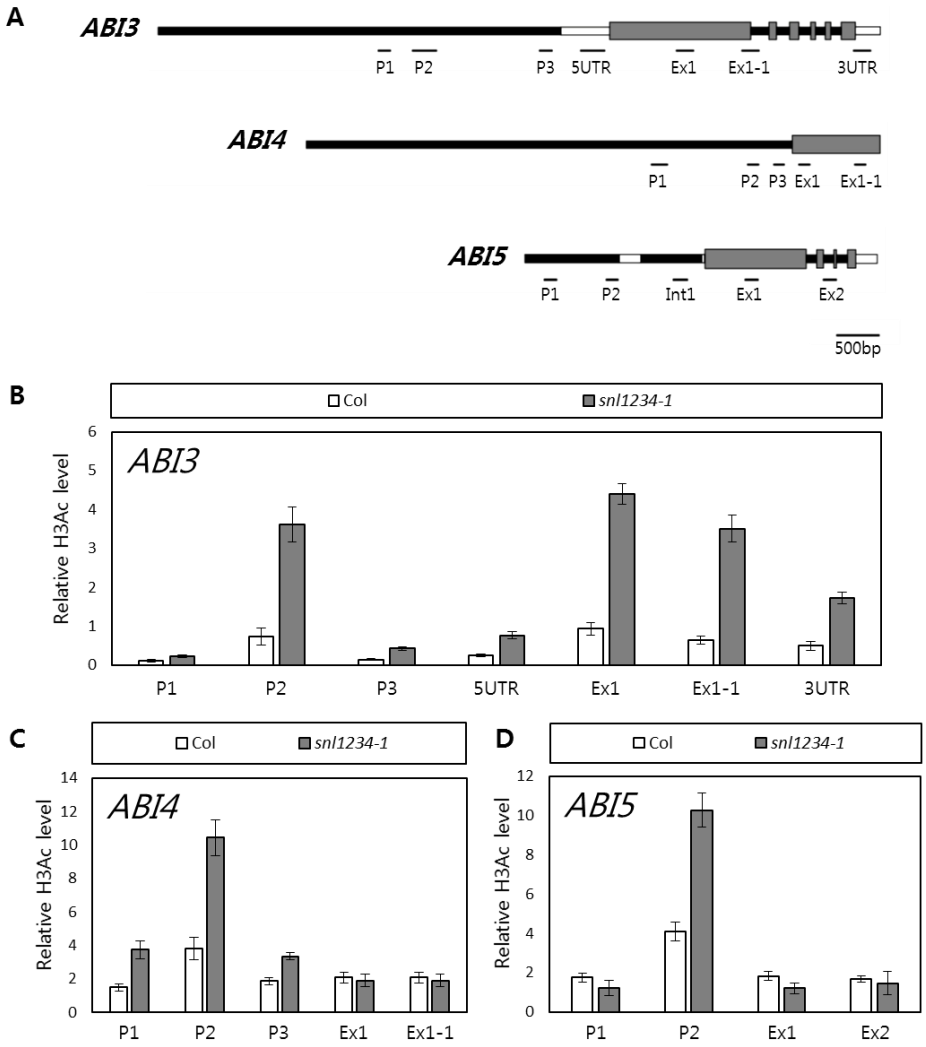

Figure 3.11 Increased Acetylation of histone 3 at *ABI3, 4,* and *5* locus by *snl1234* mutations

(A) Schematic representation of *ABI3*, *ABI4*, and *ABI5* locus showing regions amplified by the primers used for ChIP analysis. White boxes indicate 5' and 3' UTRs. Gray boxes indicate exons, black boxes indicate introns and intergenic regions. ChIP-qPCR anslyses of *ABI3* **(B)**, *ABI4* **(C)**, and *ABI5* **(D)** chromatin with

an antibody against H3Ac. Seeds of each genotype were germinated and grown under constant light for 3 days after stratification, and then harvested for ChIP assay. White bar represents H3Ac levels on each gene locus in wildtype and gray bar is that in *snl1234* mutant. The values of immunoprecipitated chromatins were normalized with that of inputs then by the internal control *UBQ11*. 'Input' indicates chromatin before immunoprecipitation. Shown are the means ±SE of three (B) and two (C and D) biological replicates.

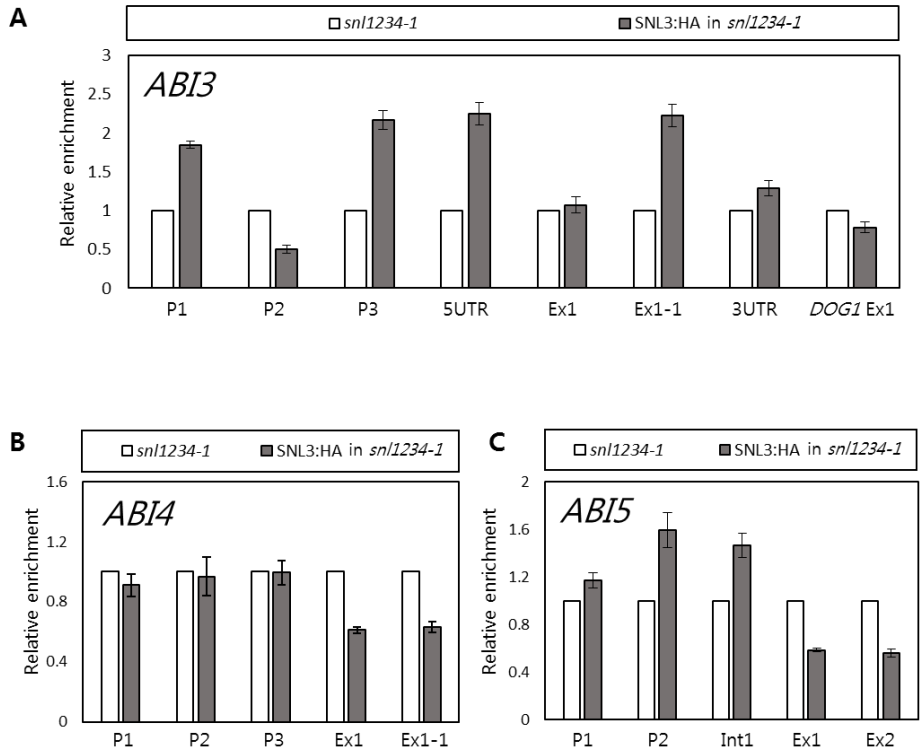

Figure 3.12 Direct association of SNL3 with *ABI3* chromatin

ChIP-qPCR analyses of relative SNL3:HA enrichment on the *ABI* genes using an anti-HA antibody. *snl1234-1* and SNL3:HA in *snl1234-1* seeds were germinated under constant light for 2 days after stratification and harvested for ChIP assay. Amplified regions of *ABI3* **(A)**, *ABI4* **(B)**, and *ABI5* **(C)** locus for the ChIP-qPCR analysis are shown in Figure 11A. Dark bar represents relative enrichment levels of SNL3 on each gene locus from SNL3:HA *snl1234* transgenic seeds. White bar stands for *snl1234-1*. The enrichment levels from Col were set to 1 after normalization by input and the internal control *UBQ11*. The ChIP-qPCR value of *DOG1* was demonstrated as a negative controls. Shown are the means ±SE of three (A) and two (B and C) biological replicates.

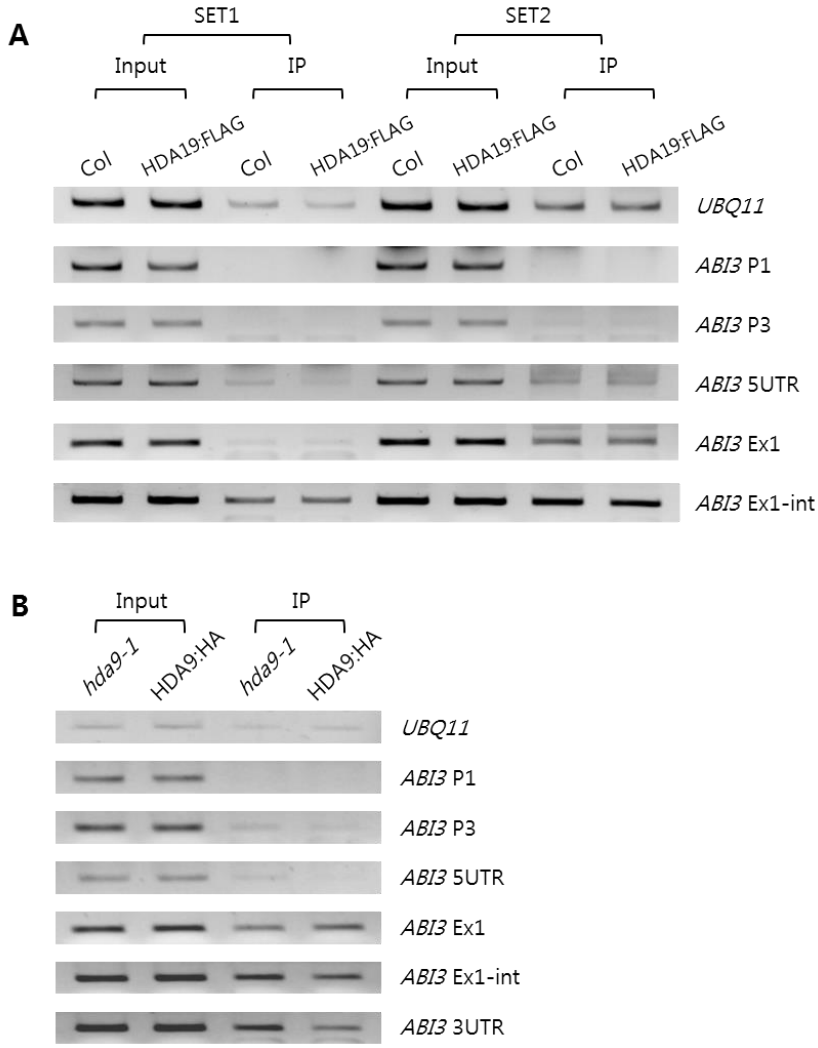

Figure 3.13 Enrichment of HDAC19 and HDA9 on *ABI3*

(A) ChIP-qPCR analysis of HDAC19 enrichment. Seeds of wildtype and *HDA19:FLAG* construct-harboring transgenic plants were germinated under constant light for 2 days after stratification and harvested. ChIP assay was performed with an antibody against FLAG. *UBQ11* was used as an internal control. Data represent two biological repeats. **(B)** ChIP-qPCR analysis of HDA9. Seeds of wildtype and *HDA9:HA* construct-harboring thransgenic plants were germinated

under constant light for 2 days after stratification and harvested. ChIP assay was performed with antibody against HA. ‘Input’ indicates chromatins before immunoprecipitation. *UBQ11* was used as an internal control. Data represent two biological repeats. Schematic view of amplified *ABI3* locus by the ChIP analysis is shown in Figure 11A.

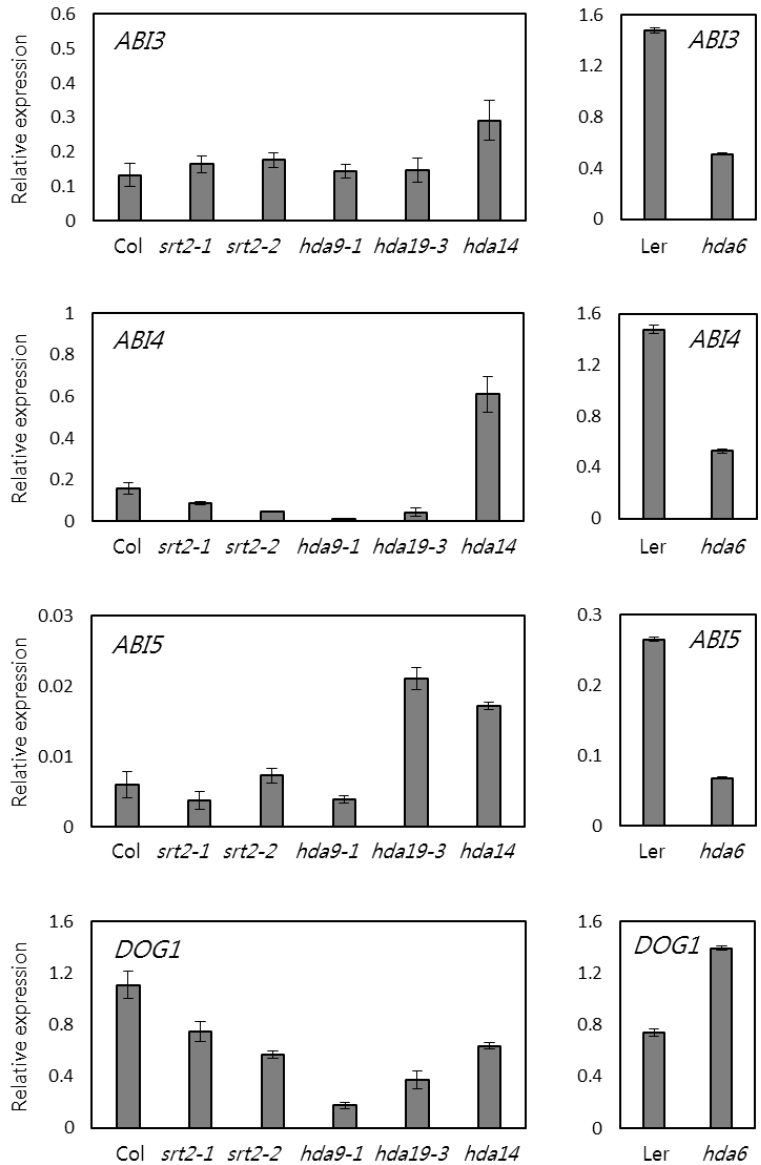

Figure 3.14 Expression of *ABI* genes in various *hdac* mutants

mRNA expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in wildtype and various *hdac* mutant seeds grown for 3 days under constant light condition. The seeds were germinated and grown on half MS media without sucrose for 7 days (3 days under

constant light condition after 4 days of stratification), and then germinated seeds were harvested for RNA extraction. Transcription levels in Columbia ecotype background mutants, *srt2-1*, *srt2-1*, *hda9-1*, *hda19-3*, and *hda14* are shown on the left, and those in Ler-background are shown on the right. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

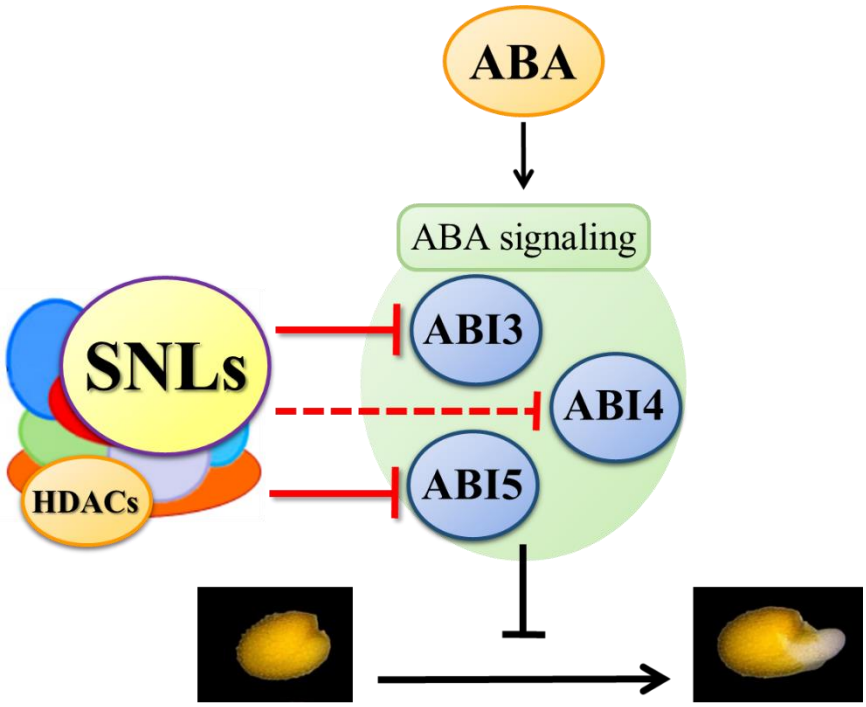

Figure 3.15 Proposed model for the role of SNLs in pathways regulating seed germination in Arabidopsis

Histone deacetylation mediated by SNLs negatively regulates the ABA signaling pathway, which inhibits seed germination.

Table 3.1. Oligonucleotides used for T-DNA flanking sequence analysis

Name	Sequence
SALKLB1	5’-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3’
SNL1-1 F	5'-GAGAGCTTGTTCAGATGCGAAGA -3'
SNL1-1 R	5'-CAGGATAAAGGAACTACTTGAATA-3'
SNL2-1 F	5'-ACTCAGAGCAGCAATGAAGCGAATTAG-3'
SNL2-1 R	5'-CAAGATGAAGAATTGTTCCCAGAGGCT-3'
SNL3-1 F	5'-CTAGGTTTTGATTTGTAGATGAACATGAAG-3'
SNL3-1 R	5'-AACCTAAACATAACAAGGAGAAG-3'
SNL3-2 F	5'-GAAGATGACTGCCGAGCG-3'
SNL3-2 R	5'-TCTGCGATCCTCCCTTGC-3'
SNL4-1 F	5'-CCTTTCTTATCAACCTAATGC-3'
SNL4-1 R	5'-TCATGTGCGAGGGGTAAACCGC-3'
SNL4-2 F	5'-CTTAGAGACATCACTGGTTTGGATATTG-3'
SNL4-2 R	5'-CTGGTTGGCTGATTATCTCC-3'

Table 3.2. Oligonucleotides used for constructs

Name	Sequence
SNL1-promoter F	5'-ACTgtcgacGATGAGGATGAGGATTAAG-3'
SNL1-promoter R	5'-TTCGCTggatccCTGCTCTGAGTAAAACAAAC-3'
SNL2-promoter F	5'-ACGgtcgacTACAATCGTTGCCTTTC-3'
SNL2-promoter R	5'-CATCTCggatccGCTTCATCACAAG-3'
SNL3-fullength F	5'-CACCTATGTTCCGTTTTTATGGTT-3'
SNL3-fullength R	5'-CAAGAAGTTATTTTTGTAATATTGTAACCTC-3'
SNL3-promoter F	5'-TCGgtcgacCGGAAAGATAGTAATG-3'
SNL3-promoter R	5'-CTATTCAggatccCATAAGACCAAAGAACG-3'
SNL4-promoter F	5'-TTTgtcgacCATCCAAACTGCATCAATG-3'
SNL4-promoter R	5'-TCCAAggatccTTTATATTTTGCCAGAGCTTC-3'

Restriction sites used for cloning are in small letters and underlined**.**

Table 3.3. Oligonucleotides used for RT-PCR analysis

Gene	Name	Sequence
Ubiquitin11	UBQ11 F	5’-GATCTTCGCCGGAAAGCAACTT-3’
	UBQ11 R	5’-CCACGGAGACGGAGGACC-3’
LEC1	LEC1 F	5'-GAGACAAACCTATGGAGGAAATGG-3'
	LEC1 R	5'-CCAACACTGGATTCATCTTGACC-3'
LEC2	LEC2 F	5'-TCCTAACAACAATCGCTCGC-3'
	LEC2 R	5'-TGAGGATAACACTCCGATAAGTAAACC-3'
FUS3	FUS3 F	5'-CTCCGACGTATGATACTCCCGAAG-3'
	FUS3 R	5'-CGCCTGTGTTTTCTAGCACGTACATT-3'
CRC	CRC F	5'-ACAACCTAGATGTTCTCCAAGCCACC-3'
	CRC R	5'-ACTCTTCCGCTGATACCCGTTC-3'
RAB18	RAB18 F	5'-CGTCTTACCAGAACCGTCCAGG-3'
	RAB18 R	5'-TCCGTATCCTTGGCCACCTG-3'
GA3ox1	GA3ox1 F	5'-CCGAAGGTTTCACCATCACT-3'
	GA3ox1 R	5'-CCCCAAAGGAATGCTACAGA-3'
GA3ox2	GA3ox2 F	5'-TAGATCGCATCCCATTCACA-3'
	GA3ox2 R	5'-TGGATAACTGCTTGGGTTCC-3'
GA20ox2	GA20ox2 F	5'-TCCCGTTCATCGATCTCTCAAGC-3'
	GA20ox2 R	5'-CTTTCCATCAAACGGTGAGCATCC-3'
RGA1	RGA1 F	5'-TACATCGACTTCGACGGGTA-3'
	RGA1 R	5'-GTTGTCGTCACCGTCGTTC-3'
RGL1	RGL1 F	5'-CAAGCATGTTGTTGGCACTT-3'
	RGL1 R	5'-GCAACAAACAACCTTCATTCTCT-3'
RGL2	RGL2 F	5'-TCAGAACATGGGCGTTGAAT-3'
	RGL2 R	5'-AAGGTTTCAGATTCGGGTCG-3'
NCED6	NCED6 F	5'-ACCGGGTCGGATATAAATTGGGTTG-3'
	NCED6 R	5'-CCCGGGTTGGTTCTCCTGATTC-3'
NCED9	NCED9 F	5'-CTGTCCCAAGATGCTCATCACTC-3'
	NCED9 R	5'-TGAAGTTGAGAAAGTTCGGTCGAGG-3'
CYP707A2	CYP707A2 F	5'-TGGTGGTTGCACTGGAAAGAGC-3'

	CYP707A2 R	5'-TTGGCGAGTGGCGAAGAAGG-3'
ABI3	ABI3 F	5'-AAGCTGAGACACACTTGCCG-3'
	ABI3 R	5'-CCAAAACCTGTAGCGCATGT-3'
ABI4	ABI4 F	5'-ATCCTCAATCCGATTCCACC-3'
	ABI4 R	5'-ATTTGCCCCAGCTTCTTTGT-3'
ABI5	ABI5 F	5'-GGTGAGACTGCGGCTAGACA-3'
	ABI5 R	5'-GTTTTGGTTCGGGTTTGGAT-3'
RD29B	RD29B F	5'-AGCAAGCAGAAGAACCAATCAG-3'
	RD29B R	5'-TGCTCGTCATACTCATCATCATC-3'
SOM	SOM F	5'-ATGGATGTCGTTTGTACGGAACATCAA-3'
	SOM R	5'-TCAAGTCAAGAGATCATTGACCCATCC-3'
PIL5	PIL5 F	5’-ATGATTTCTGCTCAGATCTTCTCTTCT-3’
	PIL5 R	5’-AGATTCACCACCTCTACCGTTATTAAA-3’
DOG1	DOG1 F	5'-AAGAAAGTCTCAAGCCTAC-3'
	DOG1 R	5'-CGAGGATCTTCGCTAAAG-3'

Table 3.4. Oligonucleotides used for ChIP assay

Gene	Region	Name	Sequence
ABI3	P1	ABI3 P1 F	5'-ACGCATAGTAAAACAAAGTTCACATG-3'
		ABI3 P1 R	5'-GCATTGATGTATATATCAGTACTAGTCG-3'
	P2	ABI3 P2 F	5'-TCACCATCGTATCCACAAACATTATCG-3'
		ABI3 P2 R	5'-CTTGTACGTCGAGATGGCATGT-3'
	P3	ABI3 P3 F	5'-GCTGCAAAGAGAAAGAGAATAACTTAAACCC-3'
		ABI3 P3 R	5'-GAGCCCATGTGTTCCAGTTTGTTCCAT-3'
	5UTR	ABI3 5UTR F	5'-ATTGGTCTTTGTTCATCTGAAGTTGGAG-3'
		ABI3 5UTR R	5'-CTAGATTGGTGGAGAGAGAAAAGTTAGGG-3'
	Ex1	ABI3 EX1 F	5'-CTAATCCCACCGTCCGAC-3'
		ABI3 EX1 R	5'-TCTGGCTGTGGCGGATAG-3'
	Ex1-1	ABI3 Ex1-1 F	5'-AGTGATGGAGACTCAGTTACCTACC-3'
		ABI3 Ex1-1 R	5'-GCTTCTTCATCAAACCAAACGAGTG-3'
	3UTR	ABI3 3UTR F	5'-TCGCTTCACCAACTTCTCAAACTG-3'
		ABI3 3UTR R	5'-GACCAAACAGCTTTAATCATGACCCTCC-3'
ABI4	P1	ABI4 P1 F	5'-ACGTGTTGTACCAGATGTTTTTCCTCC-3'
		ABI4 P1 R	5'-GAGAAAAATTTAAGCTGTTGGGAAATCACC-3'
	P2	ABI4 P2 F	5'- GAATCCTCTGAAATCTGAATGCCTTGG-3'
		ABI4 P2 R	5'-GGGTAACTATAGCAAATCATGAGCGA-3'
	P3	ABI4 P3 F	5'-AAGAAGTGAGTGAGAAGAGAGTGTAAG-3'
		ABI4 P3 R	5'-GGAGAGGACGAATCAAGAAGGAAGG-3'
	Ex1	ABI4 Ex1 F	5'-ATCCTCAATCCGATTCCACC-3'
		ABI4 Ex1 R	5'-ATTTGCCCCAGCTTCTTTGT-3'

	Ex1-1	ABI4 Ex1-1 F	5'-GCTCACTGATGTTCCGGTAACTAATTCG-3'
		ABI4 Ex1-1 R	5'-TGATAGACTCGAACCCACCGAACC-3'
ABI5	P1	ABI5 P1 F	5'-AGTTGCTGTAATCTTTAGGTCGCTGG-3'
		ABI5 P1 R	5'-CACGTGGACTATTCACTGCATAAGG-3'
	P2	ABI5 P2 F	5'-TGTCTCTGATCATGGGCCTGG-3'
		ABI5 P2 R	5'-GCGCGTGGGGTCTAAGAAG-3'
	Int1	ABI5 Int1 F	5'-TTTGTCGCTGTCACGATGTGGACC-3'
		ABI5 Int1 R	5'-ACTTGTCCCTGTTCAGCTATTCACG-3'
	Ex1	ABI5 Ex1 F	5'-GGTGAGACTGCGGCTAGACA-3'
		ABI5 Ex1R	5'-GTTTTGGTTCGGGTTTGGAT-3'
	Ex2	ABI5 Ex2 F	5'-AGTTGAAAGAAGAGAATGCGCAGC-3'
		ABI5 Ex2 R	5'-TGCTTCCTCTTCCTCTCCAACTCC-3'
DOG1		DOG1 F	5'-AAGAAAGTCTCAAGCCTAC-3'
		DOG1 R	5'-CGAGGATCTTCGCTAAAG-3'
UBQ11		UBQ11 F	5’-TCAGTATATGTCTCGCAGCAAACTATC-3’
		UBQ11 R	5’-GACGACTCGGTCGGTCACG-3’

3.6 Discussion

Breaking seed dormancy and seed germination are often viewed as a simultaneous phenomenon that initiates plant life. However, these two processes actually occur sequentially and somewhat distinctively. Generally, seed dormancy is acquired during embryo developmental stages, built up until the completion of embryo maturation, and then released as seeds undergo desiccation-accompanied ripening. After ripening, when seeds are in non-dormant state, germination is then finally in action depending on environmental cues. Radicle expansion and hypocotyl elongation required for early seedling establishment are triggered when seeds have gained the ability to readily germinate. Therefore, seed germination and early seedling establishment process, as one of the critical transition phases in plant's life cycle, has been closely examined and pinpointed for the underlying mechanisms.

Since the early 90's, the role of ABA has been described in association with seed development and germination (Meurs et al., 1992). It has been established that ABA deficiency or insensitivity results in aberrant seed development and precocious germination. More recently, defects in late embryo development have been explained by ABI-mediated pathways via genetic analyses (Nambara et al., 2000; Nakashima et al., 2006; To et al., 2006). Together with *FUS3*, *LEC1*, and *LEC2*, *ABI3* has been known to control a particular set of genes that are involved in most aspects of seed maturation. Thus, ABI3 has been considered as a paradigm for late embryogenesis and seed-dormancy establishment. However, regulation mechanisms for the master controller, ABI3, have remained abstruse.

About a decade ago, it was reported that PKL is involved in the repression of *ABI3* and *ABI5* expression (Perruc et al., 2007). Perruc et al. concluded that PKL-influence was to limit the transcriptional potential of the embryogenic genes. Later on, PKL was found to repress the expression of the seed-associated genes, such as *FUS3*, *LEC1*, and *LEC2*, through H3K27me3 enrichment during germination (Zhang et al., 2008). Despite the efforts, contradictory influences of PKL on *ABI* genes with low H3K27me3 levels compared to those on other embryonic genes with high H3K27me3 levels and low H3Ac levels could not be explained. In our results, expression patterns of embryonic genes, such as *LEC1*, *LEC2, FUS3*, and others, could not be explained in the aspect of the delayed germination phenotype of *snl1234* and the predicted function of SNLs (**Figure 3.4**). Thus, we believe that the alteration of seed-associated gene expressions, except for *ABI3*, is controlled in rather collaborative but distinct manner from *ABI*-specific regulations.

Lately, it was reported that SNL1 and SLN2 have a redundant role in mediating the antagonism between ABA and ethylene during the establishment of seed dormancy (Wang et al., 2013). To confirm whether the altered dormancy by the mutations of *SNL1* and *SNL2* may also disrupt seed germination, we carefully scrutinized the germination phenotypes of the *snl12* double mutant and other combinations of *snl* mutant seeds. However, such mutant seeds at dormancy released stage did not show altered germination phenotypes (**Figure 3.1**). On the other hand, expression levels of a dormancy marker gene, *DOG1*, were remained elevated in *snl12* double and *snl23* triple mutants compared to wt or *snl1234* quadruple mutants (**Figure 3.9B**). The inconsistency between the germination

phenotype and the *DOG1* expression level could be explained by following suppositions. First, the higher level of *DOG1* expression in the double and triple mutants was not sufficient for conferring dormancy in dormancy released AR seeds. As shown in **Figure 3.9B**, *DOG1* expression continuously decreases from FH to AR stages and further during stratification and germination processes both in wt and *snl1234* seeds. Considering that the seed samples were harvested at two days after stratification, it is possible that the residual *DOG1* expression might be insufficient to induce delayed germination. Second, although *DOG1* might be an excellent dormancy marker in FH seeds, its expression level in AR seeds might be less meaningful in representing dormancy level. Lastly but more likely, results in **Figure 3.9B** might suggest that there might be a more powerful factor that can overcome the effect of DOG1 in conferring germination. In sum, our results indicate that only the *snl1234* quadruple but none of the double and triple *snl* mutants is defective in germination, and this germination phenotype cannot be explained by *DOG1* expression level. Thus, it is strongly endorsed that seed dormancy is not exactly coincided with seed germination efficiency and that all the four SNL proteins (SNL1, SNL2, SNL3, and SNL4) share a redundant role in seed germination that is not observed in seed dormancy.

In our study, *snl1234* seeds showed delayed germination phenotype and ABA hypersensitivity. And this was correlated with the altered expression patterns of ABA signaling genes during seed germination and early seedling establishment. During the transition process, transcript levels of the key components, *ABI3, ABI4*, and *ABI5,* in the ABA-signaling pathway were more slowly decreased in *snl1234* mutant compared to wt. This indicates that the mutations of those four *SNL* genes

result in the failure of precise transcriptional repression of the *ABI* genes. To prove the involvement of the SNLs in the ABA-signaling mechanism in depth, we provided genetic evidence on the relationship between *SNLs* and *ABI* genes (**Figure 3.10**). Germination test using the *snl1234 abi3* quintuple mutant seeds demonstrated wt level germination unlike the *snl1234* mutant seeds. It indicates that *abi3* mutation can completely block the effect of *snl1234* in seed germination. In other words, *ABI3* is epistatic to *SNLs* and, therefore, it opened a possibility that *ABI3* might be the factor that powerfully controls seed germination as a target of the SNL complex.

Dormancy, which is achieved by ABA metabolism and signaling, can be expanded to primary and secondary dormancy. The primary dormancy is established during embryo maturation whereas the secondary dormancy is programmed, when unfavorable condition for seed germination is unexpectedly given, to overcome the already-released primary dormancy and to inhibit seed germination (Hilhorst et al., 1998). However, the regulation of secondary dormancy is largely unknown and only established upon environmental cues. This very year, it was reported that, for the secondary dormancy, seeds require changes in GA contents and sensitivity (Ibarra et al., 2015). Although the secondary dormancy also requires ABA, Ibarra et al. (2015) could not observe significant alterations in either endogenous ABA level or sensitivity. Although altered *DELLA* expression was suggested to promote *ABI5* expression, the relationship between *DELLA* and *ABI5* was not clearly addressed. Furthermore, a genome-wide study demonstrated that global HDAC enrichment is strongly observed where enhanced HAT enrichment is observed (Wang et al., 2009). According to this report, HDAC

enrichment overlaps with genomic loci where histone acetylation can be readily induced. Thus, repressive role of histone deacetylation is more important in curbing active genes in need of repression than in maintaining constitutive heterochromatin. Applying this idea to our results, repression of *ABI* chromatin by SNLs might be important and prominent for the suppression of *ABI* expression during seed germination. Given that ABA, especially through the ABI-mediated signaling mechanism, is prerequisite for primary and secondary dormancy acquisition, ABI should be readily activated and tightly regulated during the first critical transition period in plant development. Therefore, we suggest that SNLs might provide a master scaffold on which HDACs can directly regulate all of the three main *ABI* genes during seed germination and early seedling establishment.

Identification of an HDAC(s) that partners with SNLs for *ABI* regulation is yet to be achieved. Although, it has been reported that HDA19, HDA9, and HDA6 are involved in seed germination or dormancy via *ABI3*-mediated pathway (Chen and Wu, 2010; Song et al., 2005; Kazan, 2006), our ChIP assays indicated that there might be no direct interaction between HDA19 or HDA9 with *ABI3* chromatin (**Figure 3.13**). Moreover, under our experimental conditions, there was no significant changes of *ABI3, ABI4,* and *ABI5* transcripts were induced, with the exception of *ABI5* transcript in *hda19,* by both *hda19* and *hda9* mutations (**Figure 3.14**). In fact, role of HDA19 on *ABI* genes was described in association with brassinolide (BR) signaling (Ryu et al., 2014). Upon BR signaling, BES1-TPL-HDA19 complex is assembled and regulates *ABI3-ABI5* module (Wang et al., 2013). Moreover, it was reported that HDA19 and SNL1 have a direct interaction *in vivo* and *in planta*. Considering our results indicating that HDA19 might not

directly target *ABI3* locus, we suspect that the SNL1-HDA19 complex might primarily act on *ABI5* and then, altered *ABI5* expression might subsequently affect *ABI3* expression.

Lastly but not less importantly, our results demonstrated that SNL3 is not only enriched at *ABI5* but also at *ABI3* loci, whereas no direct targeting of HDA19 to *ABI3* chromatin was detected. Taken together, we concluded that role of HDA19 is insufficient to explain the delayed germination of *snl1234*. As our results in **Figure 3.14** showed relatively high level of all the three *ABI* genes in *hda14* mutants, HDA14 might be one of the strongest candidates to act in concert with SNLs. Until this moment, HDA14 is left as an unclassified subfamily member of the *Arabidopsis* RPD3-type HDACs. Therefore, further study is required to see if HDA14 might form a complex with SNLs to directly modulate all *ABI* genes during seed germination.

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국문초록 (Abstract in Korean)

식물의 생활사는 발달에 중요한 여러 번의 전이과정을 포함한다. 전이과정은 내부적 신호(호르몬, 생체 나이 등)와 외부의 환경적 신호(광주기, 온도, 영양 등)에 의하여 조절되며, 수많은 유전자들의 발현 변화를 동반한다. 최근 연구들을 통하여 특정 유전자들의 에피유전학적 조절이 전이과정에 관여하는 하나의 중요한 메커니즘으로 대두되고 있다. 에피유전학적 조절은 DNA 메틸화, 히스톤 변형, RNA 간섭 등에 의하여 염색질 구조변형을 유발하므로 유전자의 전사활성에 영향을 주는 것을 의미한다. 개화와 종자발아는 에피유전학적 조절을 받는 가장 잘 알려진 전이과정이다. 그러므로, 본 연구자는 개화와 종자발아 과정에서 에피유전학적 조절에 대한 보다 깊은 이해를 얻고자 하였다.

개화는 영양생장에서 생식생장으로 전환되는 중요한 전이과정이다. 특정 유전자들의 발현이 균형을 이루어 식물의 개화를 조절한다. 그 중 FT는 개화를 유도하는 신호 단백질로서 개화과정 중 그 역할이 매우 중요하다. *FT*에서 유전자 발현 억제를 유발하는 히스톤 변형 마커인 히스톤 H3 리신 27 메틸화 축적이 이미 보고된 바 있다. 그러나, 유전자 발현 활성을 유발하는 히스톤 변형 마커인 히스톤 H3 리신 4 메틸화 혹은 이에 관여된 조절요소들은 명확하게 연구되지 않은 상태이다. *Arabidopsis thaliana Jumonji 4* (*AtJmj4*)와 *EARLY FLOWERING 6* (*ELF6*) 유전자의 기능이 상실된 애기장대 돌연변이체에서 조기 개화

표현형을 보았으며, 이는 이들의 2중 돌연변이체에서 더욱 극대화되었다. 또한 *atjmj4*와 *elf6* 돌연변이체에서 *FT* 전사체 발현의 증가와 더불어 히스톤 H3 리신 4 메틸화 레벨이 높아져 있었다. Atjmj4는 히스톤 H3 리신 4를 특이적으로 탈메틸화 시킬 수 있는 능력을 지니고 있는 효소로써 *FT* 유전자의 전사 시작부위에 직접적으로 결합함을 확인하였다. ELF6도 *FT*로의 결합이 유사하게 관찰되었으며, 그 결합부위는 이들 유전자들의 돌연변이체에서 메틸화된 히스톤 H3 리신 4의 변화를 보였던 부분과 일치 하였다. 그러므로 본 학위논문의 제 2장에서는 식물의 개화과정에서 탈메틸화 효소로서 *FT* 발현 억제에 직접적으로 관여하는 AtJmj4와 ELF6의 기능을 밝혔다.

종자발아는 배아에서 후배발달과정으로 전환되는 식물의 또 다른 중요한 전이과정이다. 종자발아는 식물호르몬인 gibberellic acid (GA)와 abscisic acid (ABA)의 상호작용에 의하여 조절된다. ABA는 ABI 단백질을 통한 신호전달에 의해 종자의 발아를 억제하는 기능을 하지만, 이에 관한 분자생물학적 이해는 아직 부족하다. 애기장대 SIN3-LIKE (SNL) 단백질들은 탈아세틸화효소와 함께 유전자들의 발현 억제 기작에 관여한다. 이에 본 학위논문의 제 3장에서는 종자발아 과정 동안의 SNL단백질들의 역할을 규명하고자 하였다. SNL1, SNL2, SNL3, SNL4 유전자 모두에 돌연변이를 일으킨 4중 돌연변이체인 *snl1234* 종자에서 발아가 지연되는 표현형을 보았다. 이 표현형은 ABA 처리 시 야생종과 돌연변이체 사이에서 더욱 큰 차이로 관찰되었다. 종자발아 과

정 중에 야생종에 비하여 *snl1234*에서 *ABI3*, *ABI4*, *ABI5* 유전자들의 아세틸화 증가와 함께 발현 증가가 관찰되었다. 더불어 SNL3 단백질이 *ABI3*와 *ABI5*에 직접적으로 결합하였다. 이를 통하여, 본 연구자는 식물의 종자발아 과정에서 SNL 단백질이 *ABI* 유전자들의 억제자인 것을 밝혔다.

주요어: 에피유전학, 히스톤 변형, 개화, 종자발아

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이학박사학위논문

애기장대 발달 전이에서 히스톤 변형 효소들의 기능에 대한 연구

A Study on the Role of Histone Modifiers in Arabidopsis Developmental Transitions

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서울대학교 대학원

생명과학부

정 주 희

Abstract

A Study on the Role of Histone Modifiers in Arabidopsis Developmental Transitions

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Plants undergo a number of developmental transitions throughout their life cycle. Developmental phase transitions of plants are largely controlled by a combination of endogenous and environmental signals such as hormones, age, photoperiod, temperature, and nutrient. Recent studies have demonstrated that epigenetic control of phase-specific genes is one of major mechanisms allowing developmental transitions in plants. Epigenetic regulation is made possible by DNA methylation, histone modifications, non-coding RNAs, and crosstalks among them. These epigenetic mechanisms control transcriptional activity of target genes via changes

of their chromatin structure. Flowering and seed germination are the best-known developmental transitions controlled by epigenetic mechanisms. Thereby, I focused on understanding epigenetic mechanisms acting in the regulation of flowering and germination.

Flowering is a critical transition from vegetative to reproductive development. Flowering is genetically controlled by networks of flowering genes. FLOWERING LOCUS T (FT) plays a key role as a mobile floral induction signal during the floral transition. Deposition of a repressive mark, trimethylation at histone H3 lysine 27 (H3K27me3), has been reported as one of the mechanisms allowing for *FT* repression in *Arabidopsis*. However, the role of an active mark, H3K4me3, in *FT* regulation has not been addressed, nor have the components affecting this mark been identified. Mutations in *Arabidopsis thaliana Jumonji 4* (*AtJmj4*) and *EARLY FLOWERING 6* (*ELF6*) caused an additive early flowering correlated with increased expression of *FT* mRNA and H3K4me3 levels within *FT* chromatin. AtJmj4 protein possessed specific demethylase activity for mono-, di-, and trimethylated H3K4. AtJmj4 and ELF6 associated directly with the *FT* transcription initiation region, where H3K4me3 levels increased most significantly in the mutants. Thus, the study in the Chapter II demonstrates the roles of AtJmj4 and ELF6 as H3K4 demethylases directly repressing *FT* chromatin and preventing precocious flowering in *Arabidopsis*.

Seed germination is another pivotal transition from embryonic to post-embryonic development. Phytohormones, namely GA and ABA, play central but antagonistic roles in the regulation of seed germination. Seed germination is positively controlled by active GA and negatively controlled by ABA levels and

ABA signaling. However, ABA-dependent regulatory pathway of seed germination is not fully understood. Several *ABA INSENSITIVE* (*ABI*) genes are involved in ABA signaling. Arabidopsis SNL proteins, orthologs of yeast Swi-independent 3 (Sin3), are putative transcriptional co-repressors possibly involved in gene-silencing by acting in concert with histone deacetylases (HDACs). In the Chapter III, it is demonstrated that seed germination and early seedling establishment of *SNL*quadruple mutant (*snl1234*) mimics the germination process under hyperactive ABA signaling conditions. Dormancy released *snl1234* seeds and embryos showed delayed germination and this germination phenotype was exaggerated with exogenous ABA treatment. Moreover, different expression patterns of the ABA-signaling genes, *ABI3*, *ABI4*, and *ABI5*, were observed in the *snl1234* mutant during germination and early seedling establishment. Altered *ABI* expression in *snl1234* was associated with enriched histone acetylation within *ABI* chromatin. Further, SNL3 protein directly targets *ABI3* and *ABI5* loci. Taken together, SNLs play an important role during seed germination and early seedling establishment as bedrock for histone deacetylation-based mechanism of *ABI* gene regulation.

Keywords: epigenetic regulation, flowering, seed germination, *Arabidopsis thaliana Jumonji 4* (*AtJmj4*), *EARLY FLOWERING6* (*ELF6*), *FLOWERING LOCUS T* (*FT*), *SIN3-LIKE* (*SNL*), *ABA INSENSITIVE* (*ABI*)

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Abbreviations

ABA	Abscisic acid
ABI	ABA-INSENSITIVE
ABI3	ABA INSENSITIVE 3
ACO	ACC OXIDASE
ADA1	Alteration Deficiency in Activation 1
AFR1	SAP30 FUNCTION-RELATED 1
AG	AGAMOUS
AGL15	AGAMOUS-like15
AGL18	AGAMOUS-LIKE 18
AGO4	ARGONAUTE 4
AP1	APETALA1
AR	After-ripened
ARHs	ADP-ribosyl hydrolases
ARP6	ACITN-RELATED PROTEIN 6
ARTs	ADP-ribosyltransferases
AS1	ASYMMETRIC LEAVES1
Ash1	Absent, Small, or Homeotic Disc 1
ASHH	ASH1 homologs of which four ASH1 HOMOLOG
ASHR	ASH1-RELATED
AtJmj4	Arabidopsis thaliana Jumonji4
AtSWR1c	Arabidopsis SWR1 complex
ATXRs	ARABIDOPSIS TRITHORAX RELATED PROTEINS

ATXs	ARABIDOPSIS TRITHORAX PROTEINS
BES1	BRASSINAZOLE-RESISTANT 2
bHLH	Basic helix-loop-helix
BiFC	Bimolecular fluorescence complementation
bp	base pair
BR	Brassinosteroid
BTD	Biotinidase
$CaMV35S_{pro}$	Cauliflower Mosaic Virus 35S promoter
CBF1	C-REPEAT/DRE BINDING FACTOR 1
ChIP	Chromatin immunoprecipitation
CIB1	CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX1
CLF	CURLY LEAF
CLSY1	CLASSY1
CMT3	CHROMOMETHYLASE 3
CO	CONSTANS
COLDAIR	COLD ASSISTED INTRONIC NONCODING RNA
COMPASS	COMPLEX PROTEINS ASSOCIATED WITH SET 1
COOLAIR	COLD INDUCED LONG ANTISENSE INTRAGENIC RNA
CRY	CRYTOCHROMES
Ct	Threshold cycle
CYP707As	ABA 8’-hydroxylases
DAG1	DOF AFFECTING GERMINATION 1

DCL1	DICER-LIKE 1
DCL3	DICER-LIKE 3
DIC	Differential interference contrast
DME	DEMETER
DML2	DEMETER-LIKE proteins
DML3	DNA GLYCOSYLASE DEMETER-LIKE PROTEIN 3
DMS3	DEFECTIVE IN MERISTEM SILENCING 3
DNMTs	DNA methyltransferases
DOF	DNA-BINDING ONE ZINC FINGER
DOG1	DELAY OF GERMINATION 1
DRD1	DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1
DRM2	DOMAINS REARRANGED METHYLTRANSFERASE 2
DTT	Dithiothreitol
E(z)	Enhancer of zeste
EBS	EARLY BOLTING IN SHORT DAYS
EDTA	Ethylenediaminetetraacetic acid
EFS	EARLY FLOWERING IN SHORT DAYS
ELF6	EARLY FLOWERING 6
ELF7	EARLY FLOWERING 7
ELF8	EARLY FLOWERING 8
EMF1	EMBRYONIC FLOWER 1
EMF2	EMBRYONIC FLOWER 2

ERF4	ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 4
ERF9	ETHYLENE SIGNALING GENES
ERS1	ETHYLENE RESPONSE SENSOR 1
ESD1	EARLY IN SHORD DAYS 1
F1	First filial generation
FD	FLOWERING LOCUS D
FES1	FRIGIDA-ESSENTIAL 1
FH	Freshly harvested
FIE	FERTILIZATION-INDEPENDENT ENDOSPERM
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FLK	FLOWERING LATE KH MOTIF
FLX	FLC EXPRESSOR
FRIc	FRIGIDA complex
FRL1	FRIGIDA-LIKE 1
FT	FLOWERING LOCUS T
FUS3	FUSCA 3
FWA	FLOWERING WAGENINGEN
GA	Gibberellic acid
GAI	GA INSENSITIVE
GCN5	GENERAL CONTROL NON-DEPRESSIBLE 5
GFP	Green fluorescence protein
GID1A	GA INSENSITIVE DWARF 1A

GNAT	GCN5-related acetyltransferases
GUS	β-glucuronidase
H2Bub1	histone H2B monoubiquitylation
H3K27me3	histone H3 lysine 27 trimethylation
H3K4me3	histone H3 lysine 4 trimethylation
H3K9me2	histone H3 lysine 9 dimethylation
H4R3me2s	histone H4 arginine 3 symmetric dimethylation
HA	HUMAN INFLUENZA HEMAGGLUTININ
HATS	HISTONE ACETYLTRANSFERASES
HCS	HOLOCARBOXYLASE SYNTHETASE
HD2	HISTONE DEACETYLASE 2-RELATED PROTEIN FAMILIES
HDA1	HISTONE DEACETYLASE 1
HDACs	HISTONE DEACETYLASES
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
His	Histidine
HLS1	HOOKLESS 1
HP1	HETEROCHROMATIN PROTEIN 1
HRP	Horseradish peroxidase
HSL1	HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2-LIKE1
HUB1	HISTONE MONOUBIQUITINATION 1
IBM1	INCREASED EXPRESSION OF BONSAI METHYLATION 1

JMJs	JmjC domain–containing proteins
K	Lysine
KYP	KRYPTONITE
LD	LUMINIDEPENDENS
LDL1	LSD1-LIKE 1
LEC1	LEAFY COTYLEDON 1
LFY	LEAFY
LHP1	LIKE HETEROCHROMATIN PROTEIN 1
lncRNAs	long non-coding RNAs
LSD1	LYSINE SPECIFIC DEMETHYLASES 1
MEA	MEDEA
MEE27	MATERNAL EFFECT EMBRYO ARREST 27
MET1	DNA METHYLTRANSFERASE 1
miR402	microRNA 402
MRG1	Morf Related Gene 1
MS	Murashige-skoog
MSI1	Musashi RNA-binding protein 1
MSI1	MULTI-COPY SUPPRESSOR OF IRA 1
MSI4	MULTI-SUBUNIT SUPPRESSOR OF IRA 4
MYST	MOZ, Ybf2/Sas3, Sas2 and Tip60
NCEDs	Nine-cis-epoxycarotenoiddioxygenases
ORCs	ORIGIN RECOGNITION COMPLEXES
PAC	Paclobutrazol
PARDs	poly-ADP-ribose glycohydrolases

PARPs	poly-ADP-ribose polymerases
PcG	Polycomb group
PHD	plant homeo domain finger
PHE1	PHERES1
PhoRC	Pho repressive complex
PHYB	PHYTOCROME B
PIE1	PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1
PIFs	PHYTOCHROME-INTERACTING FACTORS
PIL5	PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5
PKL	PICKLE
Pol II	RNA polymerase II
PolIV	RNA Polymerase IV
PRC	POLYCOMB REPRESSIVE COMPLEX
PRMTs	PROTEIN ARGININE METHYLTRANSFERASES
PTMs	Post-translational modifications
PVPP	Polyvinylpolypyrrolidone
qPCR	quantitative PCR
R	Arginines of histone, including Arg2
RdDM	RNA-directed DNA methylation
RDM1	RNA-DIRECTED DNA METHYALTION 1
RDR2	RNA-DEPENDENT RNA POLYMERASE2
REF6	RELATIVE OF EARLY FLOWERING 6
RGA	REPRESSOR OF GAI

RGA1	REPRESSOR OF GA1
RGL1	RGA-LIKE1
ROS1	REPRESSOR OF SILENCING 1
RPD3	REDUCED POTASSIUM DEPENDENCE 3
RRM	RNA RECOGNITION MOTIF
SA	Salicylic acid
SAM	Shoot apical meristem
SDG8	SET DOMAIN GROUP 8
SDS	Sodium dodecyl sulfate
SE	Standard error
SHH1	SAWADEE HOMEODOMAIN HOMOLOG 1
Sin3	Swi-independent 3
SIR2	SILENT INFORMATION REGULATOR 2
siRNAs	small interfering RNAs
SMZ	SCHLAFMÜ TZE
SNL	SIN3-LIKE
SNZ	SCHNARCHZAPFEN
SOC1	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
SOM	SOMNUS
SPT	SPATULA
STM	SHOOT MERISTEMLESS
SUF3	SUPPRESSOR OF FRIGIDA 3
SUMO	Small ubiquitin-related modifier

SUVH4	SU(VAR)3-9 HOMOLOG 4
SVP	SHORT VEGETATIVE PHASE
SWN	SWINGER
TEM1	TEMPRANILLO1
TFL2	TERMINAL FLOWER 2
TOE1	TARGET OF EAT1
TPL	TOPLESS
TrxG	Trithorax group
TSS	Transcription start site
UBC1	UBIQUITIN-CONJUGATING ENZYME 1
UBQ	UBIQUITIN
VIL1	VIN3-LIKE 1
VIM	VARIATION IN METHYLATION
VIN3	VERNALIZATION INSENSITIVE 3
VIP6	VERNALIZATION INDEPENDENCE 6
VRN1	VERNALIZATION 1

Chapter I

General Introduction

1.1 Epigenetic gene regulation

Epigenetics was first defined as a branch of biology, which investigates gene expression and underlying interactions between genes and their products that bring the phenotype (Waddington, 1942). With more and more extensive studies, the term epigenetics is developed and now described as: a branch of biology that searches for heritable changes that are made on a gene locus or a gene-encompassing chromosome without alteration of the DNA sequence (Aaron et al., 2007). Over the past years, numerous efforts have been made in the field of epigenetics to understand biological phenomena that are not explained by genetics. Among diverse regulatory mechanisms of gene expression, epigenetic regulation affects the chromatin structure that subsequently controls transcriptional activity of the target gene. Epigenetic regulation is made possible by DNA methylation, histone modification, non-coding RNAs and the crosstalks among them. These mechanisms will be exclusively described in the first part of this chapter.

1.1.1 DNA methylation

DNA methylation which contributes to gametogenesis, fertilization, and vegetative developments in plant has been considerably studied. Recently, crosstalks with histone modifications are revealed to be also important. Genome-wide DNA methylation levels are regulated by combined activities of DNA methyltransferases (DNMTs) and DNA glycosylases, which may establish, maintain, and/or remove methyl groups from the cytosine bases. In both plants and animals, 5-methyl-cytosine generally results in gene silencing and heterochromatin formation (Li and

Zhang, 2014; Zhao and Garcia, 2014; Furner and Matzke, 2011; Meyer, 2011). Plant DNA methylation may occur in context of CG, CHG, or CHH, where H refers to A, T, or C. It has been identified that DNA methylation is implemented through several pathways.

The RNA-directed DNA methylation (RdDM) pathway is mainly involved in *de novo* DNA methylation on previously unmethylated cytosine residues. In the RdDM pathway, 24-nucleotide (nt) small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) act as major regulatory factors (Wierzbicki et al., 2008; Law et al., 2010). The 24-nt siRNAs, which are generated by DICER-LIKE 3 (DCL3), are loaded onto ARGONAUTE 4 (AGO4) and the siRNA-AGO4 complex then recruits the DNA methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), to the target loci (Law et al., 2010). The generation of siRNA-guide is promoted by the plant-specific RNA polymerase, Polymerase IV (PolIV) (Onodera et al., 2005; Zhang et al., 2007; Herret et al., 2005). The PolIV complex was identified to include CLASSY1 (CLSY1), RNA-DIRECTED DNA METHYLATION 4 (RDM4), SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1), and RNA-DEPENDENT RNA POLYMERASE2 (RDR2) (Law et al., 2011). LncRNAs, long nascent transcripts generated from RdDM target loci, can also physically interact with AGO4 and lead DNA methylation factors to the target loci through its complementary sequence (Wierzbicki et al., 2009). The biogenesis of lncRNAs, which is independent of siRNA biogenesis, is facilitated by another plant-specific RNA polymerase, PolV (Wierzbicki et al., 2008). A putative chromatin-remodeling complex, called the DDR complex, also contributes to the biogenesis of lncRNAs. The DDR complex

is named after its components: SWI2/SNF2-like chromatin-remodeling protein DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), and RNA-DIRECTED DNA METHYALTION 1 (RDM1) (Kannoet et al., 2004; Law et al., 2010; Gao et al., 2010; Kanno et al., 2008).

Depending on cytosine context, distinct DNA methyltransferases work through different mechanisms to maintain DNA methylation level. CG methylation is mainly sustained by DNA METHYLTRANSFERASE 1 (MET1), the plant ortholog of mammalian Dnmt1, which might also contribute to *de novo* CG methylation. VARIATION IN METHYLATION (VIM) family proteins, including VIM1, VIM2, and VIM3, are additionally discovered to associate in the maintenance of CG methylation (Yao et al., 2012; Stroud et al., 2013). CHG methylation is maintained by a plant-unique cytosine methyltransferase CHROMOMETHYLASE 3 (CMT3), which is guided by dimethylated histone H3 lysine 9 (H3K9me2) (Lindroth et al., 2001; Law and Jacobsen, 2010; Du et al., 2012). Interestingly, CHG methylation and H3K9me2 interdependently function for target gene silencing. Methylated CHG loci are preferentially bound by histone methyltransferases KRYPTONITE (KYP)/SU(VAR)3-9 HOMOLOG 4 (SUVH4). KYP/SUVH4, SUVH5, and SUVH6 catalyze H3K9me2 in *Arabidopsis* (Inagaki et al., 2010). The mutuality between DNA and histone methylation is required for further examination. In addition to CMTs and SUVH, DRM2 also maintains CHG methylation via the RdDM pathway (Stroud et al., 2013). CMT2, a CMT3 homolog, has a role in maintaining CHH methylation, presumably via crosstalk with histone modifications (Zemach et al., 2013).

Together with DNA methyltranseferases, DNA glycosylases control DNA methylation levels by counteracting the other, and vice versa. DNA glycosylases eliminate a methyl group from 5-methylcytosine by base excision (Gehring et al., 2006; Gong et al., 2002). In *Arabidopsis*, REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER-LIKE proteins, namely DML2 and DML3, are identified, and they harbor DNA glycosylase domains. DME is required for demethylation of CG dinucleotides (Xiao et al., 2003) and is thought to be especially important in gametophyte developments (Choi et al., 2002; Schoft et al., 2011). DML2 and DML3, DME paralogs, also play a role in demethylation of CG, CHG, and CHH methylation at specific genomic sites (Ortega et al., 2008). Moreover, ROS3, an RNA-binding protein, associates with ROS1 to cause DNA demethylation in its target sites (Zheng et al., 2008). Therefore, multiple DNA glycosylases play a critical role against DNA methyltransferase activities for various cellular processes *in planta*.

1.1.2 Histone modification

In eukaryotic cells, genomic DNA is compacted into the nucleus as a complex structure called chromatin. Chromatin is comprised of repeated units of nucleosome, which consists of 146 base pair (bp) DNA that is wrapped around a histone octamer. The core-protein complex is composed of pairs of each of the four histone proteins, H2A, H2B, H3, and H4, with protruding amino-tails (Kornberg., 1977; van Holde., 1988; Luger et al., 1997; Kornberg and Lorch, 1999). Post-translational modifications (PTMs) of the histone tails lead to dynamic changes in

chromatin structure, and such modifications may contribute to various epigenetic controls of cellular processes and memory (Luger K et al., 1998; Wolffe and Hayes, 1999; Strahl and Allis, 2000; Berger, 2002; Turner, 2002). General correlations between gene-transcription activity and histone modifications, such as acetylation, methylation, phosphorylation, carbonylation, ubiquitylation, sumoylation, ADP ribosylation, and biotinylation, have been studied for about half a century (Allfrey et al., 1964; Jenuwein et al., 2001; Turner, 2000 and 2002; Strahl and Allis, 2000). Histone acetylation and methylation of specific residues, directly affecting gene transcription rate, are well described (Peterson and Laniel, 2004).

Generally, histone modification influences gene activity through conformational change of chromatin. Acetylation is highly dynamic and reversible. It causes an open structure of chromatin by eliminating the positive charge on lysine. Like acetylation, histone phosphorylation is also highly dynamic and facilitates the access of transcription factors to DNA. Phosphorylations occur on serine, threonine, and tyrosine and add negative charges onto histone tails, causing structural change of chromatin. Phosphorylation is involved in the response of DNA damage and promotes chromosomal condensation and segregation (Xiao et al., 2009; Krishnan et al., 2009). Unlike acetylation or phosphorylation, histone methylation does not affect the charge of histone. This type of histone modification rather induces the structural change of chromatin in more complex manners. Lysine methylation is relatively stable than acetylation or phosphorylation, whereas arginine methylation is more or less temporary (Zee et al., 2010; Huang et al., 2013a). Histone methylation plays a pivotal role in epigenetic regulation via mitotically heritable ways, especially in the control of flowering and seed

germination (Cazzanelli et al., 2009; D'Urso and Brickner, 2014; Jeong et al., 2009; Cho et al., 2012). In the sections of below, histone acetylation and methylation will be described more in details.

Carbonylation, an irreversible modification, takes place on arginine and lysine. Histone carbonylation masks the positive charges of histones, consequently resulting in released form of chromatin and easy access of transcription factors (Wondrak et al., 2000). A reversible modification, ubiquitylation, is achieved through the sequential actions of E1-activating, E2-conjugating, and E3-ligating enzymes (Hershko and Ciechanover, 1998) and is removed by the action of isopeptidases (Wilkinson, 2000). Mono-ubiquitylation of histone H2A lysine 119 (H2AK119ub1) participates in transcriptional repression through subsequent binding of the polycomb repressive complex (PRC) (Hunt et al., 2013). H2B ubiquitylation is related to transcriptional activation by facilitating H3K4 methylation and transcription elongation (Lee et al., 2007; Kim et al., 2009; Zhang, 2003). The role of ubiquitylation in transcription regulation is still remained to be largely elusive. Sumoylation, similar to ubiquitylation, is achieved by the E1, E2, and E3 enzymatic cascades (Seeler et al., 2003). However, it is independent from ubiquitylation because sumoylation is not associated with protein degradation. Small ubiquitin-related modifier (SUMO) polypeptide has been shown to attach onto all four core histones. Moreover, a few studies have shown that sumoylation plays a role in transcriptional repression through the competition with acetylation or ubiquitylation to target substrate lysine. (Johnson 2004; Iniguez-Lluh Ί 2006). However, the molecular mechanism of SUMO-dependent chromatin structural changes is yet to be clear. In addition, other studies have suggested that histone

sumoylation causes the repression of transcriptional activity through the recruitment of HISTONE DEACETYLASE 1 (HDAC1) and HETEROCHROMATIN PROTEIN 1 (HP1) (Shiio and Eisenman 2003).

ADP-ribosylation is observed on glutamate and arginine residues as mono- or poly-ADP ribosylated forms (Hassa et al., 2006). ADP-ribosylation is also a reversible modification. ADP-ribosylation are implemented by the ADP-ribosyltransferases (ARTs) and the poly-ADP-ribose polymerases (PARPs) for mono- and poly-ADP-ribosylation, respectively (Messner and Hottiger 2011). These processes can be reversed by ADP-ribosyl hydrolases (ARHs) and poly-ADP-ribose glycohydrolases (PARDs) (Koch-Nolte et al., 2008). Mono-ADP-ribosylation participates in the regulation of cell–cell and cell–matrix interactions (Corda and DiGirolamo 2002; Hassa et al., 2006), whereas poly-ADP-ribosylation is involved in the various cellular processes such as cell differentiation, DNA damage detection and repair, and regulation of transcription via chromatin modification (Masutani et al., 2005; Hassa et al., 2006). Histone biotinylation, a covalent attachment of biotin to the ε-amino group of lysine residues, is a reversible process (Kothapalli et al., 2005). Biotinylation is achieved via two biotinyl ligases: biotinidase (BTD) (Brenner, 2002) and holocarboxylase synthetase (HCS) (Narang et al., 2004). Interestingly, it has been suggested that the alternative splicing variants of BTD might act to catalyze the debiotinylation of histones (Ballard et al., 2002; Zempleni, 2005). Biotinylation participates in gene silencing, cellular responses to DNA damage, genome stability, mitotic condensation of chromatin, and cell proliferation (Kothapalli and Zempleni 2005; Rodriguez-Melendez and Zempleni 2003; Filenko et al., 2011). Biotinylation has been shown

to influence other histone modifications such as acetylation, phosphorylation, and methylation in synergistic or antagonistic ways or vice versa (Camporeale et al., 2004; Kothapalli et al., 2005).

1.1.2.1 Histone acetylation

Histone acetylation, one of the major histone modifications, occurs on all four core histones (Sterner and Berger, 2000; Roth et al., 2001) and neutralizes the positive charge on the histone proteins. Thereby, affinity of the histones for surrounding DNA is reduced and, consequently, the accessibility of chromatin for transcription regulators is increased (Kuo and Allis, 1998). Histone acetylation is mainly catalyzed at K9, K14, K18, and K23 of histone H3 and K8, K12, K16. and K20 residues of histone H4 (Fuchs et al., 2006). Histone acetylation is dynamic and reversible through the action of histone acetyltransferases (HATs) and histone deacetylases (HDs, HDAs, or HDACs) (Kuo et al., 1998; Brownell and Allis, 1996; Roth et al., 2001)

1.1.2.1.1 Histone acetyltransferases (HATs)

HATs are classified into two categories, Type A and Type B, depending on their subcellular distributions (Brownell and Allis, 1996; Roth et al., 2001). Type B HATs, which are mostly cytoplasmic proteins, catalyze histone acetylation particularly at lysine 5 and 12 of histone H4 (Verreault et al., 1998; Parthun et al., 1996). A type B HAT, which was first characterized in maize, functions as a

heterodimeric complex acting on histone H4 and selectively acetylates lysine 5 and 12 (Eberharter et al., 1996; Kolle et al., 1998; Lusser et al., 1999). On the other hand, the type A HATs mainly catalyze acetylation of nuclear histones and influence gene transcription through changing chromatin assembly (Carrozza et al., 2003). The type A HATs, including GCN5-related acetyltransferases (GNAT), MOZ, Ybf2/Sas3, Sas2 and Tip60 (MYST), CBP/p300, and the TFIID subunit TAF250, are found in eukaryotes (Sterner et al., 2000; Pandey et al., 2002; Carrozza et al., 2003). In *Arabidopsis*, HAT-encoding genes are also grouped as the classified families: four GNATs (HAG1-HAG3, MMC1), two MYSTs (HAM1 and HAM2), five CBPs (HAC1, HAC2, HAC4, HAC5, and HAC12), and two TAFII250s (HAF1 and HAF2) (Pandey et al., 2002; Perrella et al., 2010).

Several studies have suggested that HATs have various roles in plant responses to stress and developmental changes (Bertrand et al., 2003; Long et al., 2006). In yeast, General Control Non-depressible 5 (GCN5) protein coordinates with Alteration/Deficiency in Activation 1 (ADA1) and ADA3 as a subunit of protein complexes to stimulate transcriptional activation (Grant et al., 1997; Balasubramanian et al., 2002). AtGCN5, *Arabidopsis* GCN5-type HAT, also interacts with *Arabidopsis* homologs of the yeast transcriptional adaptor proteins, ADA2a and ADA2b (Stockinger et al., 2001; Mao et al., 2006). It is reported that AtGCN5 and ADA2 interact with transcription factors such as C-REPEAT/DRE BINDING FACTOR 1 (CBF1) in cold acclimation (Vlachonasios et al., 2003).

1.1.2.1.2 Histone deacetylases (HDACs)

HDACs can remove acetyl groups from histone and non-histone proteins. In plant, HDAC proteins can be grouped into three main families, which are RPD3/HDA1 (Reduced Potassium Dependence 3/Histone Deacetylase 1), SIR2 (Silent Information Regulator 2), and HD2 (Histone Deacetylase 2)-related protein families, base on the sequence homology to yeast HDACs (Pandey et al., 2002).

Accumulating data indicate that altered expression of HDACs affect plant growth, development, and responses to abiotic and biotic stress. Based on sequence similarity, the RPD3/HDA1 superfamily is classified into three classes. Class I proteins include HDA6, HDA7, HDA9, and HDA19 which are the best characterized HDACs in *Arabidopsis*. (Hollender and Liu, 2008; Alinsug et al., 2009). HDA2, HDA5, HDA15, and HDA18 are classified as Class II, and HDA8, HDA10, HDA14, and HDA17 belong to Class III family (Pandey et al., 2002; Hollender and Liu, 2008).

HDA6 interacts with a DNA methyltransferase, MET1, in the maintenance of DNA methylation (Liu et al., 2012; To et al., 2011; Aufsatz et al., 2002). *Arabidopsis* HDA6 participates in control of flowering as a histone deacetylase (Wu et al., 2008). Flowering regulation proteins, such as FLD, a LSD1 (Lysine Specific Demethylase 1)-type histone demethylase (Jiang et al., 2007), FVE/ MULTI-SUBUNIT SUPPRESSOR OF IRA 4 (MSI4), and MSI5, are known to co-work with HDA6 (Gu et al., 2011). Especially, the physical association between HDA6 and FLD shows a crosstalk between histone deacetylation and demethylation (Yu et al., 2011). Another well-known member of HDACs is HDA19, which is the closet homolog of HDA6. HDA19 is reported as an important factor for proper vegetative development (Tian et al., 2005; Zhou et al., 2005; Long

et al., 2006). Recent studies have shown that HDA19 interacts with the HIGH-LEVEL EXPRESSION OF SUGAR-INDUCIBLE GENE2-LIKE1 (HSL1) and is enriched at target loci to directly repress seed maturation gene expressions during germination (Zhou et al., 2013). It was also clarified that HDA19 is involved in pathogen defense mechanism through salicylic acid (SA) signaling pathways (Choi et al., 2012). Additionally, both HDA6 and HDA19 redundantly regulate the expression of embryogenesis related genes, including *LEAFY COTYLEDON 1* (*LEC1*), *FUSCA 3* (*FUS3*), and *ABA INSENSITIVE 3* (*ABI3*) (Tanaka et al., 2008).

HDA7 was reported to have roles in female gametophyte development and embryogenesis (Cigliano et al., 2013). HDA9 has been reported to function in flowering time regulation through the repression of *AGL19* transcription (Kim et al., 2013; Kang et al., 2015). HDA18 has been suggested to affect cellular patternings in root epidermis (Xu et al., 2005; Liu et al., 2013a). HDA14 was reported to associate with iateed tomis (Tran et al., 2012). HDA15 forms a repression complex with PHYTOCHROME INTERACTING FACTOR 3 (PIF3), a transcription factor that is involved in photomorphogenesis, and regulates light-responsive genes in photosynthesis and chlorophyll biosynthesis (Liu et al., 2013b).

Arabidopsis has two SIR2-encoding genes which are *SRT1* and *SRT2*. SRT2 is involved in mitochondrial energy metabolism and metabolite transport (Koenig et al., 2014). SRT2 has a negative role in plant basal defense through suppression of SA biosynthesis (Wang et al., 2010). In plants, however, the functions of the SIR2 family (Sirtuins) HDACs are not fully understood.

The HD2 protein family is plant-specific. This group has four members:

HD2A/HDT1, HD2B/HDT2, HD2C/HDT3, and HD2D/HDT4. (Pandey et al., 2002). HD2A functions in seed development, and flowering and postembryonic establishment of nucleolar dominance (Wu et al., 2000; Zhou et al., 2004; Pontes et al., 2007). HD2A and HD2B control miR165/166 distribution and the formation of adaxial01baxial leaf polarity by independent association with ASYMMETRIC LEAVES1 (AS1) and AS2 (Ueno et al., 2007). In addition, the contribution of HDACs in plant responses to abiotic stresses has been revealed (Luo et al., 2012a; Yuan et al., 2013). HD2C functionally associates with HDA6 to repress the expression of *ABSCISIC ACID-INSENSITIVE 1* (*ABI1*), *ABI2*, and *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 4* (*ERF4*) (Luo et al., 2012a) and regulates rRNA gene expression through histone modifications (Kim et al., 2014). The expression of HD2-encoding gene itself is repressed by NaCl and ABA (Sridha and Wu, 2006; Luo et al., 2012b).

1.1.2.2 Histone methylation

Histone lysine residues can be methylated, and methylations are classified as mono-, di-, or tri-methylation depending on the number of added methyl groups. Different types of histone methylations, for instance histone H3 lysine 4 mono-/di-/tri-methylation (H3K4me1, H3K4me2, and H3K4me3), histone H3 lysine 27 trimethylation (H3K27me3), and histone H3 lysine 9 dimethylation (H3K9me2), have distinctive functions for epigenetic regulation of transcription.

1.1.2.2.1 H3K4 mono/ di/ trimethylation

H3K4me3 leads to gene transcription as an active mark while the correlations between gene transcription and other methylation marks on H3K4 residue, such as H3K4me1 and H3K4me2, are yet unclear (Zhang X et al., 2009). All of the H3K4 marks exist in euchromatin but not in heterochromatin which are mostly transposons and repetitive DNA sequences. While H3K4me1 is generally enriched in the gene body, H3K4me2 and H3K4me3 are enriched in the promoter region of the gene (Zhang X et al., 2009). The H3K4 methylation is highly conserved in other organisms (Liu et al., 2005; Li et al., 2008; Mikkelsen et al., 2007; Barski et al., 2007; Pokholok et al., 2005). A yeast H3K4 methyltransferase, called SET1, has a conserved SET domain and forms the COMPLEX PROTEINS ASSOCIATED WITH SET 1 (COMPASS) complex that regulates monomethylation, dimethylation, and trimethylation of H3K4 (Eissenberg et al. 2010). In *Drosophila*, homologs of yeast SET1, Trithorax group (TrxG) proteins and Absent, Small, or Homeotic Disc 1 (Ash1), mediate H3K4 methylation. All of TrxG proteins and Ash1 contain a SET domain (Byrd et al., 2003; Schulze et al., 2007).

In *Arabidopsis*, there are five ARABIDOPSIS TRITHORAX PROTEINS (ATXs), seven ARABIDOPSIS TRITHORAX RELATED PROTEINS (ATXRs), and seven ASH1 homologs of which four ASH1 HOMOLOG (ASHH) proteins and three ASH1-RELATED (ASHR) proteins are included (Baumbusch et al., 2001). ASHH2, also named as EARLY FLOWERING IN SHORT DAYS (EFS) or SET DOMAIN GROUP 8 (SDG8), is well studied as histone methyltransferase for both

H3K4 and H3K36 (Ko et al., 2010). ATX1 and ATX2 are reported to have divergent roles in the transcription regulation through H3K4me3 and H3K4me2 enrichments, respectively (Saleh et al., 2008; Alvarez-Venegas et al., 2005). ATXR3, also known as SDG2, mainly acts as a methyltransferase for H3K4me3 (Guo et al., 2010; Berr et al., 2010).

1.1.2.2.2 H3K27 tri-methylation

In *Arabidopsis*, H3K27me3 contribute to epigenetic silencing of several developmental genes such as *FLOWERINGLOCUS C* (*FLC*), *AGAMOUS* (*AG*), *SHOOT MERISTEMLESS* (*STM*), *MEDEA* (*MEA*), and *PHERES1* (*PHE1*) (Pien et al., 2007; Hennig et al., 2009). Distribution of H3K27me3 within *Arabidopsis* chromatin is restricted to short regions which only cover a single gene in contrast to animals (Zhang et al., 2007).

Polycomb group (PcG) proteins mediate tri-methylation of H3K27 in all organisms. PcGs form several complexes, including Polycomb Repressive Complex 1 (PRC1), PRC2, and Pho repressive complex (PhoRC) (Muller et al., 2009). PRC1 recognizes and supports H3K27me3 enrichment (Schwartz et al., 2007). Although any components of PRC1 are not encoded in *Arabidopsis*, Several PRC1-like activities have been reported to recognize H3K27me3 and contribute to the gene repression. In *Arabidopsis*, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)/TERMINAL FLOWER 2 (TFL2) (Zhang et al., 2007; Turck et al., 2007), VERNALIZATION 1 (VRN1) (Mylne et al., 2006), EMBRYONIC FLOWER 1 (EMF1) (Calonje et al., 2008), and AtRING1a and AtRING1b (Xu et al., 2008;

Sanchez-Pulido et al., 2008) are reported as analogues of PRC1 complex components. PRC2 has a key subunit called Enhancer of zeste (E(z)), a specific H3K27me3 methyltransferase. In *Arabidopsis*, three E(z) homologs have been reported; CURLY LEAF (CLF), MEA, and SWINGER (SWN) (Pien et al., 2007; Hennig et al., 2009).

1.1.2.2.3 H3K9 dimethylation

In general, heterochromatin formation depends on trimethylation of H3K9 and interaction of methylated H3K9 with Heterochromatin Protein 1 (HP1) which has a chromo-domain (Lachner et al., 2001; Nakayama et al., 2001; Bannister et al., 2001; Jacobs et al., 2001). In *Arabidopsis*, however, H3K9me3 is typically enriched in euchromatin (Turck et al., 2007). Instead, dimethylation of H3K9 mostly occurs in pericentromeric heterochromatin, transposons, and in small portions of euchromatic repeats (Fuchs et al., 2006). H3K9me2 closely correlates with CHG methylated regions (Bernatavichute et al., 2008).

Su(var)3-9 homologs have been shown in many organisms to play roles in heterochromatin formation and gene silencing (Nakayama et al., 2001; Peters et al., 2001). The Su(var)3-9 family proteins are identified as histone lysine methyltransferases (Rea et al., 2000; Schulze et al., 2007). The SUVH proteins are identified as Su(var)3-9 homologs in *Arabidopsis*. KRYPTONITE (KYP), also known as SUVH4, contributes to heterochromatin formation catalyzing H3K9me1 and H3K9me2 enrichments (Jackson et al., 2004; Malagnac et al., 2002). SUVH5 and SUVH6 are reported to methylate H3K9 and act as histone lysine

methyltransferases (Jackson et al., 2004; Ebbs et al., 2005 and 2006). Although epigenetic regulation mechanisms are shared in plant and other organisms, H3K9 methylation in plant is still obscure. For example, although methylated H3K9 is recognized by HP1, *Arabidopsis* LHP1 (a homolog of HP1) does not affect H3K9 methylation but recognizes H3K27me3 instead (Zhang et al., 2007).

1.1.2.2.4 Arginine methylation

Arginines of histone, including Arg2 (R2), Arg8 (R8), Arg17 (R17), Arg26 (R26) of H3, and Arg3 (R3) of H4, are easily methylated by protein arginine methyltransferases (PRMTs) (Bedford et al., 2005). PRMTs are classified as either type I or type II enzymes. Type I PRMTs catalyze asymmetric arginine dimethylation whereas type II PRMTs enrich symmetric arginine dimethylation (Bedford et al., 2009). In *Arabidopsis*, 9 PRMTs have been identified (Niu et al., 2007). As type I methyltransferases, AtPRMT1a, AtPRMT1b, and AtPRMT10, catalyze histone H4R3 asymmetric di-methylation (Niu et al., 2007; Yan et al., 2007). In addition, AtPRMT4a and AtPRMT4b catalyze asymmetric di-methylation of histone H3 R2, R17, and R26 *in vitro* and H3R17me2a *in vivo*. AtPRMT4a and AtPRMT4b are functionally redundant in the regulation of *FLC*-dependent flowering (Niu et al., 2008). AtPRMT5/SKB1 acts as a type II methyltransferase which dimethylates H4R3 symmetrically (Yan et al., 207; Pei et al., 2007; Schmitz et al., 2008; Wang et al., 2007) Taken together, AtPRMT4a, AtPRMT4b, AtPRMT5/SKB1, and AtPRMT10 are involved in regulation of flowering time, even though they show distinctive enzymatic activities from each other (Niu et al.,

2007; Niu et al., 2008; Pei et al., 2007; Schmitz et al., 2008; Wang et al., 2007).

1.1.2.2.5 Readers of histone methylation

Currently, three superfamilies are classified according to their conserved reader-module types. There are chromo-domain or chomo-like-domain-containing superfamily, plant homeo domain finger (PHD) superfamily, and proteins containing WD40 repeats of WDR5 (Ruthenburg et al., 2007; Taverna et al., 2007). In *Arabidopsis*, a few proteins have been shown to recognize methylated histone as readers. LHP1/TFL2, homologue of the Drosophila heterochromatin protein 1 (HP1) which recognizes H3K9me3, can also bind to PRC2-mediated H3K27me3 modification through its chromo-domain (Exner et al., 2009; Gaudin et al., 2001; Turck et al., 2007; Zhang et al., 2007). The binding of LHP1/TFL2 to H3K27me3 is required to maintain silencing of *FLC* (Mylne et al., 2006) and *FT* (Kotake et al., 2003; Farrona et al., 2008; Turck et al., 2007). The origin recognition complexes (ORCs), ORC1a and ORC1b, contain PHD domains and interact with H3K4me3 (de la Paz Sanchez et al., 2009). Additionally, AtING and AL, PHD proteins, are identified as readers that recognize H3K4me2 and H3K4me3 *in vitro* (Lee et al., 2009). The *Arabidopsis* WDR5a is a homolog of human WDR5 which reads H3K4me2. WDR5a is enriched at the *FLC* locus and is suggested to recognize H3K4 methylation in the presence of FRI (Jiang et al., 2009).

1.1.2.2.6 Erasers of histone methylation

Histone demethylase plays a crucial role as an eraser in histone methylation mechanism. Recently, two classes of enzymes are demonstrated as histone demethylases, which are LYSINE SPECIFIC DEMETHYLASES 1 (LSD1) and JUMONJI C (JmjC) domain–containing proteins. LSD1 and JmjC domain-containing proteins are capable of removing methyl groups from methylated lysine residues through distinct mechanisms of amine oxidation and hydroxylation, respectively. (Shi et al., 2004; Tsukada et al., 2006). LSD1 specifically demethylates mono- and di-methylated but not tri-methylated H3K4 (Shi et al., 2004). In *Arabidopsis*, four LSD1 homologs, FLOWERING LOCUS D (FLD), LSD1-LIKE 1 (LDL1), LDL2, and LDL3, are identified. FLD, LDL1, and LDL2 participate in *FLC* repression as H3K4 demethylases (He et al., 2003; Liu et al., 2007; Spedaletti et al., 2008). LDL1 and LDL2 are also involved in the regulation of *FLOWERING WAGENINGEN* (*FWA*) in vegetative tissues (Jiang et al., 2007).

Unlike LSD1, JmjC domain-containing proteins have demethylase activities towards all of the mono-, di- and tri-methylated lysines of histones (Klose et al., 2006). In *Arabidopsis*, 21 JmjC domain–containing proteins (JMJs) are classified into five subfamilies, including the KDM3/JHDM2, KDM4/JHDM3, KDM5/JARID1, JMJD6, and JmjC domain–only groups. (Hong et al., 2009; Lu et al., 2008). KDM4/JHDM3 group proteins catalyze demethylation of di- and trimethylated histone H3K9 and H3K36 in mammals (Klose et al., 2007). The homolog of KDM4/JHDM3 group proteins, EARLY FLOWERING 6 (ELF6/JMJ11) and RELATIVE OF EARLY FLOWERING 6 (REF6/JMJ12), are functionally characterized in flowering pathways (Lu et al., 2008; Noh et al., 2004). ELF6 and REF6 play divergent roles in the regulation of photoperiodic flowering

and *FLC*, respectively (Noh et al., 2004). More recently, a study suggested that ELF6 and REF6 are involved in the brassinosteroid signaling, being able to catalyze H3K9 demethylation (Yu et al., 2008). As a homolog of human KDM3/JHDM2, INCREASED EXPRESSION OF BONSAI METHYLATION 1 (IBM1/JMJ25) contributes to the repression of the cytosine methylation mechanism, possibly through demethylation of H3K9me1 and me2 (Lu et al., 2008; Miura et al., 2009; Sazeet al., 2008). However, biochemical properties of JMJs as H3K9 demethylase are still remained to be elucidated. The MATERNAL EFFECT EMBRYO ARREST 27 (MEE27/JMJ15), one of KDM5/JARID1 group protein, is reported to be involved in female gametophyte development as a H3K4 demethylase (Pagnussat et al., 2005; Lu et al., 2008). Another KDM5/JARID1 group protein *Arabidopsis thaliana* Jumonji4 (AtJmj4/JMJ14) also shows a demethlyase activity on H3K4me3, H3K4me2, and H3K4me1 and has a role in flowering regulation (Jeong et al., 2009; Yang et al., 2010; Lu et al., 2010). In spite of the efforts to discover powerful histone demethylases, there are still unsolved questions, such as how demethylases functionally regulate developmental processes in plants. Therefore, additional researches on histone demethylation are needed at biochemical and genetic levels.

1.2 Flowering

Flowering, a transition from vegetative to reproductive phases, is important in ensuring the reproductive success in plant life. Determining the proper time for floral transition is controlled by the expression of various flowering-regulatory

genes that are sensitive to internal signals and environmental cues, such as photoperiod and temperature. When a plant acquires potency to flower by endogenous signals, such as hormones, to achieve the appropriate developmental stage, external signals are then incorporated for flowering. In *Arabidopsis thaliana*, it has been shown that various molecular pathways, including photoperiod, vernalization, autonomous, and many others, integrate internal and external cues. Moreover, recent studies have demonstrated that a number of chromatin modifiers contribute to floral transition by regulating key flowering regulatory genes, such as *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T* (*FT*).

1.2.1 FLC, a central floral repressor

Determination of summer-annual accession (which flowers rapidly without prolonged period of cold exposure) or winter-annual accession (which requires vernalization prior to flowering) depends on the expression level of *FLC* (Sheldon et al., 2000; Johanson et al., 2000). A winter-annual possesses dominant *FRI* allele that activates *FLC* expression, whereas a summer-annual has a non-functional *fri* allele (Michaels and Amasino, 2001). FLC, a MADS-box transcription factor, inhibits floral transition by repressing downstream floral integrators *FT*, *FLOWERING LOCUS D* (*FD*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Michaels and Amasino, 1999; Sheldon et al., 1999; Searle et al., 2006). Previous studies have demonstrated that *FLC* is also regulated by chromatin modifications such as H3K4me3, H3K27me3, and others. (He et al.,

2004; Pien et al., 2008; Schuettengruber et al., 2007; Sheldon et al., 2006; Wood et al., 2006; Jiang et al., 2008).

1.2.1.1 Chromatin-mediated activation of *FLC*

H3K4me3 is associated with active gene expression. In *Arabidopsis*, accumulation of H3K4me3 within *FLC* chromatin leads to active *FLC* expression. (He et al., 2004; Pien et al., 2008). Former studies have demonstrated that homologs of yeast and human PAF1 complex, called PAF1-like complex in *Arabidopsis*, recruits MLL/COMPASS to activate *FLC* expression (Zhang and van Nocker, 2002; Zhang et al., 2003; He et al., 2004; Oh et al., 2004). PAF1-like complex components include EARLY FLOWERING 7 (ELF7), the homolog of yeast Paf1, EARLY FLOWERING 8 (ELF8)/VERNALIZATION INDEPENDENCE 6 (VIP6), the homolog of the yeast Ctr9, VIP3, the homolog of human hSki8, VIP4, the homolog of the yeast Leo1, and VIP5, the homolog of yeast Rtf1 (Zhang and van Nocker, 2002; Zhang et al., 2003; He et al., 2004; Oh et al., 2004). It is well-known that PAF1-like complex functions with COMPASS-like H3K4 methyltransferases, ARABIDOPSIS TRITHORAXs (ATXs), to activate *FLC* through the accumulation of H3K4m3 on near transcription start site (TSS) region of the *FLC* locus (Pien et al., 2008; Tamada et al., 2009; Berr et al., 2010; Yun et al., 2012; Guo et al., 2010).

In addition to H3K4me3, H3K36me2 and H3K36me3 are also required for *FLC* regulation. Several studies have reported that EARLY FLOWERING IN SHORT DAYS (EFS)/SET DOMAIN GROUP 8 (SDG8) catalyzes H3K36 methylation within *FLC* chromatin and is also involved in H3K4me3 enrichment

especially with functional FRI protein (Pien et al., 2008; Xu et al., 2008; Ko et al., 2010).

Moreover, histone H2B monoubiquitylation (H2Bub1) acts as an activation mak in *FLC* expression (Liu et al., 2007b; Cao et al., 2008; Gu et al., 2009; Xu et al., 2009a). In yeast, H2B-monoubiquitylating complex is composed of Rad6, an E2 ubiquitin-conjugating enzyme, and Bre1, an E3 ubiquitin ligase, and modifies H2B of target chromatin (Wood et al., 2003). This leads to transcription activation and elongation of the target gene (Pavri et al., 2006; Shilatifard, 2006). In *Arabidopsis*, ubiquitin-conjugating enzyme 1 (UBC1), UBC2, and UBC3, which are homologue of Rad6 (Sullivan et al., 1994; Kraft et al., 2005), and HISTONE MONOUBIQUITINATION 1 (HUB1) and HUB2, which are homologs of Bre1 (Fleury et al., 2007; Liu et al., 2007b), are identified as E2 and E3 ubiquitin ligases, respectively. *Arabidopsis* H2B-monoubiquitylating complex catalyzes H2Bub1 on *FLC* locus and induces either H3K4me3 accumulation or a movement of RNA polymerase II (Pol II) towards the gene body (Cao et al., 2008; Gu et al., 2009).

A histone variant H2A.Z deposition is also associated with *FLC* activation through the *Arabidopsis* SWR1 complex (AtSWR1c) (Deal et al., 2007; Choi et al., 2007). PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1) (Noh and Amasino, 2003), ACITN-RELATED PROTEIN 4 (ARP4) (Kandasamy et al., 2005), SUPPRESSOR OF FRIGIDA 3 (SUF3)/ACITN-RELATED PROTEIN 6 (ARP6)/EARLY IN SHORD DAYS 1 (ESD1) (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), and SWC6 (Choi et al., 2007), are identified as the homologous components of the yeast SWR1c in *Arabidopsis*. Several studies have suggested that AtSWR1c accumulates H2A.Z at the *FLC* locus and mediates *FLC*

transcription (Choi et al., 2007; Zilberman et al., 2008).

Besides chromatin modifiers, FRIGIDA complex (FRIc) activates *FLC* expression as well. Specific regulators, FRIGIDA-LIKE 1 (FRL1), FRIGIDA-ESSENTIAL 1 (FES1), SUPPRESSOR OF FRIGIDA 4 (SUF4), and FLC EXPRESSOR (FLX), form the transcription activator complex FRIc (Schmitz et al., 2005; Kimet al., 2006; Kim and Michaels, 2004 and 2006, Andersson et al., 2008; Choi et al., 2011). Through interacting with FRI, the complex recruits not only general transcription factors but also chromatin modifiers, such as EFS, SWR1c, and COMPASS, on *FLC* chromatin and thus leads to *FLC* activation. (Ko et al., 2010; Jiang et al., 2009; Choi et al., 2011).

1.2.1.2 Chromatin-mediated repression of *FLC*

1.2.1.2.1 The autonomous pathway

In summer-annual accession with a non-functional *fri* allele, autonomous genes, for instance *FCA*, *FPA*, *FLOWERING LATE KH MOTIF* (*FLK*), *LUMINIDEPENDENS* (*LD*), *FY*, *FVE*, and *FLOWERING LOCUS D* (*FLD*), repress *FLC* expression. Autonomous-pathway genes are also involved in various epigenetic mechanisms such as RNA-mediated silencing, demethlaytion of H3K4me2, and deacethylation of H3 acetylation (H3Ac) to repress *FLC* expression.

Several studies have suggested that RNA recognition motif (RRM) type RNA-binding proteins facilitate chromatin structural change and gene silencing (Baurle and Dean, 2008; Baurle et al., 2007). FCA and FPA, putative RNA-binding

proteins, contain RRM domains and they are involved in RNA-mediated *FLC* chromatin silencing (Liu et al., 2010). FY, a homolog of the yeast polyadenylation factor Pfs2p, interacts with FCA and acts in the 3'-end processing of *FLC* transcript (Simpson et al., 2003). FLD, an H3K4 demethylase, is necessary for the FCA- and FPA-mediated *FLC* repression (Liu et al., 2007; Baurle and Dean, 2008). In addition to FLD, FVE also plays a role in *FLC* silencing in cooperation with FPA (Veley et al., 2008). RNA metabolism and processing mechanisms cause repressive modification of chromatin and*FLC* repression (Baurle et al., 2007; Liu et al., 2010).

Moreover, histone deacetylation or demethylation of active marks, such as H3Ac, H3K4me3, H3K36me2, and H3K36me3, are associated with gene repression in general. *Arabidopsis* FVE and FLD are homologs of the musashi RNA-binding protein 1 (MSI1) and the lysine-specific histone demethylase (LSD1), respectively (Ausin et al., 2004; Sanda and Amasino, 1996; He et al., 2003; Metzger et al., 2005) Recently, the histone deacetylation complex, FVE-FLD-HDA6 complex, wasis identified, and it results in *FLC* repression (Ausin et al., 2004). Additionally, FLD has an effect on demethylation of H3K4me1 and H3K4me2 at the *FLC* locus. (He et al., 2003; Jiang et al., 2007; Liu et al., 2007a)

1.2.1.2.2 Histone arginine methylation

Furthermore, protein arginine methyltransferases (PRMTs), catalyzing histone arginine methylation, also play an essential role in *FLC* repression. In *Arabidopsis*, a type I PRMT, AtPRMT10, catalyzes histone H4 arginine 3 asymmetric dimethylation (H4R3me2a) and represses *FLC* expression (Niu et al., 2007).

AtPRMT5, a type II PRMT, contributes to histone H4 arginine 3 symmetric dimethylation (H4R3me2s) within *FLC* chromatin (Pei et al., 2007; Wang et al., 2007). AtPRMT5 has an important function in the establishment and maintenance of vernalization-induced *FLC* silencing in functional *FRI* background. In *atprmt5* mutants of FRI background, both H3K9 and H3K27 methylation levels at the *FLC* locus do not increase by vernalization (Robert et al., 2007). In addition, two type II PRMTs, AtPRMT4a and AtPRMT4b, mediate H3R17me2a and redundantly repress *FLC* expression (Niu et al., 2008).

1.2.1.2.3 Vernalization

In winter-annual plants, vernalization, a prolonged cold exposure, is required to ensure competence to flower. Vernalization mediates epigenetic changes, such as accumulation of H3K27me3 and H3K9me3, within *FLC* chromatin and results in *FLC* silencing. Once silenced by vernalization, repressed *FLC* expression and its compacted chromatin state are mitotically maintained even when the growth condition turns back warm (Kim et al., 2009; Dennis et al., 2007; Angel et al., 2011). A complete silencing of *FLC* during vernalization requires PcG or PcG-related components and long non-coding RNAs (lncRNAs) (Wood et al., 2006; Dennis et al., 2007; Angel et al., 2011; Helliwell et al., 2011; Heo and Sung, 2011).

VERNALIZATION INSENSITIVE 3 (VIN3) (Sung and Amasino, 2004), a plant homeodomain (PHD) protein, is induced by vernalization and forms a complex with PRC2 components, CURLY LEAF (CLF)/SWINGER (SWN), FERTILIZATION INDEPENDENT ENDOSPERM (FIE), MULTI-COPY

SUPPRESSOR OF IRA 1 (MSI1), VERNALIZATION 2 (VRN2), and VRN5/VIN3-LIKE 1 (VIL1) (Kim et al., 2009; De et al., 2008; Wood et al., 2006). The PHD-PRC2 complex deposits H3K27me3 around the first exon of *FLC*, and H3K27me3 subsequently spreads over the entire *FLC* locus (De et al., 2008; Angel et al., 2011; Finnegan and Dennis, 2007). The enriched H3K27me3 then recruits a component of PRC1, LHP1, and a putative PRC1 component, VERNALIZATION 1 (VRN1), to the *FLC* locus. These two repressor complexes are required for the maintenance of stable *FLC* silencing after the cold (Sung et al., 2006; Zheng and Chen, 2011; Turck et al., 2007)

1.2.1.2.4 lncRNAs *COOLAIR* and *COLDAIR*

Recent studies have reported that some RNA transcripts also participate in *FLC* repression. Cold induces two types of long non-coding RNAs (lncRNAs), cold induced long antisense intragenic RNA (COOLAIR) and COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), that function in the vernalization-mediated *FLC* silencing (Swiezewski et al., 2009; Heo and Sung., 2011). While COOLAIR produces polyadenylated antisense *FLC* transcripts, COLDAIR generates unpolyadenylated sense *FLC* transcripts from the first intron of *FLC*. Moreover, *COLDAIR* physically interacts with CLF, recruits the PHD–PRC2 complex to *FLC* chromatin, and thereby represses *FLC* expression (Heo and Sung, 2011). Whereas the presence of COLDAIR is crucial for *FLC* silencing, it has been revealed that *COOLAIR* is not critical in *FLC* silencing, i.e., removal of *COOLAIR* did not affect *FLC* expression level during vernalization (Helliwell et al., 2011).

1.2.2 FT, a key floral inducer

In addition to FLC, FT plays a key role as florigen in promotion of floral transition. *FT* is directly repressed by FLC, whereas CONSTANS (CO) activates *FT* under long day conditions (Searle et al., 2006; Helliwell et al., 2006; Li et al., 2008). FT protein moves from leaves, through phloem, to the shoot apical meristem (SAM), where FT forms a protein complex with a bZIP transcription factor, FLOWERING LOCUS D (FD) (Abe et al., 2005; Wigge et al., 2005). The FT-FD complex subsequently activates floral-meristem identity genes, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*). Functions of such genes are associated with floral meristem differentiation during flowering initiation (Wigge et al., 2005; Abe et al., 2005).

1.2.2.1 Chromatin-mediated *FT* regulation

Recent studies have revealed that *FT* is partially regulated by epigenetic mechanisms. The concurrent actions and crosstalks between the active mark H3K4me3 and the repressive mark H3K27me3 have been reported in detail (Jiang et al., 2008; Yang et al., 2010). Several chromatin-remodeling factors, such as LHP1/TFL2, PRC2, EARLY FLOWERING 6 (ELF6), AtJMJ4/JMJ14, andHDACs are also involved in the chromatin-mediated *FT* regulation.

H3K27me3 is a typical repressive mark, which is mediated by PcG proteins. It has been revealed that H3K27me3 is extensively enriched within *FT* chromatinincluding several kilobases upstream and downstream of the coding

region (Adrian et al., 2010).CLF, a component of PRC2, directly associates with *FT* chromatin and deposits H3K27me3 (Jiang et al., 2008; Farrona et al., 2011; Lopez-Vernaza et al., 2012). Additionally, other PRC2 components, for instance SWINGER (SWN), EMBRYONIC FLOWER 2 (EMF2), and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), are also involved inH3K27me3 to repress *FT* expression (Jiang et al., 2008; Farrona et al., 2011). H3K27me3 mark is recognized by PRC1. Components of plant PRC1, such as LHP1/TFL2 and EMF1, are also required for *FT* repression. LHP1 binds to *FT* chromatin and suppresses *FT* transcription via enriched H3K27me3 (Kotake et al., 2003; Farrona et al., 2008; Turck et al., 2007). Together with these findings, it is suggested that PRC1 and PRC2 cooperate in the repression of *FT* to build and maintain H3K27me3 (Jiang et al., 2008; Farrona et al., 2011; Turck et al., 2007; Moon et al., 2003; Bratzel et al., 2010). Recently, it has been reported that REF6 antagonizes H3K27me3-methyltransferase activity in the epigenetic regulation of *FT* (Lu et al., 2011).

On the other hand, H3K4me3 also participates in epigenetic regulation of *FT*. Lack of H3K27me3 owing to loss of PRC2 results in elevated level of H3K4me3 at *FT*. PRC2-dependent H3K27me3 suppresses H3K4me3 deposition (Jiang et al., 2008). Moreover, several members of the Jmj-family proteins were revealed to act as H3K4 demethylases at the *FT* locus in *Arabidopsis*. Two *Arabidopsis* Jmj-family proteins, AtJmj4 and ELF6, were identified as repressors of *FT* via demethylation of H3K4 (Jeong et al., 2009). They directly associate with *FT* locus and demethylate H3K4me2 and H3K4me3 within *FT* chromatin. (Yang et al., 2010; Jeong et al., 2009; Lu et al., 2010). The increase of H3K4me3 due to loss of *AtJmj4* results in the reduction of H3K27me3 (Yang et al., 2010). Thus, *FT*

expression *FT* is regulated by relative levels of H3K27me3 and H3K4me3.

In addition to the regulatory mechanism of bivalent H3K27me3 and H3K4me3 marks at the *FT* locus, other chromatin-mediated mechanisms, such as H3K36me3, H3Ac, and H2A.Z-related regulation, have also been revealed to influence *FT* expression. Recent studies have reported that *FT* expression is also affected by H3K36 methylation. The *Arabidopsis* Morf Related Gene 1 (MRG1) and MRG2 proteins have been identified as H3K36 methylation readers (Xu et al., 2014; Bu et al., 2014). MRG1 and 2 proteins have important roles in the CO-dependent *FT* activation under the photoperiodic flowering pathway. MRG2 directly binds to the *FT* locus and contributes to the activation of *FT* in H3K36me3-dependent manner (Xu et al., 2014; Bu et al., 2014).

Current findings also suggest that HDACs remove acetyl marks on histones at *FT* specifically at dusk. Two plant-unique functional relatives of the yeast SAP30, SAP30 FUNCTION-RELATED 1 (AFR1) and AFR2, take a part of HDAC complexes, AFR1-HDAC or AFR2-HDAC, to modulate the acetylation level of *FT* chromatin (Gu et al., 2013). AFR1/AFR2-HDAC complex binds to the *FT* promoter region and catalyzes histone deacetylation of *FT* chromatin, which requires CO activity at the end of long days (16 hr light/8 hr dark) (Gu et al., 2013). Moreover, the MADS-domain transcription factor AGAMOUS-LIKE 18 (AGL18) interacts with AFR1 and AFR2 and recruits the AFR1/AFR2-HDAC to *FT* chromatin. AGL18-AFR-HDAC acts as a repressive *FT* regulator to moderate *FT* expression.

Additionally, the SWR1 complex is participated in the *FT* regulation

mechanism through H2A.Z deposition at *FT*. (Kumar and Wigge, 2010). *FT* is regulated by H2A.Z-containing nucleosomes under the thermo-sensory pathway (Blazquezet et al., 2003). A rise of ambient temperature results in the eviction of the H2A.Z from *FT* nucleosome and causes increased *FT* transcription (Kumar and Wigge, 2010). EARLY BOLTING IN SHORT DAYS (EBS) is also involved in *FT*-chromatin remodeling mechanism which leads to *FT* repression (Castillejo and Pela; 2008; Gómez-Mena et al., 2001; Piñeiro et al., 2003).

1.3 Seed germination

Seed germination is an important process in higher plants, and it is controlled by a combination of internal and environmental factors. Environmental conditions, such as light, temperature, nutrient, and moisture, affect seed germination. Seed dormancy, one of core internal factors, prevents germination of viable seeds even if seeds are exposed to favorable external conditions. Phytohormones, including gibberellic acid (GA), abscisic acid (ABA), ethylene, brassinosteroid (BR), and auxin, which elicit response from both external and internal signals, can also influence seed germination (Holdsworth et al., 2008; Nambara et al., 2010). Among them, GA and ABA play central but antagonistic roles in the regulation of seed germination. Their molecular mechanisms have been extensively studied at physiological and molecular levels for many years (Koorneef and Karssen., 1994). GA- and ABA-mediated seed germination will be further discussed in detail. Additionally, interactions and crosstalks between different hormones have been studied in recent years (Wolters and Jurgens, 2009; Gazzarrini and McCourt, 2003).

1.3.1 Gibberellins (GA)

GA plays a central role in plant developmental processes including seed germination, leaf expansion, stem elongation, and flowering (Schwechheimer and Willige, 2009). In *Arabidopsis*, severe GA-deficient mutants, such as *ga1-3* and *ga2-1*, are shown to have a defective phenotype in seed germination (Koornneef and van der Veen, 1980). GA biosynthetic inhibitors, paclobutrazol (PAC) and uniconazole, also prohibit seed germination (Nambara et al., 1991; Jacobsen and Olszewski, 1993). GA is necessary for embryo growth and radicle protrusion in weakening tissues that surround the embryo such as endosperm and testa. (Groot and Karssen, 1987; Silverstone et al., 1997; Telfer et al., 1997). Two *Arabidopsis* GA biosynthetic genes, *GA3ox1* and *GA3ox2*, are mainly expressed during imbibition in embryo axis. They encode GA3 oxidases that catalyze the conversion of inactive GA into active form during seed germination. (Yamauchi et al., 2004; Mitchum et al., 2006).

GA recognition is also important. Interaction between GA receptors and DELLA-domain proteins affects the germination potential of seeds. GA receptors, including GA INSENSITIVE DWARF 1A (GID1A), GID1B, and GID1C, function as key components of GA signaling. Loss of all three *GID1* causes germination failure (Griffiths et al., 2006; Willige et al., 2007; Nakajima et al., 2006). Five members of DELLA-domain proteins, GA INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3, act as negative regulators in GA responses (Sun and Gubler, 2004). Especially, RGL2 plays a

major role in the regulation of seed germination. Loss of RGL2 function causes insensitivity to PAC and restores germination ability in *ga1* mutant background. (Lee et al., 2002; Tyler et al., 2004). Other DELLA proteins only have a minor function in germination (Koornneef et al., 1985; Cao et al., 2005).

1.3.2 Abscisic acid (ABA)

ABA is a key hormone in plant stress-responses, development, seed dormancy, and seed germination (Zhu, 2002; Sharp, 2002; Nambara et al., 2010; Finkelstein et al., 2002). ABA facilitates processes that link environmental changes to plant responses. Against drought and salt stresses, plant responses are controlled by the regulation of ABA synthesis and ABA-mediated signaling. (Schroeder et al., 2001; Cutler et al., 2010; Weiner et al., 2010). In addition to stress responses, several developmental processes, including seed maturation and seed germination, require ABA- dependent regulation (Finkelstein et al., 2002). A decrease of endogenous ABA level is needed for seed germination. Treatment of exogenous ABA during seed germination leads to failure of endosperm weakening and rupture (Muller et al., 2006; Karin et al., 2011) A higher level of endogenous ABA contents in imbibed seeds results in the inhibition of seed germination (Nambara et al., 2010). In addition, ABA is involved in the maintenance and reinforcement of seed dormancy (Nambara et al., 2010). Nine-cis-epoxycarotenoiddioxygenases (NCEDs) and ABA 8'-hydroxylases (CYP707As) function in ABA biosynthesis and degradation, respectively (Seo et al., 2006; Toh et al., 2008). *CYP707A2* is highly expressed in the radicle during seed imbibition and affects seed germination

(Okamoto et al., 2006). Several *ABA-INSENSITIVE* (*ABI*) genes are involved in the ABA signaling pathway. Mutations of *ABI3*, *ABI4*, and *ABI5* genes showed ABA-insensitive phenotypes during seed germination and early seedling development (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000b; Soderman et al., 2000). ABI5 transcription factor, which is positively regulated by ABI3, plays an important role in ABA signaling (Finkelstein and Lynch, 2000b; Finkelstein et al., 2002).

1.3.3 Ethylene

Ethylene positively acts on seed germination. In other words, it is antagonistic to ABA but synergistic to GA in *Arabidopsis*. (Kepczynski and Kepczynska, 1997; Beaudoin et al., 2000; Ghassemian et al., 2000) ACC OXIDASE (ACO), a key enzyme in ethylene production, is involved in endosperm rupture to oppose the ABA-mediated inhibition of the rupture. (Kucera et al., 2005; Matilla and Matilla-Va´zquez, 2008; Linkies et al., 2009). ACO activity is repressed by ABA in the radicle, while ABA level is not affected by ethylene. Thus, ethylene counteracts to ABA-mediated inhibition of seed germination via interference of ABA signaling (Linkies et al., 2009). Ethylene synthesis and response are activated by GA. It is reported that *ACO* expression is increased by exogenous GA4 in imbibed *Arabidopsis* seeds (Ogawa et al., 2003). Ethylene-inducible genes, *HOOKLESS 1* (*HLS1*) (Lehman et al., 1996) and *ETHYLENE RESPONSE SENSOR 1* (*ERS1*) (Hua et al., 1998), are up-regulated by GA4 (Ogawa et al., 2003).

1.3.4 Light signaling

As an environmental cue, light is one of the most important factors in plant growth and development, especially in seed germination. The perception of light signals by photoreceptors is crucial in the light signaling pathway (Jiao et al., 2007). Phytochromes (phyA and phyB) or crytochromes (cry1 and cry2) can perceive far-red/red light or blue light, respectively. PHYTOCHROME-INTERACTING FACTORS (PIFs), a group of basic helix-loop-helix (bHLH) transcription factors, function as signaling integrators of light and hormones (Leivar et al., 2008; Castillon et al., 2007). The light-dependent plant developmental process involves many hormonal pathways (Neff et al., 2006). PIF3 and PIF4 participate in photomorphogenesis of seedling and another member, PIF3-LIKE 5 (PIL5)/PIF1, regulates seed germination (Nemhauser et al., 2008; Alabadı´ et al., 2009).

As mentioned above, PIF1 is involved in phytochrome-mediated seed germination and affects GA and ABA signaling also. PIF1 regulates GA responses through direct targeting of *DELLA* genes. GAI and RGA functionally repress GA signaling (Oh et al., 2007). In dark, PIF1 self-activates its transcription and leads to inhibition of GA-induced seed germination. Moreover, PIF1 indirectly affects ABA biosynthesis and *ABI3* expression (Piskurewicz et al., 2009). In short, PIF1 acts as a key regulator in dark to repress seed germination through ABA- and GA-mediated metabolism and signaling pathways (Oh et al., 2006 and 2007). PIF1-downstream proteins also play important roles in seed germination. SOMNUS (SOM), a nuclear-localized CCCH-type zinc-finger protein, participates in the inhibition of seed germination by maintaining balance between GA and ABA levels.

Loss-of-function of *SOM* shows a germination phenotype in dark without any light signals (Kim et al., 2008). DOF AFFECTING GERMINATION 1 (DAG1), a DNA-binding One Zinc Finger (DOF) transcription factor, directly binds to *GA3ox1* and negatively regulates *GA3ox1* transcription (Gabriele et al., 2010).

1.3.5 Epigenetic aspects of seed germination.

Seeds of higher plants undergo several phase transitions, such as seed germination and flowering. Seed maturation and germination are largely regulated through epigenetic mechanisms. For example, DNA methylation-related genes are needed for proper embryo developments and seed viability (Xiao et al., 2006). Reduced nuclei with highly condensed chromatin is established during seed maturation and the reduced nuclear size is restored through germination process (van Zanten et al., 2011). A few studies have demonstrated that DNA methylation is involved in the regulation of seed germination. MicroRNA 402 (miR402) targets *DNA GLYCOSYLASE DEMETER-LIKE PROTEIN 3* (*DML3*) (Sunkar and Zhu, 2004). Under high salt or cold stresses, miR402 constitutively promotes seed germination, suggesting that maintenance of 5-mC level at the *DML3* locus is important in the inhibition of seed germination (Kim et al., 2010).

The DELLA protein RGL2, which plays a key role in GA signaling, also promotes ABA biosynthesis and ABI5 activity (Piskurewicz et al., 2008). Contrarily, other type of DELLA proteins, GAI and RGA, participate in the repression of seed germination in dark (Cao et al., 2005). SPATULA (SPT) and PIF1, two bHLH transcription factors, are reported to repress seed germination by

affecting GA signaling (Penfield et al., 2005; Oh et al., 2004, 2006 and 2007). SPT is also involved in the establishment of dormancy by the repression of *GA3 oxidases*. PIF1 represses not only *GA3 oxidases* but also GA responses via stimulating RGA and GAI (Oh et al., 2007). Active transcription marks (H3K4me3, H3K36me3, and H3K9Ac) are involved in the regulation of DELLA proteins such as RGL2, GAI, and RGA, and germination-related transcription factors, including SPT and PIF1 (Roudier et al., 2011; Charron et al., 2009). In addition, the repressive mark H3K27me3 is also involved in the transcriptional regulation of *GAI*, *RGA*, and *PIF1* (Zhang et al., 2007a; Charron et al., 2009). Other histone repressive marks, H4R3me2 and H3R2me2, are also reported to regulate the transcription of GA anabolic genes, *GA3ox1* and *GA3ox2*. *Arabidopsis* histone arginine demethylases, JMJ20 and JMJ22, directly target *GA3ox1* and *GA3ox2* and enhance seed germination by eliminating H4R3me2s at the *GA3ox1* and *GA3ox2* loci (Cho et al., 2012).

Repression of embryonic properties is required for germination. PICKLE (PKL), the CHD3-class SWI/SNF chromatin-remodeling factor, is needed for the repression of embryonic properties, which is regulated by seed-maturation regulators such as LECs, FUS3, and ABI3 (Ogas et al., 1997 and 1999; Rider et al., 2003). The expression level of *PKL* is low in dry seed but is increased during imbibition (Henderson et al., 2004; Li et al., 2005). In *pkl* mutant seeds, H3K27me3 level is lower compared to wild type at the *LEC1* and *LEC2* loci (Zhang et al., 2008). Moreover, it is recently reported that DNA methylation is also involved in PKL-mediated *LEC1* silencing during germination (Zhang et al., 2012a). Unfavorable environmental conditions such as osmotic stress cause a

growth arrest at the early phase of seed germination. ABI3 and ABI5 are key factors in stress-response mechanisms. In ABA-dependent manner, PKL plays a negative role in an ABA signaling during seed germination (Perruc et al., 2007). Repressive marks, H3K9me and H3K27me, are reduced in *pkl* mutant at the *ABI3* and *ABI5* loci, and the reduction of repressive marks is induced by the treatment of exogenous ABA (Perruc et al., 2007). Therefore, PKL acts as a negative regulator in ABA-dependent seed germination via enrichment of repressive histone marks at the *ABI3* and *ABI5* loci (Perruc et al., 2007; Zhang et al., 2012a).

In addition to PKL, HDA6 and HDA19 redundantly act to repress embryonic properties during early seed germination (Tanaka et al., 2008). *hda6 hda19* double mutant was shown to have various germination-related phenotypes such as growth arrest, failure to proceed cotyledon greening and expansion, and a perpetuation of embryo-like phenotypes after germination (Tanaka et al., 2008). HDA6 is partially redundant with PKL and acts in the DICER-LIKE 1 (DCL1)-mediated *LEC2* and *FUS3* repression mechanism (Willmann et al., 2011). According to recent studies, LHP1, a component of PRC1, is possibly involved in the repression of *LEC1*, *LEC2*, *FUS*, and *ABI3* (Zhang et al., 2007a and 2007b; Charron et al., 2009). FIE, a component of PRC2, is suggested to participate in the regulation mechanisms of seed dormancy and germination. *fie* mutants exhibit germination-defective phenotypes and reinforced seed dormancy (Bouyer et al., 2011). EARLY FLOWERING IN SHORT DAYS (EFS), a putative histone methyltransferase, was reported as a negative regulator of seed germination: *efs* mutant shows an early seed-germination phenotype (Bassel et al., 2011).

However, so far little is known about epigenetic regulatory mechanisms of

seed germination. As described above, efforts to prove that epigenetic regulators play a critical role in seed germination is in progress. Therefore, epigenetic regulation has been considered a promising mechanism to illuminate the veiled area of seed germination.

Chapter II

Repression of *FLOWERING LOCUS T* Chromatin by Functionally Redundant Histone H3 Lysine 4 Demethylases in *Arabidopsis*

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2.1 Abstract

FLOWERING LOCUS T (FT) plays a key role as a mobile floral induction signal during the floral transition. Therefore, precise control of *FT* expression is critical for the reproductive success of flowering plants. Coexistence of bivalent histone H3 lysine 27 trimethylation (H3K27me3) and H3K4me3 marks at *FT* locus and the role of H3K27me3 as a strong *FT* repression mechanism in *Arabidopsis* have been reported. However, the role of an active mark, H3K4me3, in *FT* regulation has not been addressed, nor have the components affecting this mark been identified. Mutations in *Arabidopsis thaliana Jumonji4* (*AtJmj4*) and *EARLY FLOWERING6* (*ELF6*), two of the *Arabidopsis* genes encoding Jmj family proteins, caused an *FT*-dependent additive early flowering correlated with increased expression of *FT* mRNA and increased H3K4me3 levels within *FT* chromatin. Purified recombinant AtJmj4 protein possessed specific demethylase activity for mono-, di-, and trimethylated H3K4. Tagged AtJmj4 and ELF6 proteins associated directly with the *FT* transcription initiation region, a region where the H3K4me3 levels were increased most significantly in the mutants. Thus, our study demonstrates the roles of AtJmj4 and ELF6 as H3K4 demethylases directly repressing *FT* chromatin and preventing precocious flowering in *Arabidopsis*.

2.2 Introduction

Flowering, a critical developmental transition in plants, is controlled by both environmental cues and internal developmental signals. The photoperiod, one of the major environmental cues for flowering, exerts profound effects on flowering in numerous plant species including Arabidopsis. The photoperiodic signal is generated mainly in the leaves by light and circadian-clock signaling and relayed through the photoperiod pathway. *GIGANTEA* (*GI*) (Park et al., 1999; Fowler et al., 1999) and *CONSTANS* (*CO*) (Putterill et al., 1995) act as upstream activators of *FLOWERING LOCUS T* (*FT*) (Kobayashi et al., 1999; Kardailsky et al., 1999) in the photoperiod pathway. On the other hand, *FT* mRNA expression is repressed by *FLOWERING LOCUS C* (*FLC*) (Park et al., 1999; Fowler et al., 1999), and this repression is mediated possibly by a protein complex between FLC and SHORT VEGETATIVE PHASE (Li et al., 2008). Thus, *FT* acts not only as a component in the photoperiod pathway but also as a floral integrator that combines the photoperiodic floral activation signal and the *FLC*-mediated floral repression signal. FT protein, as a graft-transmissible signal, is translocated from the vascular tissue of leaves to the shoot apex (Corbesier et al., 2007), where it interacts with FD and stimulates the floral transition (Abe et al., 2005; Wigge et al., 2005).

Recent studies have shown that *FT* expression is affected by histone modifications. *FT* locus was shown to be enriched with trimethylated histone H3 lysine 27 (H3K27me3) (Turck et al., 2007; Zhang et al., 2007), and loss of putative polycomb repressive complex 2 (PRC2) components results in decreased H3K27me3 within *FT* chromatin, which in turn increases *FT* expression (Jiang et al., 2008). Furthermore, lack of LIKE-HETEROCHROMATIN PROTEIN1

(LHP1), which can bind to H3K27me3 and silence the chromatin (Turck et al., 2007; Zhang et al., 2007), also causes increased *FT* expression (Kotake et al., 2003; Takada S and Goto K, 2003). Therefore, *FT* transcription is repressed by H3K27me3 and its effecter protein.

Methylation at histone residues had been considered an irreversible epigenetic modification for a long period of time. However, more recently at least two classes of enzymes have been shown to be capable of removing methyl groups from either histone lysine or arginine (R) residues. Human Lysine-Specific Demethylase1 (LSD1), a nuclear amine oxidase, specifically demethylates mono- and dimethylated but not trimethylated H3K4 (Shi Y et al., 2004). After the discovery of LSD1, a human Jumonji (Jmj) C domain-containing protein, JHDM1A, was first shown to be able to remove methyl groups from H3K36 (Tsukada et al., 2006). Soon after the identification of JHDM1A, a number of JmjC domain-containing proteins have been demonstrated to be H3K4, H3K9, H3K27, H3K36, H3R2, and H4R3 demethylases (Christensen et al., 2007; Chang et al., 2007; Klose RJ and Zhang Y, 2007). Unlike LSD1, JmjC domain-containing proteins are capable of demethylating all of the mono-, di- and trimethylated lysines of histones (Klose et al., 2006). Thus, JmjC family proteins are considered the major histone demethylases in eukaryotic cells.

Arabidopsis has twenty-one genes encoding JmjC family proteins (*Arabidopsis thaliana Jumonji* (*AtJmj*) *1~21*) (Hong et al., 2009). To date, three of these genes have been functionally characterized. *EARLY FLOWERING6* (*ELF6*; *AtJmj1*) and *RELATIVE OF EARLY FLOWERING6* (*REF6*; *AtJmj2*) were shown to be involved in photoperiodic flowering and *FLC* regulation, respectively (Noh et

al., 2004). INCREASED EXPRESSION OF BONSAI METHYLATION 1 (IBM1; AtJmj15), represses genic cytosine methylation, possibly through demethylation of H3K9me (Saze et al., 2008). In this report, we show that ELF6 and another Arabidopsis JmjC family protein (AtJmj4) directly repress *FT* expression via demethylation of H3K4me. Thus, our study demonstrates the presence of an H3K4me demethylation-mediated mechanism in addition to the previously characterized H3K27 methylation-mediated mechanism in the chromatin repression of a key flowering time regulator, *FT*.

2.3 Materials and Methods

2.3.1 Plant materials and growth

atjmj4 T-DNA insertion lines in the Col background were obtained from the SALK collection (http://signal.salk.edu/; *atjmj4-1*, SALK_135712; *atjmj4-2*, SALK_136058). The following mutants are in the Col background and were described previously: *elf6-4* (Noh et al., 2004), *vin3-5* (Mylne et al., 2006), *flc-3* (Michaels et al., 1999), *gi-2* (Park et al., 1999), *co-101* (Takada S and Goto K, 2003), *ft-10* (Yoo et al., 2005), *lhp1-4* (Larsson et al., 1998), *fld-3* (He et al., 2003), *fve-4* (Ausin et al., 2004), *FRI* (Lee et al., 1994). All plants were grown under 100 $\mu E\ m^{-2}\ s^{-1}$ cool white fluorescent light at 22℃.

2.3.2 T-DNA flanking sequence analysis

The T-DNA borders of *atjmj4-1* and *atjmj4-2* alleles were defined by sequencing PCR products obtained using a T-DNA border primer (SALKLB1; Table 2.1) and gene-specific primers. For *atjmj4-1*, AtJmj4-1-R and AtJmj4-1-F primer pair (Table 2.1) was used to detect wt allele while AtJmj4-1-R and SALKLB1 primer pair was used to detect *atjmj4-1* allele. For *atjmj4-2*, AtJmj4-2-F and AtJmj4-2-R primer pair (Table 2.1) was used to detect wt allele while AtJmj4-2-F and SALKLB1 primer pair was used to detect *atjmj4-2* allele.

2.3.3 RT-PCR and qPCR analyses

Total RNA was isolated from seedlings using TRI Reagent (Molecular Research Center, INC.) according to the manufacturer's instructions. RT was performed with M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's

instructions using 3 µg of total RNA. PCR was performed on first strand DNA with i-Taq DNA polymerase (iNtRON Biotechnology). Primers used for RT-PCR and qPCR analyses are listed in Table S2. qPCR was performed in 96-well blocks with an Applied Biosystems 7300 real-time PCR system using the SYBR Green I master mix (Bio-Rad) in a volume of 20 µl. The reactions were performed in triplicate for each run. The comparative $_{\Delta\Delta}$CT method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (Ct) was automatically determined for each reaction by the system set with default parameters.

2.3.4 GUS and GFP assays

The *FT$_{pro}$::GUS* (Yoo et al., 2005) and the *ELF6::GUS* (Noh et al., 2004) were described previously. For the construction of the *AtJmj4::GUS* translational fusion construct, a 6-kb genomic DNA fragment of *AtJmj4* containing 1.5-kb 5' upstream region and the entire coding region was generated by PCR amplification using AtJmj4GUS-F and AtJmj4GUS-R as primers (Table 2.3). After restriction digestion with *Sal*I-*Sma*I, the PCR product was ligated into pPZP211-GUS (Noh et al., 2003) at *Sal*I-*Sma*I sites. The final construct was introduced into wt Col plants by the floral dip method (Clough et al., 1998) through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 50 µg ml^{-1} kanamycin. Histochemical GUS staining was performed as described (Schomburg et al., 2001).

AtJmj4 cDNA obtained from Col RNA through RT-PCR using AtJmj4OE-F and AtJmj4OE-R as primers (Table 2.3) was used for the construction of the

CaMV35S$_{pro}$*::AtJmj4::GFP*. The cDNA was cloned into the *Sal*I site of p35SsGFP/pGEM which has the open reading frame of *sGFP* (Niwa et al., 1999) behind the Cauliflower Mosaic Virus 35S promoter (*CaMV35S*$_{pro}$). Mesophyll protoplasts were isolated from rosette leaves of Col plants grown for 4 weeks in LD as described (Yoo et al., 2007). The LHP1::RFP (Choi et al., 2005) was included as a nuclear protein control. Protoplasts were co-transformed with the *CaMV35S*$_{pro}$*::AtJmj4::GFP* and the *LHP1::RFP* constructs, each with 10 μg of plasmid DNA prepared with Nucleo Bond Xtra Midi Kit (Macherey-Nagel). After 16 h incubation at 22℃ in dark, protoplasts were observed with LSM 510 confocal microscope (Zeiss). The GFP and RFP fusion proteins were excited at 488 nm and 543 nm, respectively. The autofluorescence of chlorophylls, GFP, and RFP were analyzed with LP650, BP500-530IR, and BP565-615IR filters, respectively. The merged image was obtained using the LSM Image Browser (Zeiss).

2.3.5 AtJmj4::FLAG

The *AtJmj4::FLAG* construct is consisted of a 0.8 kb 5' upstream region of *AtJmj4* (*AtJmj4*$_{pro}$), the sequence for 3xFLAG tags, and the full coding sequence of *AtJmj4* cDNA. *AtJmj4* cDNA was obtained from Col RNA through RT-PCR using AtJmj4OE-F and AtJmj4OE-R1 as primers (Table 2.3), digested with *Sal*I, and cloned into the *Sal*I site of a construct containing *3xFLAG* behind the *CaMV35S*$_{pro}$ in pPZP211 vector. The *CaMV35S*$_{pro}$ was replaced with the *AtJmj4*$_{pro}$ obtained from Col genomic DNA through PCR using Atjmj4FLAG-F and Atjmj4FLAG-R as primers (Table 2.3) at *Pst*I site. Then the *AtJmj4*$_{pro}$*::3xFLAG::AtJmj4 cDNA* was PCR-amplified using AtJmj4FLAG-F1 and Atjmj4FLAG-R1 as primers (Table

2.3). After restriction digestion with *Nhe*I, the PCR fragment was ligated into the *Sma*I-*Xba*I sites of the binary vector pPZP221B (Kang et al., 2001). The final construct was introduced into *atjmj4-1* mutants by the floral dip method through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 25 μg ml^{-1} glufosinate ammonium.

Protein samples were extracted using 2xloading buffer from wt Col and transgenic plants harboring the *AtJmj4::FLAG* construct, and their concentrations were determined by Protein Assay (Bio-Rad). 3.75 μg of protein samples were size-fractionated on a 7% SDS-PAGE gel, transferred to Pure Nitrocellulose (GE Water & Process Technologies), and blocked with 10% skim milk power in TTBS (0.1% tween 20, 20 mM Tris-HCl pH7.4, 150 mM NaCl). AtJmj4::FLAG protein was detected using Anti-FLAG M2-Peroxidase (HRP) antibody (Sigma), ECL Western Blotting Detection Kit (GE Healthcare), and JP/LAS-3000 Luminescent Image Analyzer (Fujifilm).

2.3.6 AtJmj4 protein expression and purification

For the expression of AtJmj4 protein in insect cells, the full length *AtJmj4* coding region was PCR amplified from a cDNA clone using JMJ4_pENTR_For and JMJ4_pENTR_Rev as primers (Table 2.3) and ligated into the Klenow-filled *Eco*RI site of pFastBac HT A vector (Invitrogen). The resulting *AtJmj4::pFastBac HT A* construct with amino-terminal 6xHis tag was used to transform DH10Bac *E. coli* competent cells (Invitrogen), and the recombinant baculovirus DNA was selected and used for the infection of *sf9* cells following the Bac-to-Bac system instructions (Invitrogen). Cells positive for the recombinant-protein expression as

tested by western blot with anti-His antibody (Santa Cruz) was used to infect cells to produce *6His-AtJmj4* baculovirus stocks. Viral stocks were stored at 4 ℃. For protein expression, 2.5 ml of viral stock was used to infect approximately 2×10^6 adherent *sf*9 cells in 400 ml of sf-900 II SFM serum free medium (Gibco) and cultured at 27℃ for 48 h. Then, cells were harvested and washed with PBS and frozen at -80℃ until further purification. Frozen cells were thawed on ice and resuspended with 20 ml equilibration buffer (80 mM Na_2HP0_4, 20 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 100 μM PMSF, 10% glycerol). Cells were disrupted by sonication, and the lysate was clarified by centrifugation at 10,000x g for 20 min at 4℃. The supernatant was applied into a Ni-NTA-Agarose (Qiagen) chromatography column and washed with 10x column volume of equilibration buffer. Protein was eluted from the column with 3x bed volume of elution buffer (80 mM Na_2HP0_4, 20 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 100 μM PMSF, 10% glycerol). Purified recombinant 6His-AtJmj4 protein was dialyzed against dialysis buffer (40 mM HEPES-KOH pH 7.9, 50 mM KCl, 10% glycerol, 1 mM DTT, 0.2 mM PMSF) overnight at 4℃. Next-day, dialyzed 6His-AtJmj4 proteins was quantified and stored at -20℃.

2.3.7 Histone demethylase assay

In vitro histone demethylation assay was performed as previously described (Whetstine et al., 2006) with minor modifications. Briefly, two or four μg of purified 6His-AtJmj4 protein was incubated with 4 μg of calf thymus histones type II-A (Sigma) in the DeMTase reaction buffer 1 (20 mM Tris-HCl pH 7.3, 150 mM

NaCl, 50 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6(H_2O)$, 1 mM α-ketoglutarate, 2 mM ascorbic acid) for 5 h at 37℃. Histone modifications were detected by western blot analysis with antibodies as follows: anti-H3K4me1 (Upstate 07-436), anti-H3K4me2 (Upstate 07-030), anti-H3K4me3, (Abcam ab8580), anti-H3K9me3 (Upstate 07-442), anti-H3K36me2 (Upstate 07-369), anti-H3K27me3 (Upstate 07-449), and anti-H3 (Abcam ab1791-100).

2.3.8 ChIP assay

ChIP was performed as described by Han et al. (Han et al., 2007) using 55- to 60-d-old plants grown in SD. Briefly, leaves were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin was isolated and sonicated into ~0.5 to 1 kb fragments. Specific antibody against GUS (Invitrogen A5790), FLAG (Sigma A8592-0.2MG), H3K4me3 (Upstate 07-473), or H3K27me3 (Upstate 07-449) was added to the chromatin solution, which had been precleared with salmon sperm DNA/Protein A agarose beads (Upstate 16-157). After subsequent incubation with salmon sperm DNA/Protein A agarose beads, immunocomplexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immunocomplexes were removed by incubation with proteinase K, followed by phenol/chloroform extraction. DNA was recovered by ethanol precipitation. The amount of immunoprecipitated *FT*, *CO*, and *Actin1* chromatins was determined by PCR with primer pairs in Table S4.

2.4 Results

2.4.1 Mutations in *AtJmj4* cause early flowering

To address the biological roles of Arabidopsis genes encoding JmjC domain-containing proteins, we obtained T-DNA insertion lines of these genes from the SALK T-DNA collection and tested their phenotypes. Two independent homozygous T-DNA insertion mutants of *Arabidopsis thaliana Jumonji4* (*AtJmj4* or *At4g20400*) (Hong et al., 2009) showed an early flowering phenotype both in long days (LD; 16 h light/8 h dark) and short days (SD; 8 h light/16 h dark; **Figures 2.1A-C**). The early flowering phenotype was not due to an accelerated leaf initiation rate (**Figure 2.8**) but resulted from accelerated transition of the shoot apical meristem (SAM) from the vegetative to the reproductive phase as characterized by a lower number of rosette and cauline leaves at the onset of flowering (**Figure 2.1C**). No other phenotypic traits were noticed in *atjmj4* mutants. Plants heterozygous for the T-DNA insertions displayed a wild-type (wt) flowering time (data not shown), indicating that *atjmj4-1* and *atjmj4-2* are recessive mutations. Because both alleles displayed similar early flowering behaviors, *atjmj4-1* was chosen to carry out all the rest of genetic and molecular analyses.

2.4.2 Early flowering of *atjmj4* is due to increased expression of *FT*

Because AtJmj4 might have a role in histone modification and affect the transcription of its target gene(s), we tested the mRNA expression of key flowering time regulators, namely *GI, CO, FT, SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Samach et al., 2000; Lee et al., 2000), and *FLC*, in *atjmj4-1* to understand its early flowering phenotype (**Figure 2.2**). In LD, the mRNA levels of

GI and *CO* were not affected by the *atjmj4-1* mutation. However, the mRNA expressions of the floral integrators, *FT* and *SOC1*, were up-regulated in *atjmj4-1* and that of *FLC* was slightly reduced (**Figure 2.2A**). To further confirm these observations, we monitored the expression of these genes in SD (**Figure 2.2B**). *FT* mRNA expression showed a stronger up-regulation by the *atjmj4-1* mutation in SD than in LD, but the expression of *SOC1* mRNA was not affected significantly. Because *CO* expression was not increased by the *atjmj4-1* mutation in SD, the up-regulation of *FT* might be caused by de-repression instead of induction. The mRNA level of *FLC* was slightly reduced in *atjmj4-1* mutants compared to in wt as was in LD.

Then we compared the mRNA expressions of *FLC*, *FT*, and *SOC1* in wt versus *atjmj4-1* at different developmental stages both in LD and SD (**Figures 2.2C** and **2.2D**). Notably, *FT* mRNA levels were higher in *atjmj4-1* than in wt throughout the developmental stages tested. *FT* promoter showed an increased activity both in LD and SD when the construct containing the *FT* promoter fused with *β-glucuronidase* (*FT::GUS*) (Takada S and Goto K, 2003) was introduced from wt Col into *atjmj4-1* homozygous mutants (**Figure 2.2E**). *FT::GUS* expression which was detected in the marginal minor veins of wt leaves were also observed in the central minor veins of *atjmj4-1* mutant leaves in LD. In SD, *FT::GUS* expression was robust in the major veins of *atjmj4-1* mutant leaves, although its expression was weak in the same wt leaf tissue. Thus, the *atjmj4* mutation leads to increased expression of *FT* mRNA through enhanced activity of *FT* promoter. However, the specific expression of *FT* in leaf veins was not affected by the *atjmj4* mutation.

Since the *atjmj4* mutation also caused a slight reduction in *FLC* mRNA levels (**Figures 2.2A-D**), we tested if the decrease in *FLC* expression is the major cause for the increased expression of *FT* in the mutants. For this, we first compared *FT* mRNA levels between in an *flc* null mutant (*flc-3*) (Michaels et al., 2001) and in *atjmj4-1* (**Figure 2.9**). Although the expression levels of *GI*, *CO*, and *SOC1* mRNAs were similar in the two genotypes, *FT* mRNA level was clearly higher in *atjmj4-1*. Furthermore, when we compared the flowering times between *atjmj4-1* single and *atjmj4-1 flc-3* double mutants both in LD and SD, the double mutants flowered earlier than the single mutants in both photoperiodic conditions (**Figures 2.3A** and **2.3B**). *atjmj4* showed normal response to vernalization as wt, while a double mutant between *atjmj4* and the vernalization unresponsive *vernalization insensitive3* (*vin3*) (Sung et al., 2004) did not (**Figure 2.10**). Further, *atjmj4 vin3* flowered earlier than *vin3* without or with vernalization (**Figure 2.10**) which acts largely through *FLC* (Sheldon et al., 1999; Michaels et al., 1999). Taken together, these data indicate that the *atjmj4* mutation causes an early flowering independently of *FLC* expression.

The idea for the *FLC*-independent activity of AtJmj4 was reinforced by our studies on genetic interactions between *atjmj4* and several autonomous-pathway mutants (Koornneef et al., 1991) (**Figure 2.3C**). Double mutants between *atjmj4* and *flowering locus d* (*fld*) (He et al., 2003) or *fve* (Ausin et al., 2004) represented intermediate flowering time of each single mutant. When a functional *FRIGIDA* (*FRI*) (Lee et al., 1994; Koornneef et al., 1994) allele was introduced into *atjmj4*, *atjmj4-1 FRI* flowered also at intermediate time between *atjmj4-1* and *FRI*. These

results indicate that AtJmj4 controls flowering process mainly through an *FLC*-independent pathway.

To test interactions between *AtJmj4* and genes acting in the photoperiod pathway, we made double mutants, namely *atjmj4-1 gi-2*, *atjmj4-1 co-101*, *atjmj4-1 ft-10*. The early-flowering phenotype of *atjmj4-1* was attenuated by LD-specific late flowering phenotypes of *gi-2* and *co-101* (**Figures 2.3D** and **2.3E**). However, the early flowering of *atjmj4-1* was fully suppressed by the *ft-10* mutation (**Figure 2.3F**). Therefore, AtJmj4 might affect *FT* expression independently of GI and CO. Consistent with this hypothesis, *FT* mRNA level in *atjmj4-1 gi-2* or *atjmj4-1 co-101* double mutants was higher compared to that in *gi-2* or *co-101* single mutants, respectively (**Figure 2.11**). LHP1 directly represses *FT* chromatin (Turck et al., 2007; Zhang et al., 2007) such that *FT* is strongly de-repressed in *lhp1* mutants (Kotake et al., 2003). Consistent with the repressive roles of AtJmj4 and LHP1 in *FT* expression, *atjmj4-1 lhp1-4* double mutants flowered at similar times with the severe early flowering mutant *lhp1-4* in both LD and SD (**Figures 2.3G** and **2.3H**).

Because all the data above indicated that AtJmj4 acts as an *FT* repressor, we were tempted to test if AtJmj4 affects *FT* expression indirectly through controlling the expression of *FT* regulators. To address this, we compared the mRNA levels of known *FT* regulators, namely *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3* (Jung et al., 2007; Aukerman et al., 2003), *SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) (Jung et al., 2007; Schmid et al., 2003), *CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX1* (*CIB1*) (Liu et al., 2008), *TEMPRANILLO1* (*TEM1*), *TEM2* (Castillejo et al., 2008), *SHORT VEGETATIVE PHASE* (*SVP*) (Li et al., 2008; Lee et al., 2007; Hartmann et al., 2000), *AGAMOUS-like15* (*AGL15*),

and *AGL18* (Adamczyk et al., 2007), in between wt and *atjmj4* mutants, but none showed detectable differences (**Figure 2.12**).

2.4.3 AtJm4 is a nuclear protein preferentially expressed in vascular tissues and shoot/root apices

When we compared the mRNA expression of *AtJmj4* between in SD- and in LD-grown seedlings at a similar developmental stage, we could observe higher expression of *AtJmj4* mRNA in SD-grown seedlings, although the expression of *FT* mRNA was clearly higher in LD-grown seedlings (**Figure 2.4A**). This observation was consistent with the higher *AtJmj4* promoter activity in SD than in LD as studied using an *AtJmj4 promoter::GUS* fusion construct (Hong et al., 2009). Because the results might mean a preferential repressive role of AtJmj4 in *FT* expression in SD, we studied further to see if the expression level of AtJmj4 protein is also higher in SD than in LD. For this, we made an *AtJmj4::FLAG* fusion construct that contains an *AtJmj4* promoter fragment, 3 copies of FLAG tags, and the *AtJmj4* cDNA with the entire coding sequence. The construct fully rescued the early flowering phenotype of *atjmj4-1* when was introduced into the mutants (**Figure 2.4B**). When we measured the expression levels of the AtJmj4::FLAG fusion protein in transgenic plants at the same developmental stage with the one used to study the expression of *AtJmj4* mRNA (**Figure 2.4A**), however, there was no difference in the expression level between in SD- and LD-grown seedlings (**Figure 2.4C**). Thus, AtJmj4 expression might be further controlled at posttranscriptional level(s), although its promoter activity *per se* is regulated by a

day-length signal, and AtJmj4 protein exerts its repressive role for*FT* in both LD and SD.

Since AtJmj4 protein expression is distinct from its mRNA expression, we studied the spatial expression pattern of AtJmj4 protein using a construct harboring the entire genomic region of *AtJmj4* including 1.5 kb promoter in frame with *GUS* (*AtJmj4::GUS*). In seedlings, AtJmj4::GUS expression pattern was similar in SD and LD (**Figures 2.4D** and **2.4E**). The GUS activity was detected in most organs, but strong activity was observed in the shoot apex (**Figure 2.4J**), primary root tip (**Figure 2.4H**), trichomes of young leaves (**Figure 2.4F**), and leaf vascular tissues (**Figure 2.4G**). In floral organs, strong GUS activity was detected in anther filaments and styles (**Figure 2.4I**). Importantly, the *AtJmj4::GUS* expression domain showed an overlap with the *FT* expression domain (Takada S and Goto K, 2003) in leaves.

The subcellular localization of AtJmj4 protein was evaluated by protoplast transfection assay using a fusion protein between AtJmj4 and green fluorescence protein (GFP; AtJmj4::GFP) expressed by the *Cauliflower Mosaic Virus 35S* (*CaMV35*) promoter. The fusion protein between LHP1 and red fluorescence protein (RFP; LHP1::RFP), which was known to be localized into the nucleus (Choi et al., 2005), was co-expressed with the AtJmj4::GFP. Both RFP and GFP signals were detected only in the nucleus (**Figure 2.4K**). This result is in agreement with the possible role of AtJmj4 as a chromatin and/or transcriptional regulator.

2.4.4 AtJmj4 and ELF6 play redundant roles in *FT* repression as H3K4-specific demethylases

In our previous study, we reported that *ELF6* (*At5g04240*), a gene encoding an Arabidopsis Jmj-domian protein, acts as a repressor in the photoperiodic flowering pathway (Noh et al., 2004). Therefore, we were tempted to study the relationship between *AtJmj4* and *ELF6* in the regulation of photoperiodic flowering. For this, *elf6-4 atjmj4-1* double mutant was generated and assayed for flowering time. The double-mutant plants flowered earlier than the either single-mutant as well as the wt Col plants both in LD and SD (**Figures 2.5A-C**). Since both *ELF6* and *AtJmj4* have repressive roles in the photoperiod pathway, then we tested the mRNA expression of genes acting in the photoperiod pathway, namely *GI*, *CO* and *FT*, using RNAs isolated from SD-grown 56-d old plants. mRNA expressions of *GI* and *CO* were similar among the wt, the *elf6-4* and *atjmj4-1* single mutants, and the *elf6-4 atjmj4* double mutants at ZT4 and ZT11 (**Figure 2.5D**). However, *FT* mRNA level was increased in the *elf6-4* and the *atjmj4-1* single mutants by at least 3 folds compared to that in the wt, and this increase was more significant in the *elf6-4 atjmj4-1* double mutants (**Figures 2.5D** and **2.5E**). Therefore, the data for *FT* mRNA expression as well as the flowering time data (**Figures 2.5A-C**) indicate that *ELF6* and *AtJmj4* have redundant repressive roles in photoperiodic flowering through negatively regulating *FT* mRNA expression.

Our unpublished phylogenetic analysis on the JmjC domains of ELF6, AtJmj4, and human Jmj proteins showed that the JmjC domains of ELF6 and AtJmj4 are clustered along with the JmjC domains of human JARID1 family which is known to specifically demethylate H3K4me3 and H3K4me2 (Christensen et al., 2007;

Klose RJ and Zhang Y, 2007). Hence, we studied if the level of H3K4me within *FT* chromatin is affected by *elf6* and *atjmj4* mutations through chromatin immunoprecipitation (ChIP) assay. Sets of primers covering different regions of *FT* locus were used for the ChIP assay (**Figure 2.6A**). H3K4me3 levels were increased in G, I, and EX1 regions of *FT* locus by the *elf6-4* and *atjmj4-1* mutations, and the increase was more significant when both the mutations were combined (**Figures 2.6B-D**). However, H3K4me3 levels in regions F and N were not affected significantly by these mutations. Level of another histone methylation, H3K27me3, which was reported to be enriched within *FT* chromatin (Turck et al., 2007; Zhang et al., 2007), was slightly reduced by the *elf6-4* but not by the *atjmj4-1* mutation in some of the *FT* regions tested (**Figure 2.6C**). These results indicate that ELF6 and AtJmj4 repress *FT* mRNA expression by negatively affecting the methylation of H3K4 but not H3K27 within *FT* chromatin.

To test if ELF6 and AtJmj4 are active histone demethylases, we tried to express the full-length ELF6 and AtJmj4 proteins in several expression systems. Although we could not express the full-length ELF6 in any systems employed, we could express the full-length AtJmj4 in insect *sf9* cells as an amino-terminal 6 histidine-tagged protein (6His-AtJmj4) with a molecular mass of 130 kilo-daltons. 6His-AtJmj4 was purified near homogeneity (**Figure 2.6E**) and subjected for *in vitro* histone demethylase activity assay. 6His-AtJmj4-mediated histone demethylase activity was analyzed by decreased signals in western blots with antibodies specific to methylated histone H3 residues (**Figure 2.6E**). Incubation of the recombinant 6His-AtJmj4 protein with histone substrates in the demethylase assays resulted in reduced levels of H3K4me1, H3K4me2, H3K4me3, but not of H3K9me3,

H3K36me2, and H3K27me3 (**Figure 2.6E**). The levels of H3K4me3 and H3K4me2 were deceased more than the level of H3K4me1. These results indicate that AtJmj4 is an intrinsic H3K4-specific demethylase which has higher activity for H3K4me3 and H3K4me2 than H3K4me1.

The results in **Figure 2.5** and **Figure 2.6** suggested that AtJmj4 and ELF6 might directly target *FT* chromatin and repress the transcription activity of *FT* by reducing the methylation level of H3K4. To test if *FT* chromatin is directly targeted by ELF6 and AtJmj4 proteins, we preformed ChIP assays using transgenic plants expressing functional *ELF6::GUS* (Noh et al., 2004) and *AtJmj4::FLAG* (**Figures 2.4B** and **2.4C**) as demonstrated by the complementation of the *elf6-4* and *atjmj4-1* mutant phenotypes, respectively. PCR was then carried out using primers amplifying various regions of *FT* locus (**Figure 2.7A**). ELF6::GUS showed binding with broad regions of the *FT* locus around the transcription start site with strongest binding with region I (**Figures 2.7B**). However, ELF6::GUS did not show binding with region N which is a part of the first intron of *FT* and the transcription initiation region of *CO*. AtJmj4::FLAG showed a similar binding pattern with ELF6::GUS to *FT* chromatin (**Figures 2.7C**). It showed strong bindings with regions I to EX1 like ELF6::GUS, but its binding was not detected with regions F and G unlike ELF6::GUS. AtJmj4::FLAG binding to region N and the *CO* region was not detected as for the case of ELF6::GUS. In summary, both ELF6::GUS and AtJmj4::FLAG can associate directly and specifically with the transcription initiation region of *FT* locus where the H3K4me3 levels showed the largest increase in *elf6, atjmj4,* and *elf6 atjmj4* mutants. Thus, ELF6 and AtJmj4 proteins directly target *FT* chromatin and regulate flowering time via

demethylation of H3K4me.

2.5 Figures and Tables

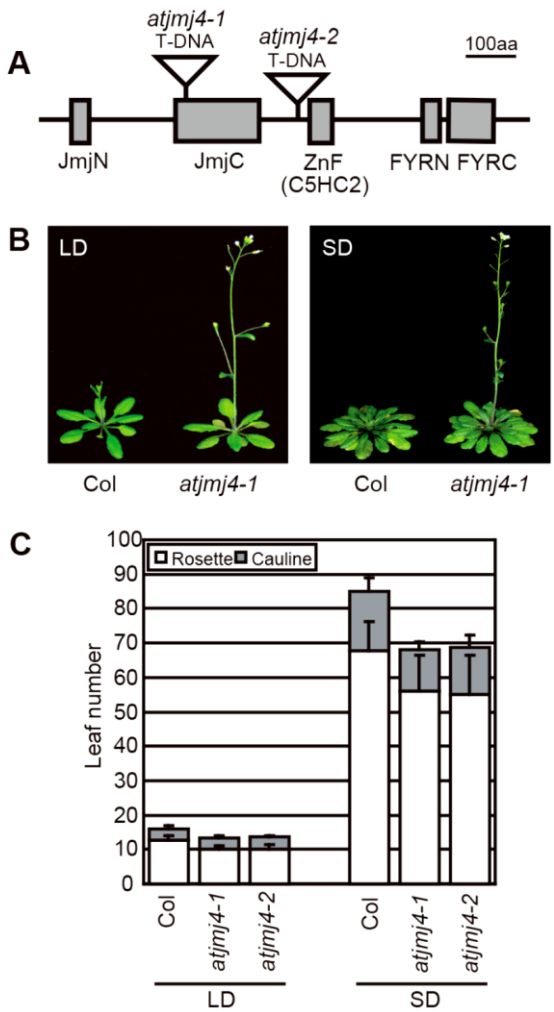

Figure 2.1 Early flowering of *atjmj4* mutants.

A) Domain organization of AtJmj4. Domains were predicted by SMART (http://smart.embl-heidelberg.de/). Lines indicate interdomain regions. T-DNA insertion sites on the genomic sequence of *AtJmj4* in *atjmj4-1* and *atjmj4-2* are marked on the corresponding positions of their translated protein products. **B)** Early flowering phenotype of *atjmj4-1* mutant plants grown in either LD or SD. **C)**

Flowering time of *atjmj4* mutants. Wt Col and *atjmj4* mutant plants were grown in either LD or SD and their flowering times were determined as the number of primary rosette and cauline leaves formed at bolting. At least 12 individuals were scored for each genotype. Error bars represent sd.

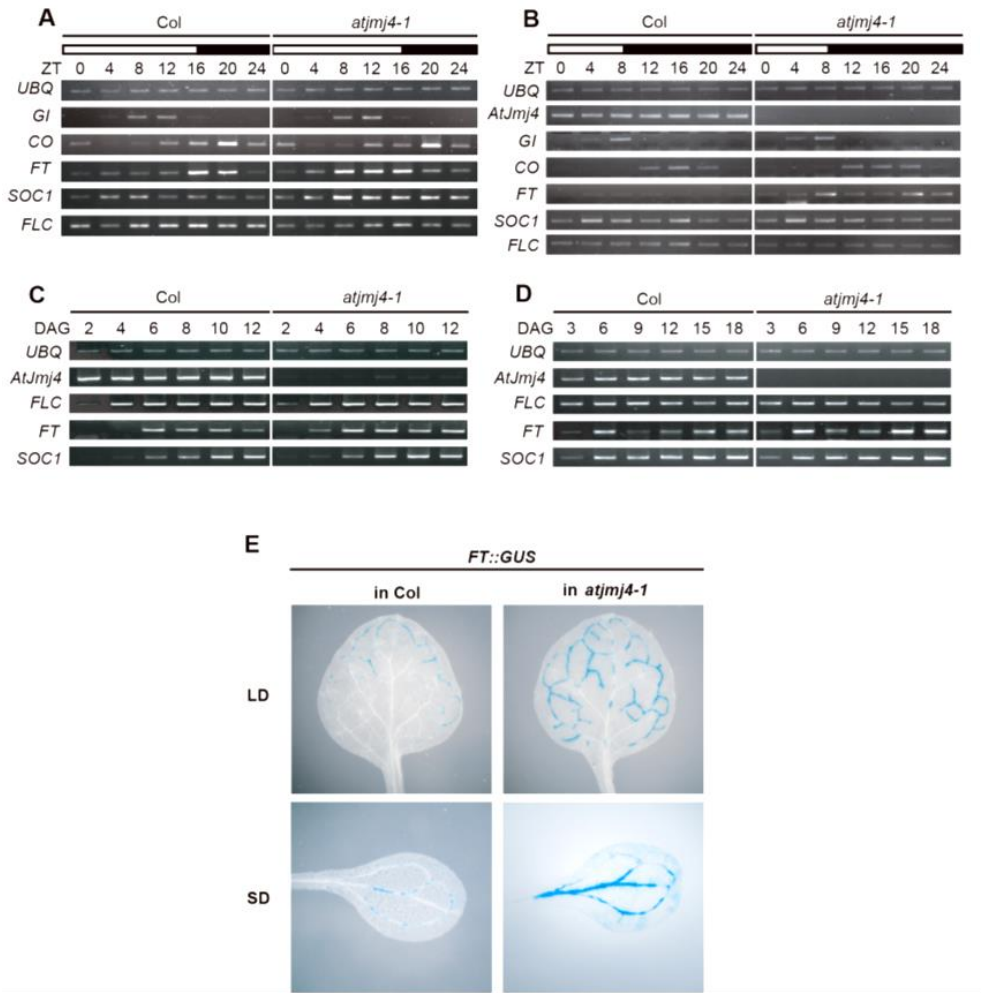

Figure 2.2 Increased expression of *FT* in *atjmj4* mutants.

A and B) Expression of flowering genes in *atjmj4-1* mutants. Col and *atjmj4-1* plants were grown in LD (A) for 10 d or in SD (B) for 15 d, harvested every 4 hours (h) at indicated zeitgeber (ZT; h after light-on) for one d, and used for RT-PCR analyses. *Ubiquitin* (*UBQ*) was included as an expression control. Identical results were obtained from two independent experiments, and one of them is shown. White and black bars represent light and dark periods, respectively. **C and D)**

Temporal expression of flowering genes in *atjmj4-1* mutants. Col and *atjmj4-1* plants were grown for up to 12 days after germination (DAG) in LD (C) or 18 DAG in SD (D). Plants were harvested during the growth period at ZT14 (LD) or ZT8 (SD) of designated DAG and used for RT-PCR analyses. **E)** Histochemical GUS staining of transgenic plants harboring *FT::GUS* fusion construct in Col or *atjmj4-1* plants. Plants were grown for 16 d either in LD or SD before GUS staining.

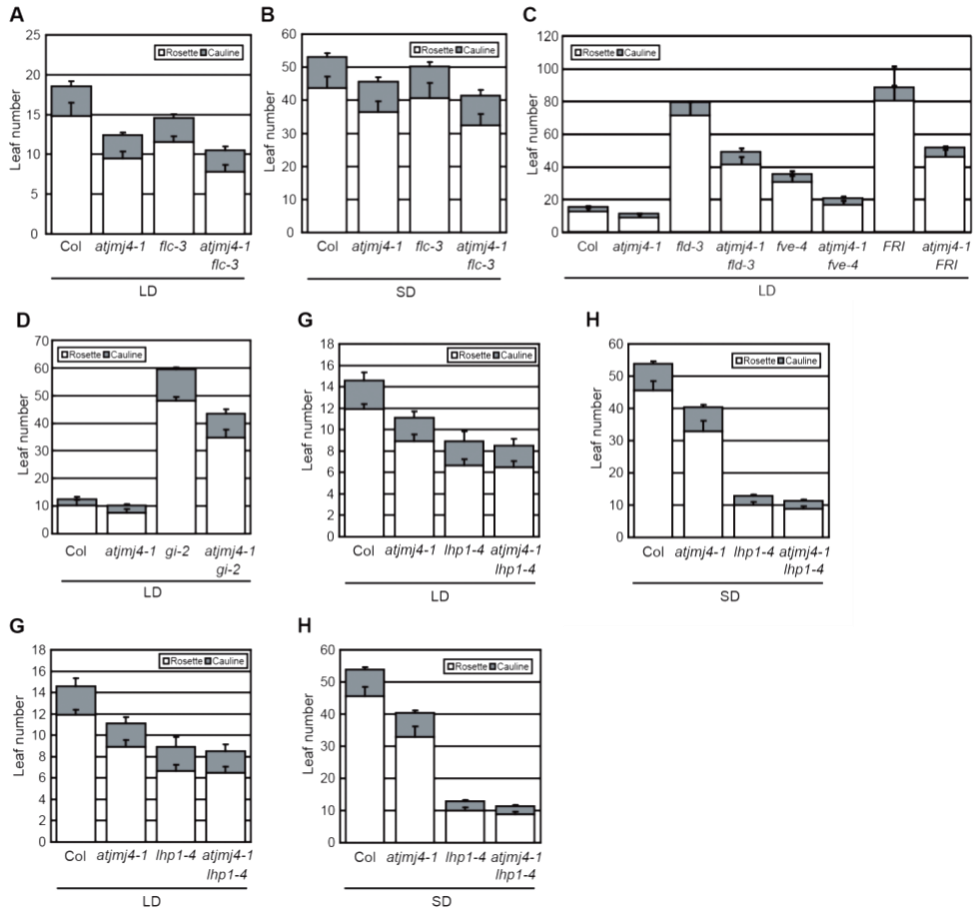

Figure 2.3 Genetic interaction between *Atjmj4* and other flowering genes.

A and B) Flowering time of *atjmj4-1 flc-3* double mutants. **C)** Genetic interaction between *AtJmj4* and *FLC* regulators. **D to F)** Genetic interaction between *AtJmj4* and photoperiod-pathway genes. **G and H)** Genetic interaction between *AtJmj4* and *LHP1*. Flowering times were determined in LD (A and C to G) or SD (B and H). At least 12 individuals were scored for each genotype (A to H). Error bars represent sd (A to H).

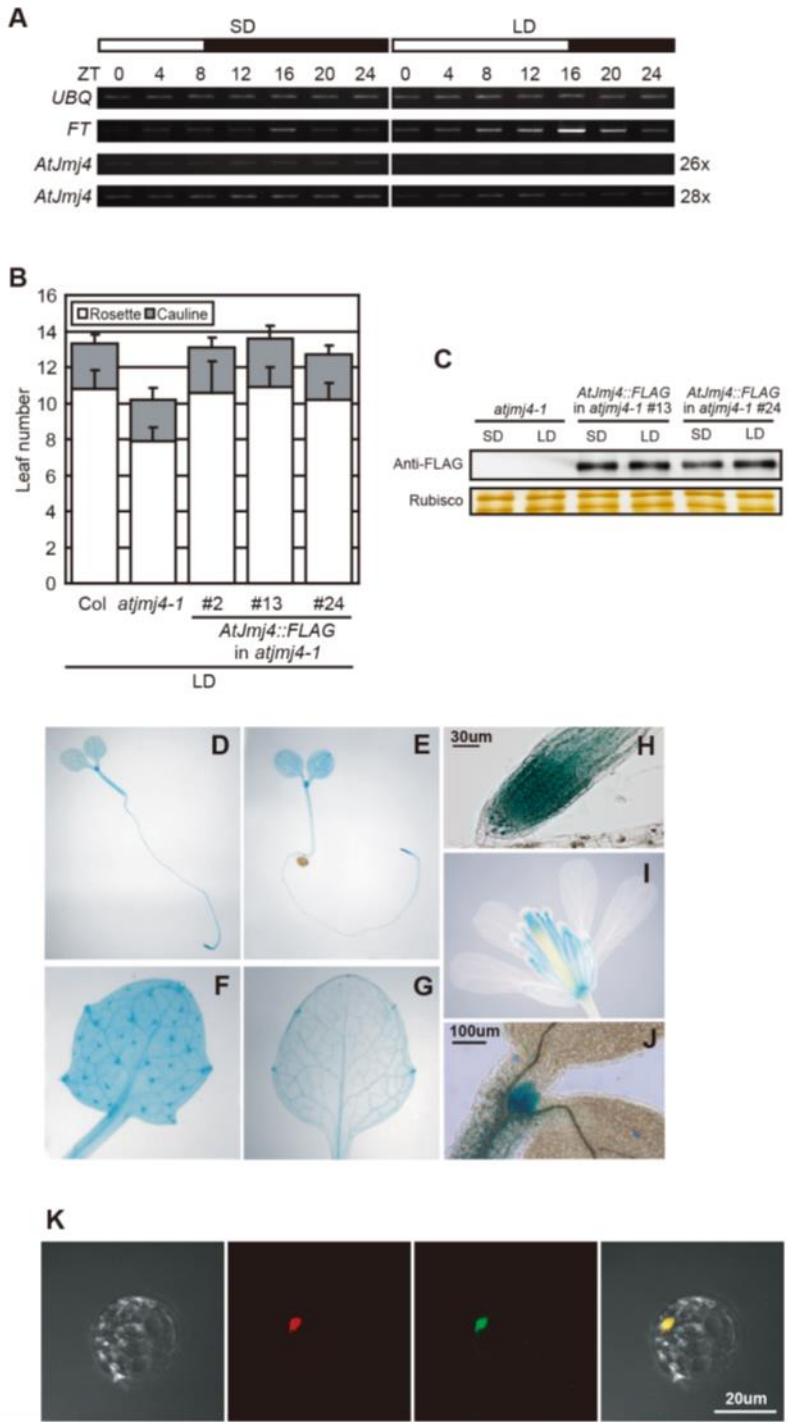

Figure 2.4. *AtJmj4* expression.

A) mRNA expression of *AtJmj4* in SD and LD. Wt Col plants were grown in SD for 12 d or in LD for 8 d and used for RT-PCR analyses. *UBQ* was used as an expression control. Number of PCR cycles used for *AtJmj4* is indicated on the right. **B)** Genomic complementation of *atjmj4-1*. Three independent transgenic lines of *atjmj4-1* containing *AtJmj4::FLAG* (see text for details) were grown in LD and their flowering times were determined as the number of rosette and cauline leaves formed at bolting. At least 12 individuals were scored for each genotype. Error bars represent sd. **C)** Expression of the AtJmj4::FLAG fusion protein in SD and LD. Plants of *atjmj4-1* and two of the complementation lines shown in (B) were grown for 12 d in SD or 8 d in LD, harvested at ZT12, and used for Western blot analyses. Upper panel: Western blot with anti-FLAG antibody. Lower panel: Silver stained gel image of rubisco subunits. **D to J)** Histochemical GUS staining of transgenic Col plants harboring *AtJmj4::GUS*. Plants grown in LD (D and I) or SD (E, F, G, H, and J) were used for GUS staining. (D) In 4 d-old seedling. (E) In 6-d old seedling. (F) In trichomes. (G) In leaf. (H) In root tip. (I) In floral organs. (J) In shoot apex. **K)** Subcellular localization of AtJmj4 in Arabidopsis mesophyll protoplast. From left to right; bright-field image, LHP1::RFP fusion protein, AtJmj4::GFP fusion protein, merged image of the left three images.

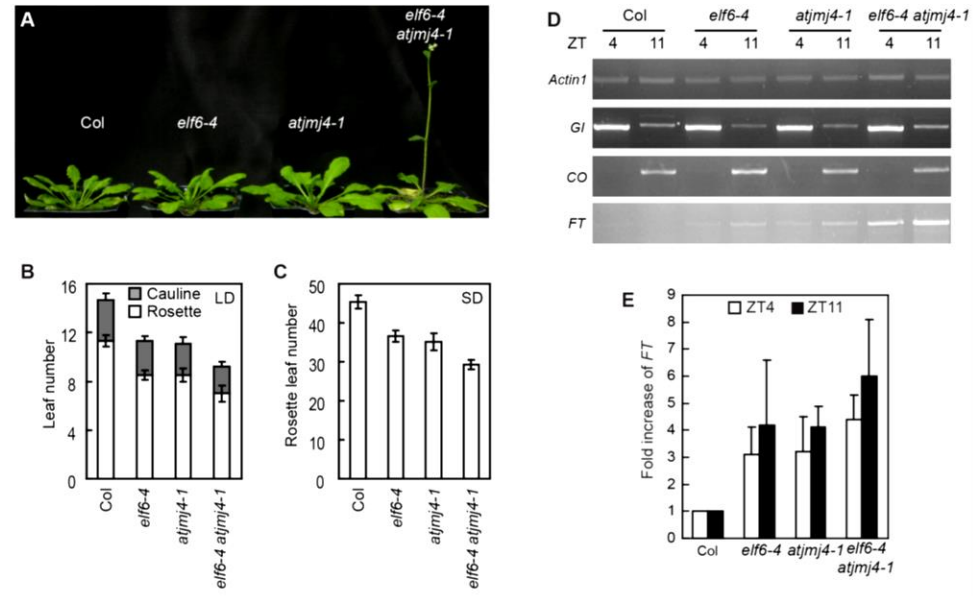

Figure 2.5 Additive effect of *elf6* and *atjmj4* mutations on *FT*-dependent early flowering.

A) Early flowering phenotype of *elf6-4 atjmj4-1* double mutant. All plants were grown in SD for 63 d before taken picture. **B and C)** Flowering time of wt Col, *elf6-4*, *atjmj4-1*, and *elf6-4 atjmj4-1* double mutants in LD and SD as determined by number of leaves formed at bolting. At least 15 individuals were scored for each genotype. Error bars represent sd. **D)** Expression of flowering genes in *elf6-4 atjmj4* double mutants. Plants of each genotype were grown in SD for 57 d and harvested at ZT4 or ZT11 for RT-PCR analyses. *Actin1* was included as an expression control. Identical results were obtained from two independent experiments and one of them is shown. **E)** qPCR analysis of *FT* expression. The same RNAs used in (D) were evaluated. The wt Col levels were set to 1 after normalization by *Actin1* for qPCR analysis. Error bars represent sd.

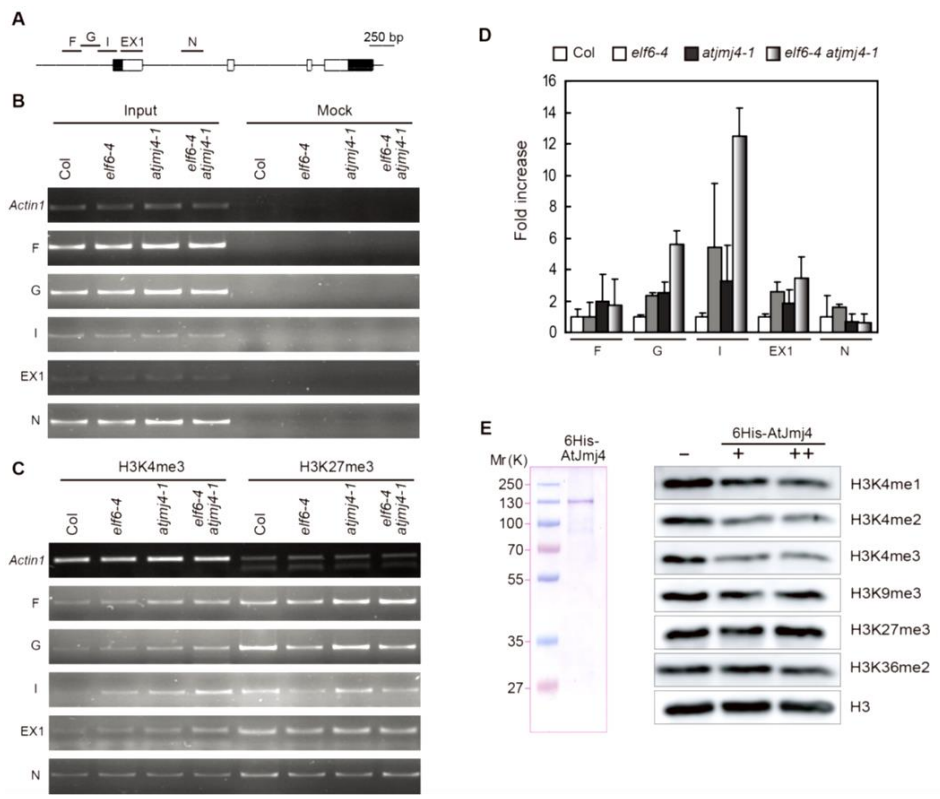

Figure 2.6 Increased trimethylation of H3K4 at *FT* locus by *elf6* and *atjmj4* mutations.

A) Schematic of *FT* locus showing regions (F, G, I, EX1, and N) amplified by the primers used for ChIP analysis. The front and the rear black boxes indicate 5' and 3' UTRs, respectively. White boxes indicate exons, while lines indicate introns and intergenic regions. **B and C)** ChIP assay of *FT* chromatin with antibody against H3K4me3 or H3K27me3. Plants of each genotype were grown in SD for 57 d and harvested for ChIP assay. 'Input' indicates chromatins before immunoprecipitation. 'Mock' refers to control samples lacking antibody. *Actin1* was used as an internal control. **D)** qPCR analysis of the ChIP assay for H3K4me3 described in (B and C).

The wt Col levels were set to 1 after normalization by input. Error bars represent sd. **E)** Coomassie-blue stained 6His-AtJmj4 protein purified from *sf9* cells (left), and *in vitro* histone demethylation activity assay using the purified protein (right). Assays were performed without (-) or with either two (+) or four (++) µg of purified 6His-AtJmj4 protein. Mr (K), molecular mass in kilo-daltons.

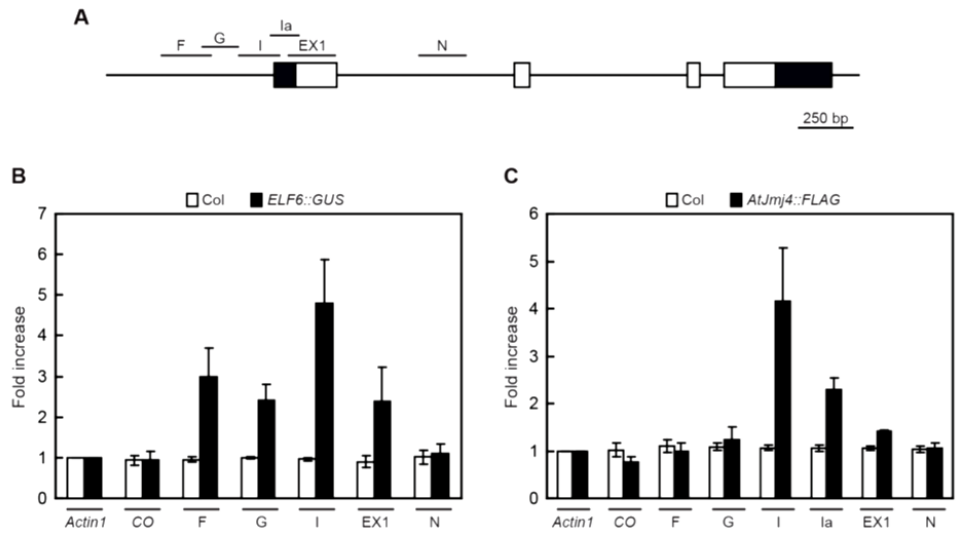

Figure 2.7 Direct association of ELF6 and AtJmj4 with *FT* chromatin.

A) *FT* regions tested for ChIP assay. Schematic is as described in **Figure 6A** except for the region Ia, which was added in assays in (C). **B)** ELF6 binding to *FT* chromatin. LD grown 16 d-old wt Col and *ELF6::GUS*–containing transgenic *elf6-4* plants [22] were harvested and used for ChIP assay using GUS-specific antibody. Amount of immunoprecipitated chromatin was measured by qPCR (B and C). *Actin1* and *CO* were used as internal controls, and the level of *Actin1* in each sample was set to 1 for normalization (B and C). Error bars represent se of three independent biological replicates (B and C). **C)** AtJmj4 binding to *FT* chromatin. LD grown 16 d-old wt Col and *AtJmj4::FLAG*–containing *atjmj4-1* plants were harvested and used for ChIP assay using FLAG-specific antibody.

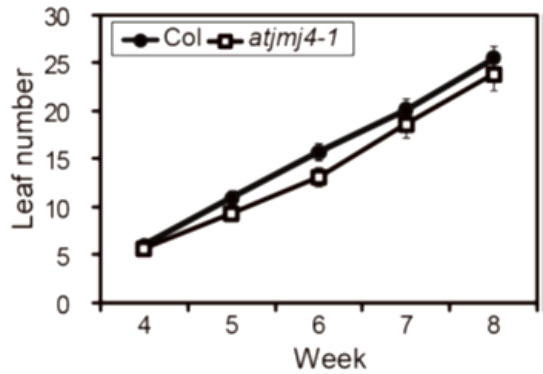

Figure 2.8 Leaf initiation rate of *atjmj4-1* mutants.

Wt Col (black circles) and *atjmj4-1* mutant plants (white squares) were grown in SD and their leaf numbers were scored every week from four weeks after planting. At least 10 individuals were scored for each genotype. Error bars represent sd.

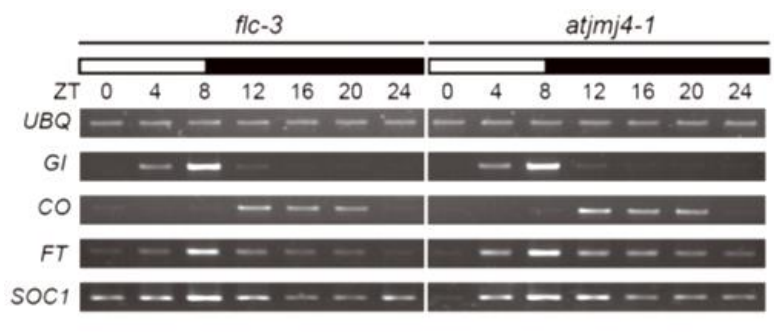

Figure 2.9 *FLC*-independent function of *AtJmj4*.

Expression of flowering genes in *flc-3* and *atjmj4-1* mutant plants grown in SD for 12 d as determined by RT-PCR analysis. *UBQ* was used as an expression control.

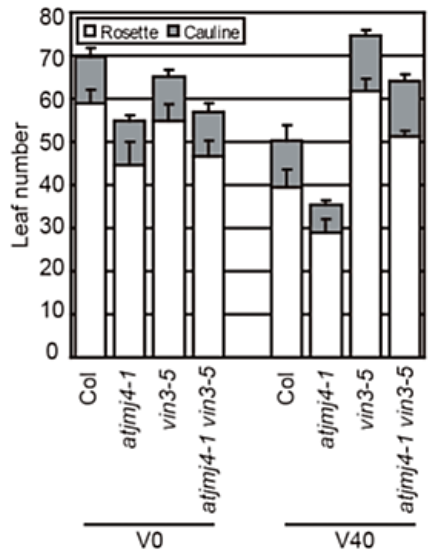

Figure 2.10 Vernalization response of *atjmj4* mutants.

Plants of each genotype were treated with vernalization for 40 days (d) as described previously [56]. Flowering time was scored as leaf number for plants either without (V0) or after (V40) vernalization treatment. At least 12 individuals were scored for each genotype. Error bars represent sd.

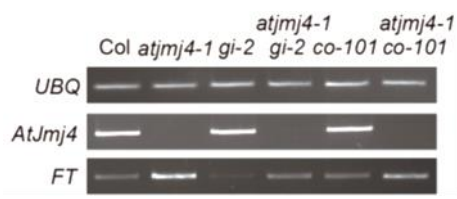

Figure 2.11 *CO*- and *GI*-independent increase of *FT* expression in *atjmj4*.

Plants of each genotype were grown in LD for 14 d and harvested at ZT8 for RT-PCR analyses. *UBQ* was used as an expression control.

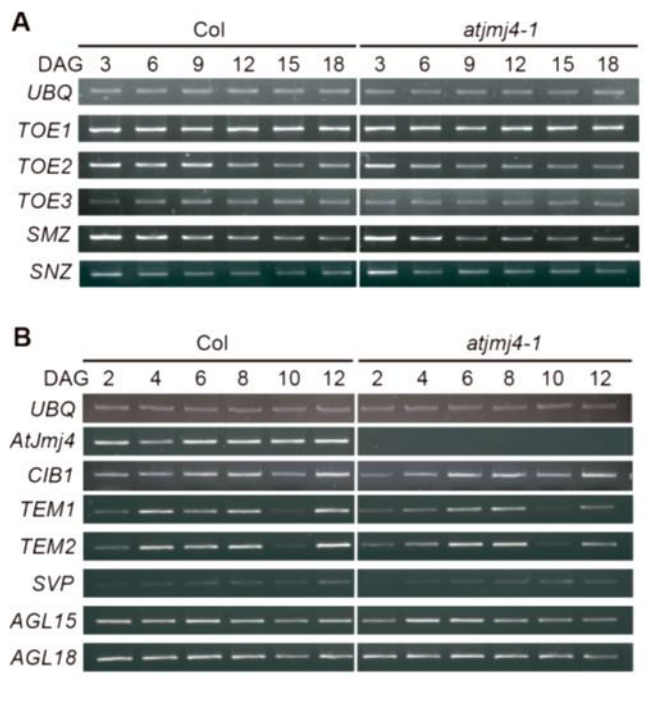

Figure 2.12 Expression of *FT* regulators in *atjmj4*.

A and B) Temporal expression of *FT* regulators in *atjmj4-1*. Col and *atjmj4-1* plants were grown in SD (**A**) or in LD (**B**) until indicated DAG and harvested at ZT14 (LD) or ZT8 (SD) for RT-PCR analyses. *UBQ* was used as an expression control.

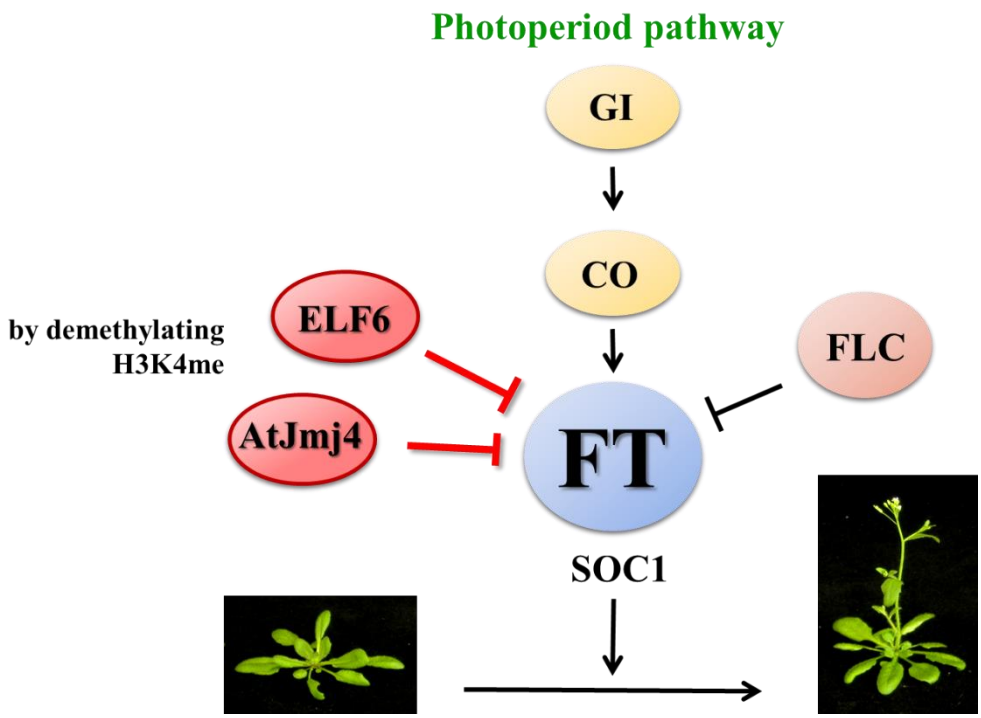

Figure 2.13 Proposed model for the role of AtJmj4 and ELF6 in pathways regulating flowering time in Arabidopsis

ELF6 and AtJmj4 have redundant repressive roles in flowering through negatively regulating *FT* expression via demethylation of H3K4me.

Table 2.1. Oligonucleotides used for T-DNA flanking sequence analysis

Name	Sequence
SALKLB1	5’-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3’
AtJmj4-1-F	5’-GAGAAGTTGCGCTCTAAAGCAGAATC-3’
AtJmj4-1-R	5’-TGGATCACCTGTGTGTAAGTAGTTCATGG -3’
AtJmj4-2-F	5’-AATATGTATCTCACTCTGCACC-3’
AtJmj4-2-R	5’-AAATCACCCTCCATCTTTCGC-3’

Table 2.2. Oligonucleotides used for RT-PCR analysis

Gene	Name	Sequence
Ubiquitin	UBQ-F	5’-GATCTTTGCCGGAAAACAATTGGAGGATG GT-3’
	UBQ-R	5’-CGACTTGTCATTAGAAAGAAAGAGATAAC AGG-3’
AtJmj4	AtJmj4-F	5’-GCTTGACCCAACAAACCTAACC-3’
	AtJmj4-R	5’-TCTCACCACACAGAAGTCCATGC-3’
GI	GI-F	5’-GTTGTCCTTC AGGCTGAAAG-3’
	GI-R	5’-TGTGGAGAGC AAGCTGTGAG-3’
CO	CO-F	5’-AAACTCTTTCAGCTCCATGACCACTACT-3’
	CO-R	5’-CCATGGATGAAATGTATGCGTTATGGTTA-3’
FT	FT-F	5’-GCTACAACTGGAACAACCTTTGGCAAT-3’
	FT-R	5’-TATAGGCATCATCACCGTTCGTTACTC-3’
SOC1	SOC1-F	5’-TGAGGCATACTAAGGATCGAGTCAG-3’
	SOC1-R	5’-GCGTCTCTACTTCAGAACTTGGGC-3’
FLC	FLC-F	5’-TTCTCCAAACGTCGCAACGGTCTC-3’
	FLC-R	5’-GATTTGTCCAGCAGGTGACATCTC-3’
TOE1	TOE1-F	5’-ACTCAGTACGGTGGTGACTC-3’
	TOE1-R	5’-CGAGGATCCATAAGGAAGAGG-3’
TOE2	TOE2-F	5’-CACTTTCTATCGGAGGACAG-3’
	TOE2-R	5’-CTTCCACATACGGAATTGTT-3’
TOE3	TOE3-F	5’-GTTACGTTTTACCGACGAAC-3’
	TOE3-R	5’-TGCTTGCAATATCAGACTTG-3’
SMZ	SMZ-F	5’-AATGGTGAAGAAGAGCAGAA-3’
	SMZ-R	5’-CTTTCCGATGATGATGAAAT-3’
SNZ	SNZ-F	5’-TTTGGAATCCTTAAACGAAA-3’
	SNZ-R	5’-TATCTCATTGCATTTTGCTG-3’
CIB1	CIB1-F	5’-GCATAGCAGAACGAGTTAGAAGAG-3’
	CIB1-R	5’-ATCAGAACTGGTATTCACTTGCTG-3’
TEM1	TEM1-F	5’-GCGTGTTGTTTCGGTATCACTA-3’
	TEM1-R	5’-ATTCAGAGAACGGCGTCGA-3’

TEM2	TEM2-F	5’-TTCCTCAGCCTAACGGAAGAT-3’
	TEM2-R	5’-TCCTTGACGAATCGACTCCAT-3’
SVP	SVP-F	5’-CGCTCTCATCATCTTCTCTTCCAC-3’
	SVP-R	5’-GCTCGTTCTCTTCCGTTAGTTGC-3’
AGL15	AGL15-F	5’-TTATCTAGATGGGTCGTGGAAAAATCGAG-3’
	AGL15-R	5’-TTAGCGGCCGCAGAGAACCTTTGTCTTTTG GCTTC-3’
AGL18	AGL18-F	5’-ATGGGGAGAGGAAGGATTGAGATTAAGA A-3’
	AGL18-R	5’-TCAATCAGAAGCCACTTGACTCCCAGAGT-3’

Table 2.3. Oligonucleotides used for constructs

Name	Sequence
AtJmJ4GUS-F	5’-gtcgacGTCTCCTCTCTATCGCCATTCTTG-3’
AtJmJ4GUS-R	5’-CTTcccgggAAAGGACTTATCTCCATC-3’
AtJmJ4OE-F	5’-CAgtcgacATGGATCAGCTTGCATCTC-3’
AtJmJ4OE-R	5’-TGCgtcgacAAGGACTTATCTCCATC-3’
AtJmJ4OE-R1	5’-TGCgtcgacTTAAGGACTTATCTCCATC-3’
AtJmj4FLAG-F	5’-TTctgcagGTCTTCGTCTCCTCTCTATCGC-3’
AtJmj4FLAG-R	5’-GGGctgcagCATTTACAGTGAGATTAAGTTC-3’
AtJmj4FLAG-F1	5’-GAgctagcTCCTCTCTATCGCCATTCTT-3’
AtJmj4FLAG-R1	5’-GTCTCGAGGAATTCCCAAACATATAGTAGATG-3’
JMJ4_pENTR_For	5’-CACCATGGATCAGCTTGCATCTCTAG-3’
JMJ4_pENTR_Rev	5’-AGGACTTATCTCCATCTTATC-3’

Restriction sites used for cloning are in small letters and underlined**.**

Table 2.4. Oligonucleotides used for ChIP assay

Gene	Region	Name	Sequence
FT	F	FF	5’-ACTTGGCGGTACCCTACTT-3’
		FR	5’-ATATCTCCCACTTGGTAG -3’
	G	GF	5’-GTCGAGAGAGGTATCTTGTTAAAG-3’
		GF	5’-ATCATAGGCATGAACCCTCTACAC-3’
	I	IF	5’-TATGTGTAGAGGGTTCATGCCTATG-3’
		IR	5’-TGGCCATAACCTTTAGAGTG -3’
	Ia	IaF	5’-CCACCTGTTTGTTCAAGATC-3’
		IaR	5’-GAAGGCCTTAGATCCAAGCC-3’
	EX1	EX1F	5’-ATGTCTATAAATATAAGAGACCCTC-3’
		EX1R	5’-CTTCTCCACCAATCTCAACTCTTG-3’
	N	NF	5’-TCCACCAACTTCTTGCATAAGTGA-3’
		NR	5’-CCACAACAGAGATTCATCAATATAT-3’
Actin1		Actin1 F	5’-CGTTTCGCTTTCCTTAGTGTTAGCT-3’
		Actin1 R	5’- AGCGAACGGATCTAGAGACTCACCTTG-3’
CO		CO F	5’-GGCACTCAGGATTCGATCTCC-3’
		CO R	5’-CCGGCATGTGTCACAGGGTCG-3’

2.6 Discussion

Recent studies have shown that the expression of some key flowering genes, such as *FLC* and *FT*, are regulated through chromatin modifications and have also discovered factors involved in the chromatin modification processes (Farrona et al., 2008; Schatlowski et al., 2008). In this study, we showed that AtJmj4 and ELF6 play a role in the repression of *FT* transcription by removing methyl groups from H3K4 at *FT* locus.

FT chromatin contains bivalent marks, thus the active mark (H3K4me3) and the repressive mark (H3K27me3) exist simultaneously (Jiang et al., 2008). Of these, only H3K27me3 has been studied. It plays a critical role in preventing precocious floral transition by establishing and maintaining the repressive *FT* chromatin as default state. The PRC2-like complex comprised of CURLY LEAF, SWINGER, EMBRYONIC FLOWER2, and FERTILIZATION INDEPENDENT ENDOSPERM, are required for H3K27me and the repression of *FT* (Jiang et al., 2008).

Unlike H3K27me3, H3K4me3 has been known to be positively associated with transcriptional activities (Li et al., 2007). H3K4me3 can be recognized by the TFIID complex via the PHD finger of TAF3, which in turn recruits RNA polymerase II, leading to transcription activation (Vermeulen et al., 2007). Therefore, H3K4me3 might also affect the chromatin state and expression of *FT*. In this study, we demonstrate that AtJmj4 is involved in *FT* repression as an H3K4-specific demethylase directly targeting *FT* locus with the following data: 1) The loss of *AtJmj4* function increased *FT* expression via enhanced *FT* promoter activity (**Figure 2.2**); 2) The loss of *AtJmj4* function increased H3K4me3 level in the

transcription initiation region of *FT* (**Figures 2.6C** and **2.6D**); 3) The purified AtJmj4 protein can specifically demethylate H3K4me1, H3K4me2, and H3K4me3 *in vitro* (**Figure 2.6E**); 4) The AtJmj4::FLAG binds to the transcription initiation region of *FT*. Up-regulation of *FT* expression could also be responsible, at least in part, for the increased H3K4me3 level in *atjmj4* at the *FT* locus.

It is notable that the increase of H3K4me3 at *FT* locus caused by the loss *AtJmj4* is not substantial. This suggests that there might be other histone demethylases having redundant roles with AtJmj4 for the demethylation of H3K4 at *FT* locus. ELF6 is turned out to be one of those histone demethylases from our following data: 1) The loss *ELF6* function increased *FT* expression and H3K4me3 level in the transcription initiation region of *FT*, and these increases were more significant when *AtJmj4* and *ELF6* functions were lost together (**Figures 2.5D**, **2.5E**, **2.6C**, and **2.6D**); 2) The ELF6::GUS binds to the promoter and transcription initiation region of *FT*. Previously we reported that both AtJmj4 and ELF6 belong to the same group (Group I) of Arabidopsis Jmj family proteins (Hong et al., 2009). Our unpublished phylogenetic analysis indicates that eight Arabidopsis Jmj family proteins belonging to this group have JmjC domains clustered together with the JmjC domains of human JARID1 family that are H3K4 demethylases (Christensen et al., 2007; Klose RJ and Zhang Y, 2007). Therefore, not only AtJmj4 and ELF6 but also other members of the Arabidopsis Group I Jmj family proteins have a potential to be H3K4 demethylases acting at *FT* locus. However, their genetic and biochemical roles need yet to be addressed in the future study.

According to recent studies, the antagonistic histone marks, H3K27me3 and H3K4me3, are coordinately regulated by protein complexes containing both

histone methyltransferase and histone demethylase (Lee et al., 2007; Pasini et al., 2008). Pasini et al (Pasini et al., 2008) reported that the RBP2 H3K4 demethylase is recruited by the PRC2 to repress the expression of target genes in mouse embryonic stem cells, and the loss of RBP2 increases the expression of the target genes. At this moment, it is not clear if a similar interaction between AtJmj4/ELF6 and the Arabidopsis PRC2 components occurs for the repression of *FT*. However, the report that the level of H3K4me3 within *FT* chromatin is increased in the absence of CURLY LEAF activity (Jiang et al., 2008) suggests such scenario is plausible. In this study, we could not observe a significant reduction of H3K27me3 level within *FT* chromatin in *elf6 atjmj4* double mutants. Thus, H3K4 demethylases might be recruited by PRC2, but the PRC2 recruitment might not be affected by H3K4 demethylases.

The coexistence of bivalent H3K27me3 and H3K4me3 marks at the same locus has been proposed to poise genes for the activation upon appropriate developmental cues (Bernstein et al., 2006; Azuara et al., 2006). Thus, the bivalent chromatin marks within *FT* chromatin might be a strategy for plants to achieve reproductive success by a precise regulation of *FT* expression and flowering time. It might be possible that enriched H3K27me3 favors constitutive *FT* repression, while a proper level of H3K4me3 provides appropriate accessibility for transcription factors controlled temporally such that *FT* expression can be regulated by changing developmental or environmental cues. Interestingly, the region I of *FT* locus, in which both AtJmj4::FLAG and ELF6::GUS showed strongest binding (**Figures 2.7A-C**) and the largest increase of H3K4me3 by the *elf6* and *atjmj4* mutations was observed (**Figures 2.6A**, **2.6C**, and **2.6D**), contains binding sites for *FT*

transcriptional regulators, namely TEM1/TEM2 (Castillejo et al., 2008) and a CO-containing protein complex (Wenkel et al., 2006). Thus, it would be of interest in the future to test if the binding of these *FT* transcriptional regulators is altered by the activity of AtJmj4 and ELF6.

Chapter III

Arabidopsis SIN3 Homologs Negatively Regulate *ABI* genes in Seed Germination and Early Seedling Establishment

3.1 Abstract

Seed germination accompanies a number of morphological changes representing developmental transitions. During seed germination, expression of a number of genes is transcriptionally and post-transcriptionally modulated. As a major factor that links and converts environmental signals to plant responses, abscisic acid (ABA) has a key role in seed maturation and germination. Especially, precise regulation of *ABA INSENSITIVE* (*ABI*) genes, which assist ABA-signaling, is required for adequate germination. During germination, histone deacetylation-based mechanisms antagonize histone acetyltransferase activity to regulate ABA signaling-related genes on a platform of SIN3-LIKE (SNL) in *Arabidopsis*. In this study, I demonstrate that seed germination and early seedling establishment of *SNL* quadruple mutant (*snl1234*) mimics the germination process under hyperactive ABA-signaling conditions. SNL1, SNL2, SNL3, and SNL4 protein expressions are spatially overlap each other in developing and mature embryos. This is consistent with the idea that SNL proteins have redundant roles during germination. Dormancy released *snl1234* seeds and embryos showed delayed germination, and this phenotype was exaggerated with exogenous ABA treatment. Although, endogenous ABA content was not altered in *snl1234*, different expression patterns of the ABA-signaling genes, *ABI3*, *ABI4*, and *ABI5*, were observed in the mutant during germination and early seedling establishment. Moreover, attuned *ABI* expression in *snl1234* was associated with enriched histone acetylation within *ABI* chromatin. Furthermore, SNL3 directly targets *ABI3* and *ABI5* loci. Taken together, SNLs play an important role during seed germination and early seedling establishment as bedrock for histone deacetylation-based mechanism of *ABI* gene

regulation.

3.2 Introduction

Proper transition from seed germination to early seedling establishment in the life of higher plants is both ecologically and economically important. Seed germination begins with imbibition of after-ripened seed and finishes with radicle protrusion or endosperm rupture (Bewley, 1997; Bewley et al., 2013). Seed germination is also a successive process from seed development to early seedling establishment (Angelovici et al., 2010). If a favorable condition for a plant to begin its life cycle is given, the after-ripened seed germinates and subsequently develops to become a seedling. For the control of seed germination and early seedling development, various environmental factors, such as light, temperature, water and nutrient availability, are incorporated into hormonal regulatory pathways (Holdsworth et al., 2008). At molecular level, seed germination is largely influenced by the regulation of phytohormones, such as gibberellic acid (GA), abscisic acid (ABA), ethylene, and auxin. Moreover, a number of gene-expression changes through transcriptional and post-transcriptional modulations are also required to complete the seed germination (Nakabayashi et al., 2005; Soeda et al., 2005).

The signaling networks that incorporate light signals to hormonal responses are crucial in seed development, dormancy, germination, and post-germinative developmental processes (Bai et al., 2012; Finkelstein et al., 2008; Oh et al., 2012; Rajjou et al., 2012). Especially, the signaling mechanisms of phytohormones, namely GA and ABA, are essential in the regulation of seed dormancy, germination, and early seedling development. Since these phytohormones function antagonistically in the seed germination process, the

metabolism and signaling balance between GA and ABA should be tightly regulated (Koornneef et al., 1982; Razem et al., 2006; Holdsworth et al., 2008). GA breaks dormancy and promotes seed germination, while ABA induces and maintains seed dormancy (Koornneef and Karssen, 1994; White et al., 2000). During germination processes, GA-related germination mechanisms are well-characterized at both physiological and molecular levels, whereas ABA-related mechanisms have mostly been examined in relation only with physiological responses. Therefore, more detailed studies are necessary for the understanding of ABA-related molecular mechanisms acting during seed germination and early seedling establishment.

ABA-mediated inhibition of seed germination occurs through various signaling components, including ABA-INSENSITIVE 3 (ABI3) (Giraudat et al., 1992), ABI4 (Finkelstein et al., 1998; Soderman et al., 2000), and ABI5 (Finkelstein and Lynch, 2000). *ABI3*, *ABI4*, and *ABI5* encode transcription factors which are highly expressed in seeds and repressed in vegetative tissues (Giraudat et al., 1992; Finkelstein et al., 1998, 2002; Finkelstein and Lynch, 2000). It has been demonstrated that mutations in *ABI3*, *ABI4*, and *ABI5* cause ABA-insensitivity in seed and yield viviparies (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Soderman et al., 2000). To more specifically describe, ABI3 encodes a member of B3-domain transcription factor. During seed maturation, it acts in the embryo for the accumulation of storage-proteins or lipids and, more importantly, induces seed dormancy (Stone et al., 2001; Kroj et al., 2003; To et al., 2006). An ERF/AP2-type transcription factor ABI4 was revealed to participate in the regulation of seed maturation-related genes and also in the acquisition seed

dormancy (Liu et al., 2007). A basic leucine-zipper transcription factor ABI5, of which expression is enhanced by ABI3 and ABI4, stimulates the expression of many critical genes that are involved in the arrest of seedling growth after germination (Bossi et al., 2009; Lopez et al., 2002). It is clear that the network of ABA signaling components, namely ABI3, ABI4 and ABI5 as the key regulators, is critical in seed development, especially for ABA-mediated inhibition of germination (Park et al., 2011). However, more comprehensive regulatory mechanisms of such genes are yet to be revealed.

Only recently, some studies have reported that a germination-arrest mechanism is mediated by chromatin-remodeling factors (Perruc et al., 2007; Li et al., 2005). Since then, more and more studies have demonstrated that various epigenetic regulations are entailed for gene-expression changes during seed germination and very early phase of seedling establishment (Liu et al., 2011; Cho et al., 2012). Histone methylation is a renowned epigenetic control for these processes. In *Arabidopsis*, histone arginine demethylases, JUMONJI 20 (JMJ20) and JMJ22, promote seed germination in red light-dependent manner by eliminating histone H4 arginine 3 symmetric dimethylation (H4R3me2s) at the *GA3 oxidase 1* (*GA3ox1*) and *GA3ox2* loci via the PHYB-PIL5-SOM pathway (Rider et al., 2003; Cho et al., 2012). When PHYTOCROME B (PHYB) is inactive, PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5 (PIL5)/PIF1 directly upregulates the expression of *SOMNUS* (*SOM*), a repressor of seed germination, which in turn represses *GA3ox1* expression. In a previous report, SOM was shown to be enriched in the promoter of *JMJ20* or *JMJ22*, and these JMJs directly target *GA3ox1* and *GA3ox2* chromatin (Cho et al., 2012). When seeds are exposed to red-

light, H4R3me2s levels at *GA3ox1* and *GA3ox2* are decreased in wild type (wt) but not in *jmj20 jmj22* double mutants. Moreover, it has been proven that a crosstalk between histone methylation and other epigenetic modifications exists in seed germination. Although how H4R3me2s influences the level of other histone marks is not yet clear, histone H3 lysine 9 trimethylation (H3K9me3) or H3K4me3 levels are increased or decreased, respectively, in *jmj20 jmj22* compared to those in wt. Another example of epigenetic control reported is the role of PICKLE (PKL), a SWI/SNF-type chromatin remodeling factor, in the repression of embryonic trait genes, *LEAFY COTYLEDON* (*LEC1*), *LEC2*, and *FUSCA 3* (*FUS3*), during seed germination and at multiple developmental points (Rider et al., 2003). It was reported that *pkl* mutant seeds fail to develop into seedling phase because seed-associated traits are continually expressed after germination and GA biosynthesis is up-regulated in *pkl* mutant. It was recently informed that repressive role of PKL on those embryonic genes was implemented via H3K27me3 enrichment (Zhang et al., 2008).

Interestingly, among many histone modifier-mutant seeds we tested, a few mutants including non-functional *SIN3-LIKE* (*SNL*) mutants exhibited delayed germination phenotypes. *Arabidopsis* SNLs are the orthologs of yeast Swi-independent 3 (Sin3). The molecular mechanism of Sin3 in other organisms has been widely studied. In 1987, Sin3 of budding yeast was first identified as a negative transcriptional regulator (Sternberg et al., 1987). Since then, a number of researches have ascertained that Sin3 is a transcriptional co-repressor involved in gene-silencing mechanism in concert with a histone deacetylase (HDAC), Reduced potassium dependency 3 (Rpd3) (Kasten et al., 1997; Vidal and Gaber, 1991). It

has also been revealed that Sin3 itself does not possess an intrinsic DNA-binding domain. Instead, it is considered a master scaffold which serves as a platform for the association of HDACs and transcription factors (Zhang et al., 1997).

In *Arabidopsis*, six homologs of yeast Sin3 (SNL1, SNL2, SNL3, SNL4, SNL5, and SNL6) are identified by the analysis of protein sequence similarity. Moreover, a few studies have identified the roles of SNL proteins in various biological processes in plants. It has been revealed that SNL3 induces APETALA2/EREBP-type transcription factor AtERF7, which functions as an important transcriptional repressor in ABA responses upon drought stress (Song et al., 2005). Very recently, it was reported that SNL1 and SNL2 participate in the acquisition of seed dormancy via antagonistic mechanisms on the ethylene- and ABA-mediated pathways (Wang et al., 2013). In *snl1 snl2* double mutants, histone acetylation levels of ethylene synthesis-related genes (*ACO1* and *ACO4*), ethylene signaling genes (*ERF9*, *ERF105*, and *ERF112)*, and ABA hydrolase-encoding genes (*CYP707A1* and *CYP707A2*) were slightly enriched. However, these enrichment levels were less than two-fold, and these observations were made during the acquisition of seed dormancy.

Since former findings have suggested that epigenetic control of phytohormone signalings might be associated with various developmental processes such as plant stress responses and the acquisition of seed dormancy, using *snl1234* quadruple mutant seeds, here we attempted to demonstrate that all or some SNL proteins may function in seed germination and early seedling establishment via phytohormone signaling pathway,. Because both endogenous hormone level and its signaling greatly influence seed germination and early

seedling establishment, we aimed to clarify whether SNL proteins are associated with hormonal metabolism or signaling. Especially, as direct relationship between histone acetylation and seed germination is poorly understood, epigenetic aspects of hormone-related seed germination mechanism have to be more clearly elucidated. Therefore, we intended to focus on the roles of SNLs on ABA-mediated regulation during seed germination and early seedling establishment. In addition, as SNLs may function as a platform for Sin3-HDAC complex repressing target gene expression, the purpose of this study was to identify the role of SNL proteins as co-repressors of their target genes as well as to test a possibility of SNL-mediated role of histone acetylation in seed germination and early seedling establishment.

3.3 Materials and Methods

3.3.1 Plant materials and growth

snl3 and *snl4* T-DNA insertion lines in the Col background were obtained from the SALK collection (http://signal.salk.edu/; *snl3-1*, SALK_920633; *snl3-2*, SALK_028140; *snl4-1*, SALK_042565; *snl4-2*, SALK_053319). The genotype of each mutant allele was defined by gene-specific primers (listed in **Table 3.1**). The following mutants are in the Col background and were described previously: *snl1* and *snl2* (Wang et al., 2013), *abi3* (Michaels & Amasino, 1999). Double, triple and quadruple mutants were obtained by cross. All seeds were sown after After-ripening (3 months later from seed harvested) and were grown under 100 umol/m^2s, long day (16 hr light/ 8 hr dark).

For generation of the *SNL3pro::SNL3:HA* translational fusion construct, a 7.3kb genomic DNA fragment of *SNL3* containing 1.27kb 5' upstream region and the entire coding region was generated by PCR amplification using SNL3-fullength F and SNL3-fullength R primers (listed in **Table 3.2**). The amplified region was cloned into the vector pEarleyGate 301 (Earley et al., 2006). The fusion construct was introduced into *snl1234-1* by the floral dip method (Clough and Bent, 1998) through *Agrobacterium tumefaciens* strain C58C1, and transformants were selected on MS media supplemented with 1% sucrose and 25 μg ml^{-1} glufosinate ammonium. The *HDA19pro::HDA19:FLAG* (Choi et al, 2012) and *HDA9pro:HDA9:HA* (Kang et al, 2015) were described previously.

3.3.2 Germination Assay

For the Germination assays, seeds were selected that were grown as side by side at the same time. Seeds were surface-sterilized and plated on half Murashige-skoog (MS) media without sucrose (0.8% phytoagar, pH 5.7). Then, the seeds were placed in a growth chamber at 22℃ under white light. Germinated seeds, which were completely penetrated the seed coat (protrusion of the radicle) were scored at the indicated times. At least 50-100 seeds were used for each germination assay and three biological replicates were performed for statistical analyses. To determine the effect of GA3 or ABA, Seeds were placed on half MS Media supplemented with variable concentrations of GA3 or ABA.

3.3.3 Gene Expression Analyses

Total RNA was isolated from seed with previously described but modified method. (Ling Meng and Lewis Feldman, 2010). The RNA purified using Qiagen RNeasy Plant mini kit with on-cloumn DNase I digestion step (Qiagen). The 3 ug of total RNA was reverse transcribed using MMLV Reverse Transcriptase) according to the manufacturer's instructions (Fermentas, USA). The qRT-PCR analysis was performed using Rotor-Gene Q (Qiagen) with the SYBR Green Fast qPCR master mix (Kappa Biosystems) in a total volume of 20ul. Quantification of amplified products was performed by generating standard curves using serial dilutions of all cDNA sample to be analyzed. Expression level of each genes were calculated as fold change compared to *UBQ11*. All qRT-PCR results were performed in triplicate reactions of three- biological repeats as means±STD. Primer used for qRT-PCR

analysis are listed in **Table 3.3**.

3.3.4 GUS and GFP assays

For the construction of the *SNL1pro::GUS*, *SNL2pro*::*GUS*, *SNL3pro*::*GUS*, and *SNL4pro*::*GUS* transcriptional constructs, each promoter regions of *SNL1*, *2*, *3*, and *4* were amplified by specific primer sets (listed in **Table 3.2**) and then subcloned in pPZP211 vector (Hajdukiewicz et al. 1994). The final construct were introduced into Col by the floral dip method. For GUS assay, *SNL1pro*::*GUS*, *SNL2pro*::*GUS*, *SNL3pro::GUS*, and *SNL4pro::GUS* transgenic plants were reciprocally crossed with Col. 7days after pollination seeds and completely maturated embryo were used to GUS assay. Seeds were fixed by acetone on the ice for 30 min, kept in the dark wrapped with foil and washed three times with KPO4 buffer. The fixed seeds were dissected by forceps, and the extracted embryos were stained with X-Gluc solution for 2hr 30 min or overnight. The stained embryos were photographed using AxioVison under optical microscope (Carl Zeiss, Germany).

For the *SNL3pro::SNL3:GFP* transgenic line, the *SNL3* genomic locus (including 1.27kb upstream of the start codon) were amplified with the primer fullength-SNL3 F and fullength-SNL3 R (listed in **Table 3.2**). The PCR product was ligated into modified pEarleygate301 that has GFP and His tag from pEarleygate103. The construct was transformed to *snl234 triple mutant* plants following the floral dip method. Subcellular localization of SNL3::GFP was determined by LSM 510 confocal microscope (Zeiss). Embryos of *SNL3pro::SNL3:GFP* are rescued from seed coat by forceps 1hr after dark

imbibition.

3.3.5 Quantification of Endogenous ABA level

Endogenous ABA was extracted with previously described. (Francisco et al. 2000). To quantify the ABA content of *snl1234-1,-2,* and wild-type plants, 100-200 mg of dry or imbibed seeds were homogenized in 10 ml of ABA-extraction buffer (10 mm HCL, 1% PVPP in methanol). Sample was shaken overnight at 4℃, and then a supernatant was collected. Add 150μl of 1M NaOH for neutralization as described (Peña-Cortes et al. 1989). ABA was quantified with a Phytodetek-ABA-kit (AGDIA Inc., IN) using the protocol provided.

3.3.6 Chromatin immunoprecipitiation (ChIP) assay

ChIP was performed as described previously described (Jeong et al. 2009) with modifications. 500mg seeds were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin was isolated by using lysis buffer (containing 50mM HEPES, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 1mM PMSF, and Protease inhibitor with 0.1% SDS) and sonicated into ~0.5 to 1 kb fragments. Specific antibody against H3Ac (Millipore, 06-599), HA (Abcam, ab9110). FLAG (Sigma A8592-0.2MG) was added to the chromatin solution, which had been pre-cleared with salmon sperm DNA/Protein A agarose beads (Upstate 16-157). After subsequent incubation with salmon sperm DNA/Protein A

agarose beads, immune-complexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immune-complexes were removed by incubation with proteinase K, followed by DNA extraction. DNA was recovered by a DNA purification kit (Qiagen). The amount of immune-precipitated chromatins was determined by PCR with primer pairs in **Table 3.4**.

3.4 Result

3.4.1 Mutations in *SNL1, SNL2, SNL3,* and *SNL4* Cause Delayed Seed Germination

As seed germination should require turn-off and turn-on of numerous embryonic and post-embryonic genes, respectively, we hypothesized that seed germination might involve an epigenomic reprogramming. Hence, we have searched for epigenetic mutants defective in seed germination. Six arabidopsis SNL peptide sequences were identified from the *Arabidopsis* genomic DNA sequence database. It is known that SNL genes have sequence similarity to each other and share HDAC-interaction domains (Bowen et al, 2010). A phylogenic tree was generated with human, mouse, and yeast Sin3 peptide sequence with six arabidopsis SNL peptide sequences using ClustalW (Thompson et al., 1994; http://align.genome.jp/). The resultant phylogenetic tree, six SNL proteins were subdivided into three groups: SNL1 (AT3G01320) and SNL2 (AT5G15020); SNL3 (AT1G24190) and SNL4 (AT1G70060); and SNL5 (AT1G59890) and SNL6 (AT1G10450). Because Sin3 homologs of other organisms, yeast, human, and mouse, are not clustered with *Arabidopsis* SNL, it is proposed that these proteins may function in plant-specific biological contexts (**Figure 3.1A**). To address if SNLs have a functional redundancy, we searched for collections of T-DNA insertional lines of the corresponding genes and series of double, triple, and quadruple mutants of the *snl1*, *snl2*, *snl3*, and *snl4* are generated. One or two allelic homozygous T-DNA insertional mutant lines for corresponding *SNL1*, *2*, *3*, or *4* genes are obtained from the SALK collection and T-DNA insertions for each gene were depicted in

Figure3.1B. All combinations of double and triple mutants were generated by using *snl1-1*, *snl2-1*, *snl3-1*, and *snl4-1*. *snl1234-1* quadruple mutant is generated by using *snl1-1*, *snl2-1*, *snl3-1*, and *snl4-1*. Additionally, independent quadruple mutant *snl1234-2* is generated by using *snl1-1*, *snl2-1*, *snl3-2*, and *snl4-2*. The germination test of these double, triple and quadruple mutants was subsequently performed using non-dormant seeds. In the end of embryo maturation, seeds are highly dormant, which are marked by a very high mRNA expression level of *DELAY OF GERMINATION 1*(*DOG1*). Immediately after maturation, freshly harvested (FH) seeds have not yet acquired the competence for seed germination. The measurement of germination ability at FH seed stage is used to indicate the level of seed dormancy. On the other hand, germination-competence is obtained after endurance of a certain period of time to release dormancy. During dry storage, several physiological changes are followed in seeds so that greenish color become brown and seeds hold adequate amounts of moisture and oil contents, and endogenous phytohormone levels. When the seed state is optimized for germination, seeds become non-dormant and ready to germinate upon a signal, which are called after-ripened (AR) seeds. For all the germination tests, non-dormant AR seeds, which now have a germination-competence, are used. In the **Figure 3.1C**, the result has shown that the quadruple mutants *snl1234-1* and *snl1234-2* have a delayed seed germination phenotype compared with the wildtype. In **Figure 3.1E** and **F**, all of double and triple mutants did not show any notable germination phenotype. Differences during germination and early seedling establishment process between wildtype and *snl1234* mutant seeds were depicted in **Figure 3.1D**. *snl1234-1* mutant allele was shown to have a little stronger

phenotype than *snl1234-2*, so we used *snl1234-1* allele for all of followed experiments. As shown in **Figure 3.1E and F**, wildtype and each combination of double and triple mutant seeds were completely germinated within 4 days after sowing. However, *snl1234-1* and *snl1234-2* quadruple mutant seeds were not fully germinated until 10 days after sowing. These results suggest that SNL 1, 2, 3, and 4 have a functional redundancy in seed germination process. In addition, transgenic *snl1234-1* containing the *SNL3pro::SNL3:HA* construct showed a restored germination ability at wildtype level (**Figure 3.1C**). Therefore, it is clear that *Arabidopsis* SNLs have functional redundancy in seed germination processes.

To assess the roles of SNLs in seed germination in more details, we generated *SNLs pro::GUS* transgenic plants which have a SNL1, 2, 3, or 4 transcriptional fusion with β-glucuronidase (GUS) construct. The spatial expression patterns of each SNL protein were examined by histochemical GUS staining (**Figure 3.2A**). Since the seed germination is affected by both the maternal and zygotic-originated tissues, we examined the SNLs expression in distinguished tissues, using seeds and mature embryos of F1 progeny, which were obtained by reciprocal cross between Col and each *SNLs pro::GUS*. In maternal tissue seed coat, expression of SNL2pro::GUS and SNL3pro::GUS were visualized but those of SNL1pro::GUS and SNL4pro::GUS were not shown. For zygotic-originated tissues embryo and endosperm, all of SNLs pro::GUS were clearly observed in embryos under both stages of developmental and mature embryos but none of them were visible in endosperm of developing seed. This indicates that SNL1, 2, 3, and 4 expressions are overlapped in embryo from developmental stage to after progression of mature seed (**Figure 3.2A**). Previously SNLs were reported that they function as a

platform of HDAC-associated complex. Additionally, it was demonstrated that SNL1 colocalizes with HDA19 in the nucleus of tobacco leaf cell by a ratio metric BiFC Assay (Perrella et al., 2013). To confirm the expression patterns of SNLs in the nucleus at embryonic stage, the subcellular localization of SNL3 protein was depicted. The subcellular localization of SNL3 protein was demonstrated by transgenic plants containing the construct that have its own promoter and SNL3 protein fused to green fluorescence protein (GFP; *SNL3pro::SNL3:GFP*) (**Figure 3.2B**). GFP signals were detected in the nucleus of *SNL3pro::SNL3:GFP* transgenic embryo. *snl234-1* is used as a negative control and no GFP signal was detected. Altogether, it is suggested that SNLs may function redundantly in embryo, within the nucleus, and be involved in the regulation of seed germination.

We next investigated whether the *snl1234* mutant embryo can grow without defects and to reach the typical seedling stage (**Figure 3.3**). Embryos were separated from the seed coats and grown. Embryo-germination tests were performed by counting the emerged green cotyledons at 7days after sowing on half MS media with sucrose (**Figure 3.3A**). In comparison with seed germination test, embryo germination test was depicted in **Figure 3.3A** and the percentages of green cotyledons among scattered embryos or seeds were represented in graph as in **Figure 3.3B**. While more than 90% wildtype embryos were developed into seedlings with green cotyledons, less than 80% *snl1234* mutant embryos showed green cotyledons (**Figure 3.3B**). On the other hand, more than 80% wildtype seeds were germinated and expanded green cotyledons, whereas less than 50% *snl1234* mutant seeds showed green cotyledons. This result suggests that seed germination is controlled more acutely than embryos germination and the delayed germination

by *snl1234* is more prominent with the seed coat. This result supports that SNLs are involved in seed germination process, which represents the development of embryo to seedling, better accomplished in the presence of the seed coats. Interestingly, sizes of seeds and embryos are slightly reduced in *snl1234* compared with wildtype and the small seedling phenotype was continuously observed after germination (**Figure 3.3C**).

In addition, it is reported that the regulation of embryo-specific genes, such as *LEC1*, *LEC2*, and *FUS3*, are not only critical for seed developmental stages but also in seedling developmental stages (Dean Rider et al, 2003; To et al, 2006). Ectopic expression of embryo-specific genes during embryo development and de-repression of those gene expressions in subsequent stages cause the failure of seedling growth after germination. Moreover, it is reported that HDACs, candidates of SNL-functional partner, might be involved in the repression embryo-specific genes during germination (Tanaka et al., 2008; Rider et al., 2003; Zhou et al., 2004; Tai et al., 2005). To address such embryo-specific genes' expression level in *snl1234*, quantitative realtime (qRT)-PCR was performed with gene specific primer sets listed in **Table 3.3**. The mRNA levels of each embryo-specific genes were monitored from dry seeds, through 4 days of stratification until 7 days on MS under constant light (**Figure 3.4**). As the result of qRT-PCR analyses, there was neither relevance nor consistency between altered expression patterns of embryo-specific genes and the phenotype of *snl1234*. Expressions of *LEC1* and *FUS* in *snl1234* were more suppressed than those in wildtype. Although expression patterns of *LEC2*, *CRC*, and *RAB18* were altered in *snl1234* at specific time points during stratification until the first day after light treatment but they were not

constantly maintained. Rather, delayed germination of *snl1234* was not caused by alteration of embryo-specific gene expression and expression alteration of embryonic genes may be the result of the delayed germination in *snl1234*.

3.4.2 SNL1, SNL2, SNL3, and SNL4 Are Independent to GA Metabolism, Bioactive GA Levels, or Expression of GA Signaling Mediators

To determine whether the delayed germination phenotype of *snl1234* is caused by the deficiency or the insensitivity of endogenous GA hormone, we performed germination tests with exogenous GA treatment on the media. First, 100μM of GA was added in the media (**Figure 3.5A**). As a result, germination in wildtype was little shifted to earlier dates after sowing. This means 100μM of exogenous GA was sufficient to induce early germination and show seed germination rate in GA-dependent manner. However, *snl1234* seeds still showed delayed germination phenotypes as in the germination test without GA. Therefore, the delayed germination phenotype of *snl1234* was not due to lack of endogenous GA. Moreover, GA signaling is also important in seed germination and it is known that the disruption of GA signaling pathway leads to the failure of seed germination. To test if SNLs are involved in the GA signaling, we determined the GA sensitivity in *snl1234* mutant. GA sensitivity was represented by the germination rate on media containing paclobutrazol (PAC), GA biosynthesis inhibitor, with various GA concentrations indicated in the **Figure 3.5B**. As in the GA-only supplied media, *snl1234* mutant seeds were germinated less efficiently than wildtype in the

condition of 100μM GA supplemented with 80μM of PAC. In addition, the germination rates of *snl1234* mutant seeds were elevated in accordance with the increase of GA concentration. This result suggests that the delayed germination of *snl1234* quadruple mutants is not associated with GA-sensitivity.

Additionally, we measured the expression levels of GA metabolic and signaling genes in different stages of germination process using dried or stratified seeds and seeds under constant light treatment for seven days. First, the expression patterns of *GA20ox2*, *GA3ox1*, and *GA3ox2* are measured as GA biosynthetic genes. GA20ox2 catalyzes a multi-step oxidation to produce immediate precursors of active GA and, GA3ox1 and GA3ox2 convert these GA precursors to their bioactive forms in plant. As in **Figure 3.5C**, the expressional peak of *GA3ox1* in *snl* quadruple mutant is shifted to earlier day, at day 1 after stratification instead of day 3 in wildtype. Without any shifts in time, the peak of *GA3ox2* mRNA level of was higher in *snl1234* than in wildtype during germination. In addition, although *GA20ox2* expression was low during stratification in the mutant, it became almost the same as that in wildtype during germination. On the other hand, to consider negative regulators of GA signaling into our account, we measured mRNA expression patterns of genes that encode DELLA proteins such as *REPRESSOR OF GA1 (RGA1)*, *RGA-LIKE1 (RGL1)* and *RGL2*. Expression levels of the repressive genes were increased both in wildtype and *snl1234* mutant during germination to repress GA responses and integration of GA signaling during the process. However, after germination, expressions of these DELLA protein-encoding genes were decreased. This is consistent in both wildtype and *snl1234* mutant. Overall, these results suggest that GA metabolism and signaling pathway are not associated with

the delayed germination phenotype of *snl1234*. In other words, GA-mediated germination pathway is not affected by mutations of *SNLs*.

3.4.3 SNL1, SNL2, SNL3 and SNL4 Do Not Affect the Endogenous ABA Level

ABA is one of the key hormones in sustaining seed dormancy. In order for seeds to germinate, ABA level must be down-regulated. Thus, biological regulations of ABA synthesis and signaling are also critical in seed germination mechanism (Schroeder et al., 2001; Cutler et al., 2010; Weiner et al., 2010). It is known that the mutations of *SNL1* and *SNL2* cause alteration of ABA contents in dormant seeds (Wang et al., 2013). To test whether endogenous ABA contents are altered by SNL-mutations during seed germination, ABA concentration levels in wildtype and the quadruple mutants were measured in non-dormant dried or imbibed seeds (**Figure 3.6A**). As known in wildtype that endogenous ABA level is decreased by imbibition, the ABA level in *snl1234* was also decreased by imbibition but maintained at the same level of wildtype in both dried and imbibed samples. Unlike in dormant seeds, endogenous ABA contents were not affected by *SNL* mutation, even in the condition where four members of *SNL* subfamily members were disrupted. This result suggests that the delayed germination phenotype of *snl1234* was not caused by endogenous ABA contents. To add more, mRNA expression levels of ABA biosynthetic and metabolic genes were measured during germination (**Figure 3.6B**). *9-cis-EPOXYCAROTENOID DIOXYGENASE6 (NCED6)* and *NCED9* are known to be seed-specifically expressed and represent ABA

biosynthetic genes during seed development. During germination, from dried seeds until the endosperms are ruptured, the transcription level of *NCED6*, encoding the rate-limiting enzyme in ABA biosynthesis, fluctuates and hits a peak at the end of stratification in wildtype seeds. In *snl1234* mutant seeds, varied pattern is similar but with smaller amplitudes and likely a slowed rate of decrease until the germination is completed. The mRNA expression level of *NCED9* also undulates during germination in both wildtype and *snl1234* mutant seeds. The re-repression rate of *NCED9* expression in *snl1234* during germination, after the expressional peak at day1, seemed to be little slowed down than in wildtype. Furthermore, the transcription level of *CYP707A2*, which encodes ABA 8'-hydroxylase an enzyme that degrades ABA, was also measured during germination. Its expression in wildtype seeds peaks at the end of stratification while that in the mutant seeds is highest on the day 1 after stratification. To recapitulate the results, up-and-down patterns of both ABA biosynthetic and degrading genes exist in both wildtype and *snl1234* and it is obvious that the expressional peak of the critical ABA biosynthetic gene, *NCED6*, is concurrently overlapped with that of ABA degrading gene, *CYP707A2*. However, it is interesting that the expressional peak in both mechanism was delayed and the fluctuation of transcriptional level is dulled in *snl1234* than in wildtype. Altogether, this suggest a possibility that, although total endogenous ABA level is sustained in *snl1234* mutant seeds compared with that in wildtype, ABA-related metabolism is somewhat affected in the *snl* quadruple mutant during germination. It suggests that the rate of ABA metabolism and correspondingly the ABA sensitivity in *snl1234* mutant is altered.

3.4.4 SNL1, SNL2, SNL3 and SNL4 Play a Role in ABA Signaling During Germination

To verify and confirm the roles of SNLs in ABA-mediated signaling pathway, we tried to evaluate ABA sensitivity of *snl1234* seeds. Wildtype and *snl1234* seeds were sown on half MS media containing 0, 0.5, 1, 3, or 8 μM of ABA and their germination efficiency was measured. At 48 hours after sowing, wildtype seeds were almost completely germinated on the media containing up to 3 μM of ABA. In *snl1234*, however, the percentage of seed germination was notably decreased on the media containing only 0.5 μM of ABA and the germination was practically inhibited by 1 μM of exogenous ABA (**Figure 3.7A**). To observe the germination dynamics, we measured germination rates at more specific time points under the indicated ABA conditions (**Figure 3.7B**). Under low ABA concentration conditions, which do not affect germination of wildtype seeds, *snl1234* showed a delayed germination pattern. 0.5 μM ABA in the media was sufficient to delay the germination rate of *snl1234* mutant seeds. Therefore, we could infer that *snl* quadruple mutant is hypersensitive to ABA. Obviously, more severe phenotype in *snl1234* with more reduced rate and efficiency in seed germination was exhibited according to the increased ABA treatments. In addition, at 78 hours after sowing, wildtype seeds were fully germinated even with exogenous ABA treatments while *snl1234* seeds were germinated less than 70%, 50%, or 25% on 1 μM, 3 μM, or 8 μM ABA-containing media, respectively. These results support that *snl1234* is hypersensitive to ABA and SNLs are involved in the ABA-mediated signaling pathway during seed germination.

To identify how SNLs are associated in ABA signaling mechanism, we primarily examined mRNA expression of the central ABA signaling component genes, *ABI3, ABI4,* and *ABI5,* during seed germination and seedling establishment. Transcriptional levels of *ABI* genes were determined by qRT-PCR in wildtype and *snl1234* seeds during germination in the same condition as other previous observations were made (**Figure 3.8**). In wildtype, mRNA expression pattern of *ABI3* and *ABI4* dramatically increase after stratification. This pattern is also kept in *snl1234*. Interestingly, on the third day after stratification when all the *ABI* genes become completely re-repressed in wildtype, the expression is still remained and the extent of the decrease is less steep in the mutant throughout the germination process. Although the overall expression pattern of *ABI5* during germination process is little different from other *ABI* gene patterns, uncontrolled expression on day3 in *snl* mutants is also consistent. It is remarkable that induced *ABI* expression levels were tardily decreased in *snl1234* than in wildtype. Representing as ABI-downstream genes, mRNA expression levels of *RD29B* and *SOM*, were also elevated in *snl1234*. Moreover, un-repressed expression of these genes on day3 was observed as well. Again, these results show that ABA hypersensitivity mediated by SNL mutations were most significant on day3 during germination or early seedling establishment.

As shown in Figure 1, *snl* single, double, or triple mutants did not have defects in seed germination and it was implied as that SNLs may have a functional redundancy during seed germination. To confirm whether the redundancy is elaborated within the ABA-signaling, the expression patterns of *ABI* genes were observed from the seeds of various combinations of *snl* double mutants. The

mRNA levels of *ABI* genes from germinating seeds at day2 after the stratification were shown in **Figure 3.9**. Consistent with the germination phenotypes, *ABI* expressions were not induced in any of the *snl* double mutant seeds compared to the *snl* quadruple mutants. Moreover, it was reported that, for seed dormancy, SNL1 and SNL2 function in redundant manner in dormant seeds through the regulation of ABA-ethylene antagonism. These results support the idea that all of four SNLs, SNL 1, 2, 3, and 4, have a functional redundancy in the ABA-signaling regulation mechanism during seed germination and early seedling establishment. Furthermore, this results approve that ABA-mediated regulation mechanism for seed dormancy and seed germination is through separate and distinctive pathways.

To ensure the function of SNLs within ABA signaling pathway, we generated quintuple mutant *snl1234-1 abi3*. Mutation in *ABI3* exhibits aberrant seed development and *abi3* seeds fail to desiccate during seed maturation, resulting in loss of viability upon matured dry seed (Huang et al., 2008). Thus, the germination test involving *abi3* mutant seeds were implemented using FH seeds of Col, *abi3-16*, *snl1234-1* and *snl1234-1 abi3-16* (**Figure 3.10**). Because FH seeds have elevated dormancy, seeds were no to germinate even if the proper condition for germination is given. To partially break the seed dormancy, seeds were stratified for a longer period of time than usual before performing the germination test. Due to the raised dormancy, germination in wildtype was reduced to less than 40 % and that in *snl1234-1* seeds were also reduced but still with the delayed phenotype. As expected, in *abi3-6,* where the dormancy was not acquired at all, showed early germination phenotype. Interestingly, however, *snl1234-1 abi3-16* seeds germinated in equal rate with *abi3-16* single mutant seeds. This result

indicates that *ABI3* mutation masks the effects of *snl1234* on seed germination and supports that SNLs function in seed germination through the ABI genes. It also suggests that *ABI* genes may be the targets of SNL complex during seed germination and early seed establishment.

3.4.5 SNL1, SNL2, SNL3, and SNL4 Affect *ABI* Transcription via Regulation of H3Ac Level

In yeast and mammals, Sin3 is involved in the transcriptional inactivation by interacting with HDACs. (Lai et al., 2001; Grzenda et al., 2009) It proposes that SNLs may affect the transcription of *ABI* genes during germination via regulation of histone acetylation. To evaluate whether the H3Ac levels of *ABI* genes were affected by *snl1234*, we performed chromatin immunoprecipitation (ChIP) assay using specific antibody against acetylated histone H3 (H3Ac) (**Figure 3.11**). Seeds were collected when differential transcript levels were observed between wildtype and *snl1234* mutant in the previous experiments, at 3d during germination. The relative H3Ac levels were determined by quantitative PCR (qPCR) amplifying various regions of *ABI* genes depicted in **Figure 3.11A**. Increased H3Ac levels in *snl1234* relative to wildtype were detected at promoter regions of all *ABI* genes, *ABI3*, *ABI4*, and *ABI5* (**Figure 3.11B and C**). In particular, a strong ABA-signaling factor, *ABI3*, also has an increased acetylation levels on exonic regions as well as the promoter region. This result approves that SNLs repress *ABI* genes by mediating the regulation of histone deacetylation levels.

To authenticate whether *ABI* genes are directly targeted by SNLs, we

performed a binding assay using functionally expressed *proSNL3::SNL3:HA* in *snl1234-1* transgenic line (**Figure 3.12**). Enrichment of SNL3:HA was observed on the 5'UTR and promoter regions of *ABI3* and *ABI5*. Concisely, SNL3 is strongly bound on the 5'UTR and a promoter regions of the *ABI* genes to directly repress their transcription. It is coherent with the fact that the 5'UTR region of *ABI3* is essential for the negative regulation of its transcription expression (Ryu et al, 2014; Zhou et al, 2013). Moreover, similarly with the H3Ac enrichment levels, enhanced SNL3 enrichment was also observed on the exonic regions of *ABI3* locus. These results support that *ABI* transcriptional regulation is mediated by direct involvement of SNLs through modification of histone acetylation mechanism. Alternatively, association of SNL3 was not detected within *ABI4* locus despite of that H3 hyperacetylation was retained on *ABI4* promoter region in *snl1234*. In other words, direct interaction of SNL3 is executed only on *ABI3* and *ABI5* but not on *ABI4* chromatin. This result implies that, although there exists a high redundancy among the SNL proteins, there also exists a target specificity among SNLs. Further study is required to clarify the specificity of each SNL for their target in ABA signaling mechanism during germination.

Then, it was indispensable to find a SNL-complex components since SNLs do not have a DNA-binding domain themselves and it is notorious that metazoan Sin3 forms a complex with Class I HDACs to regulate H3Ac level of its target. Thus, to find an ally, among various HDACs, that is correlated with the germination phenotype of *snl1234*, ChIP analyses were performed (**Figure 3.13**). HDA19 or HDA9 enrichment on *ABI3* loci was displayed using *HDA19pro::HDA19:FLAG* or *HDA9pro:HDA9:HA* transgenic line and a FLAG- or HA-antibody, respectively.

Recently, it was reported that HDA19 form a complex with brassinosteroid (BR)-activated BES1 (BRASSINAZOLE-RESISTANT 2) and WD40 domain-containing TPL (TOPLESS) to mediate an inhibitory action of BR-signaling to repress transcription of *ABI3* and *ABI5* (Ryu et al., 2014). Moreover, another latest study demonstrated that HDA9 negatively influences germination and represses seedling traits in dry seeds (Zanten et al., 2014). Unfortunately, either HDA19 or HDA9, the best characterized HDACs in *Arabidopsis* associated with ABA signaling and germination, does not show prominent enrichments on all examined regions of *ABI3* locus, representing that there is no direct interaction between the tested HDACs and *ABI* genes for SNL-mediated germination and early seedling establishment processes.

With an inquisitiveness to identify a component of SNL-HDAC complex, various candidate *hdac* mutants were examined for *ABI* mRNA expression levels (**Figure 3.14**). Different classes of HDAC mutants were tested. Among the classified HDACs, *had6, hda9* and *hda19* as a Class I RPD3-type, *hda14* as an unclassified of RPD3-type, and *srt2-1* and *srt2-2* as SIR2-type HDAC were used in the experiment using the appropriate ecotype for control. Within abiotic stress or ABA response mechanism, a model was proposed in which HDA6 and HDA19 redundantly function through a binding of ERF4 and ERF7 to their target genes, possibly recruited by AtSin3 (Chen et al., 2010). However, expression of ABA signaling genes are not altered in *hda6* or *hda19* mutants implicating that HDA6 or HDA19 are not recruited by SNLs during seed germination for ABA signaling. Interestingly, it was observed that, in *hda14* mutant seeds, the expression all of *ABI* genes was increased compared with wildtype. Currently, HDA14 is unclassified

within RPD3-type subfamilies in *Arabidopsis*, and only one report was made on *HDA14* (Tran et al., 2012). Therefore, more profound studies are required on HDA14, as a candidate for SNL-partner, to elucidate the distinctive acetylation modulation mechanism in ABA signaling during seed germination and early seedling establishment processes.

3.5 Figures and Tables

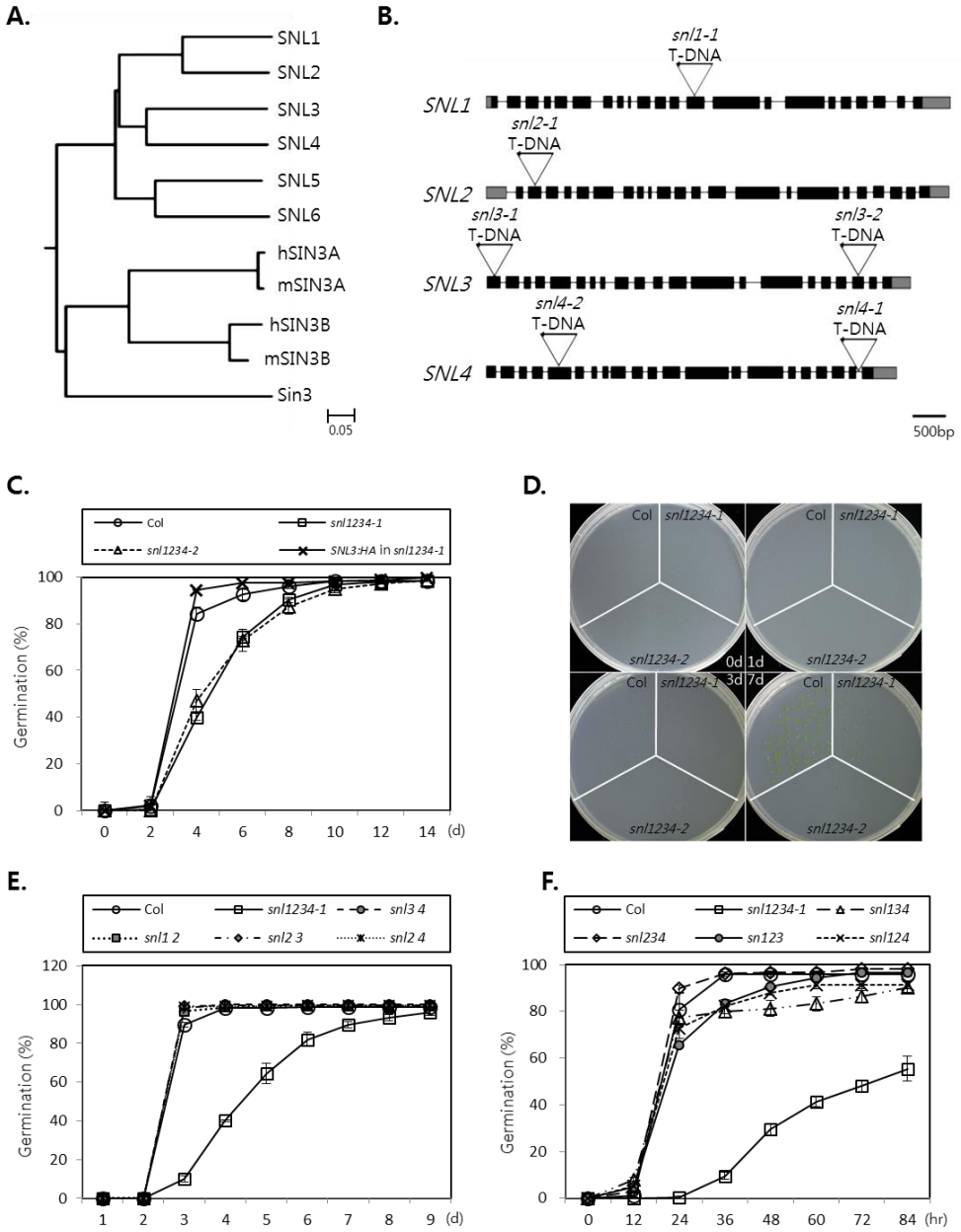

Figure 3.1 Delayed seed germination phenotype of *snl1234* quadruple mutant

(A) Phylogenic analysis of yeast Sin3 and its homolog proteins of Arabidopsis, human, and mouse. Analysis of the six SNL peptide sequences was performed by

ClustalW (Thompson et al., 1994; http://align.genome.jp/). **(B)** Schematic representation of the *SNL1*, *2*, *3*, and *4* gene structures, indicating the positions of the T-DNA insertions. Exons are represented as black boxes, UTR regions as gray boxes and introns as black lines. **(C)** Seed germination of *snl1234* quadruple mutants and a complementation line generated by an introduction of *SNL3pro::SNL3:HA* construct into *snl1234-1*. Germination was scored by radicle protrusion at the indicated time after sowing on half MS media without sucrose. Seed germination assays were performed in three replicates using at least 100 seeds for each genotype and the average value is graphed with ±Standard error (SE). **(D)** Photographs of Col, *snl1234-1*, and *snl12334-2* at 0, 1, 3, and 7 days after sowing on half MS medium without sucrose. **(E and F)** Seed germination of different combinations of double or triple mutants of *SNL1*, *2*, *3*, and *4*. Seed germination was scored at the indicated time on half MS media without sucrose. All seed germination assays were performed in three replicates using more than 100 seeds for each genotype and the average value is shown with ±SE.

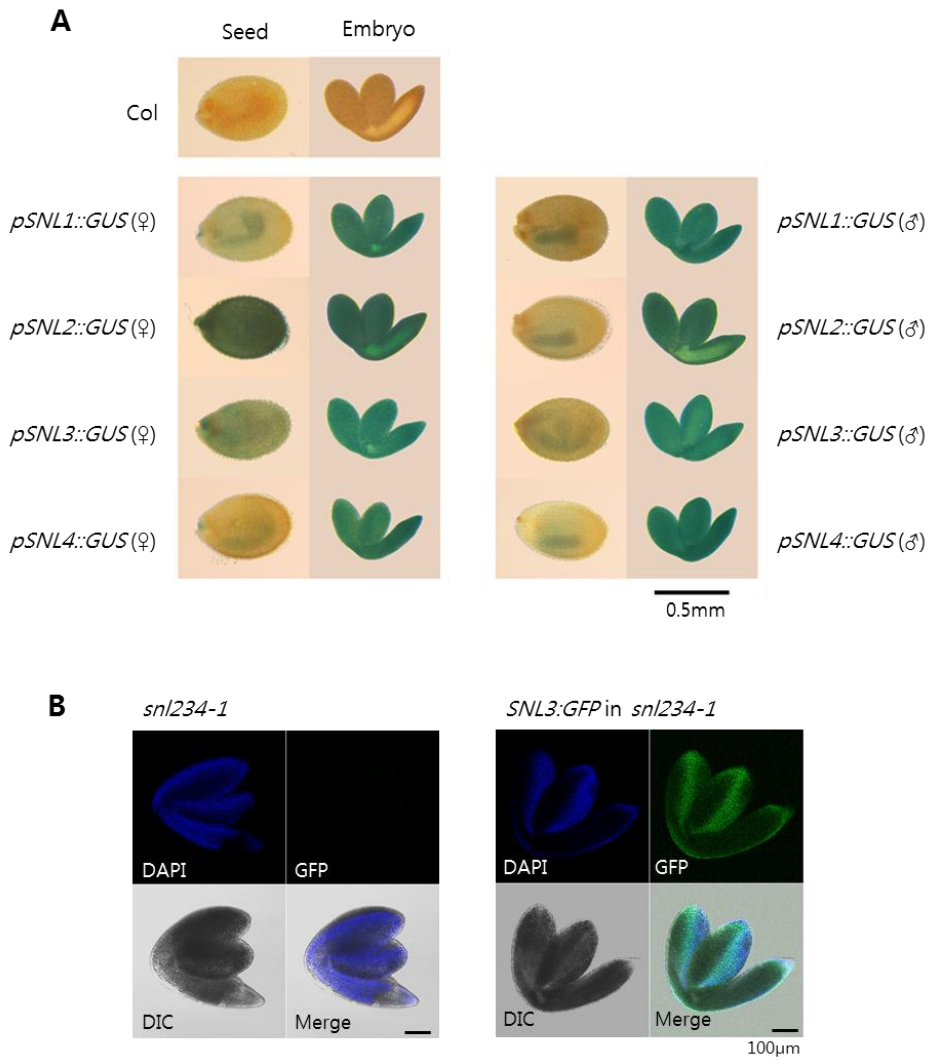

Figure 3.2 Spatial or subcellular expression patterns of the SNL1, 2, 3, and 4 in seeds or embryos

(A) Histochemical GUS staining of F1 whole seeds or embryos. Female (left panel) or male (right panel) plants harboring *SNL1, 2, 3, or 4pro::GUS* constructs was used for reciprocal cross with Col. 7 days after pollination, seeds with seed coats and rescued embryos from mature seeds were used in GUS staining. The bar

represents 0.5mm **(B)** Subcellular localization of SNL3 protein. Transient expression of *SNL3pro::SNL3:GFP* showed localization of SNL3 in the nucleus. Images were obtained by confocal laser scanning microscopy. The bar represents 100µm. Nuclear-specific DAPI staining was also depicted in addition to SNL3:GFP image, using differential interference contrast (DIC) image, the merged image of all three images clearly showed SNL3 localizes in nucleus.

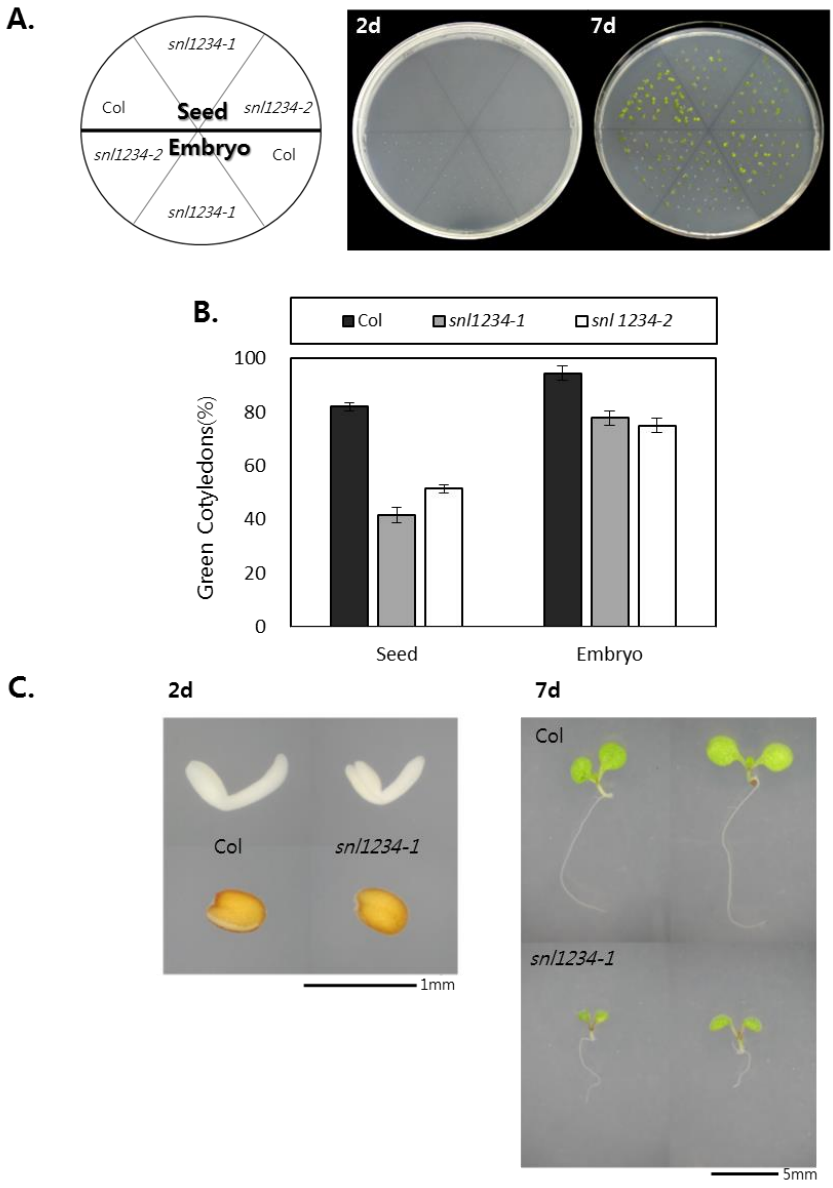

Figure 3.3 Delayed embryo germination of *snl1234* quadruple mutant

(A) Photographs of embryo germination with or without the seed coat at 2 days or 7 days after sowing. Whole seeds and rescued embryos without seed coats from wildtype, *snl1234-1* and *snl1234-2* were germinated and grown on half MS media

without sucrose for 7 days and depicted at the indicated time. **(B)** Embryo germination scored by appearance of green cotyledons at 7 days after sowing. Experiments were performed using embryos with or without seed coats and the scoring was graphed from duplicates of at least 30 embryos or seeds. The average value is shown with ±SE. Embryos were dissected from 20 min of imbibed seeds then sown on half MS medium with 1% sucrose. **(C)** Morphological phenotype of *snl1234* mutant seeds, embryos and seedlings. Seeds and embryos were germinated and grown on half MS media without sucrose for 7 days. Pictures were taken in detail on day 2 or 7 during germination. Left panel illustrates embryos and seeds of wildtype and *snl1234-1* at 2 days. Radicle protrusion is obvious in wildtype seeds. Right panel illustrates seedlings of wildtype and *snl1234-1* at 7 days. Bar represents 1mm (left) or 5mm (right).

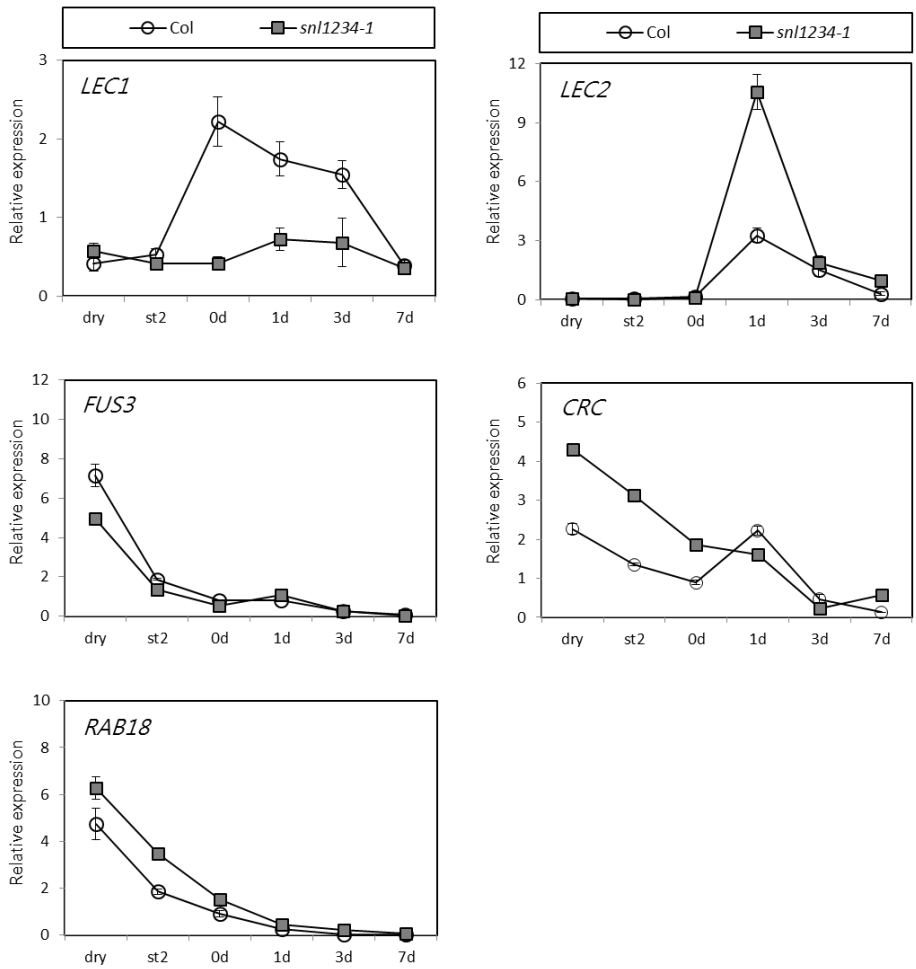

Figure 3.4 Relative transcript levels of embryo-related genes during germination

qRT-PCR analyses of transcript abundances of various embryo-related genes in wildtype and *snl1234* mutant seeds. *LEC1*, *LEC2*, *FUS3*, *CRC*, and *RAB18* were measured. The seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification), and harvested at the indicated time for RNA extraction. In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period

of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents reanscript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were quantified, in relative to UBQ11. Error bars represent ±SE of three independent biological replicates.

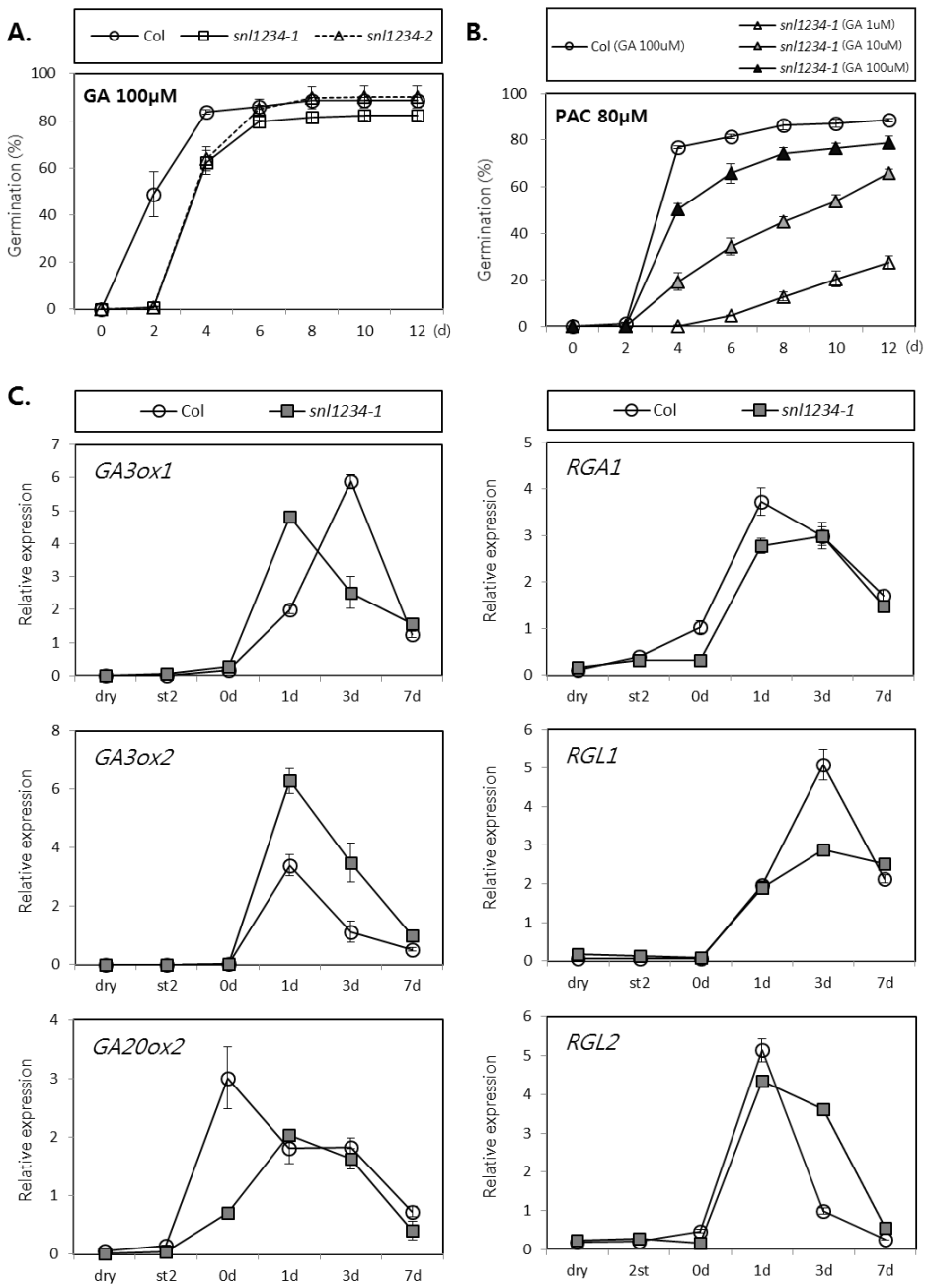

Figure 3.5 Effect of GA in *snl1234* quadruple mutant

(A) Germination of Col, *snl1234-1*, and *snl1234-2* seeds scored upon exogenous

GA. Germination assay was performed on 100μM GA-supplemented media. Each circle, square, or trigngle represents wildtype, *snl1234-1*, or *snl1234-2* mutant seeds, respectively. Germination assays were examined in triplicates with more than 100 seeds each genotype. The average value is shown with ±SE. **(B)** Percentage of germination in wildtype, *snl1234-1*, *snl1234-2* seeds measured in response to GA supplemented with 80μM of PAC. Different concentrations of 1, 10, or 100 μM GA in the media was used to determine GA response in *snl1234*. Open circle represents germination of wildtype seeds under 100 μM GA condition and white, gray, or black triangle is germination of *snl1234-1* mutant seeds on 1, 10, or 100 μM GA supplemented media, respectively. All germination assays were examined in triplicates of more than 100 seeds from each genotype. The average value is shown with ±SE. **(C)** Relative transcript levels of GA metabolic genes (*GA3ox1*, *GA3ox2*, and *GA20ox2*) and GA signaling-related genes (*RGA1*, *RGL1*, and *RGL2*) in Col and *snl1234-1* mutant seeds. Seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification), and then seeds and seedlings were harvested at the indicated time for RNA extraction. On the horizontal axis in the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

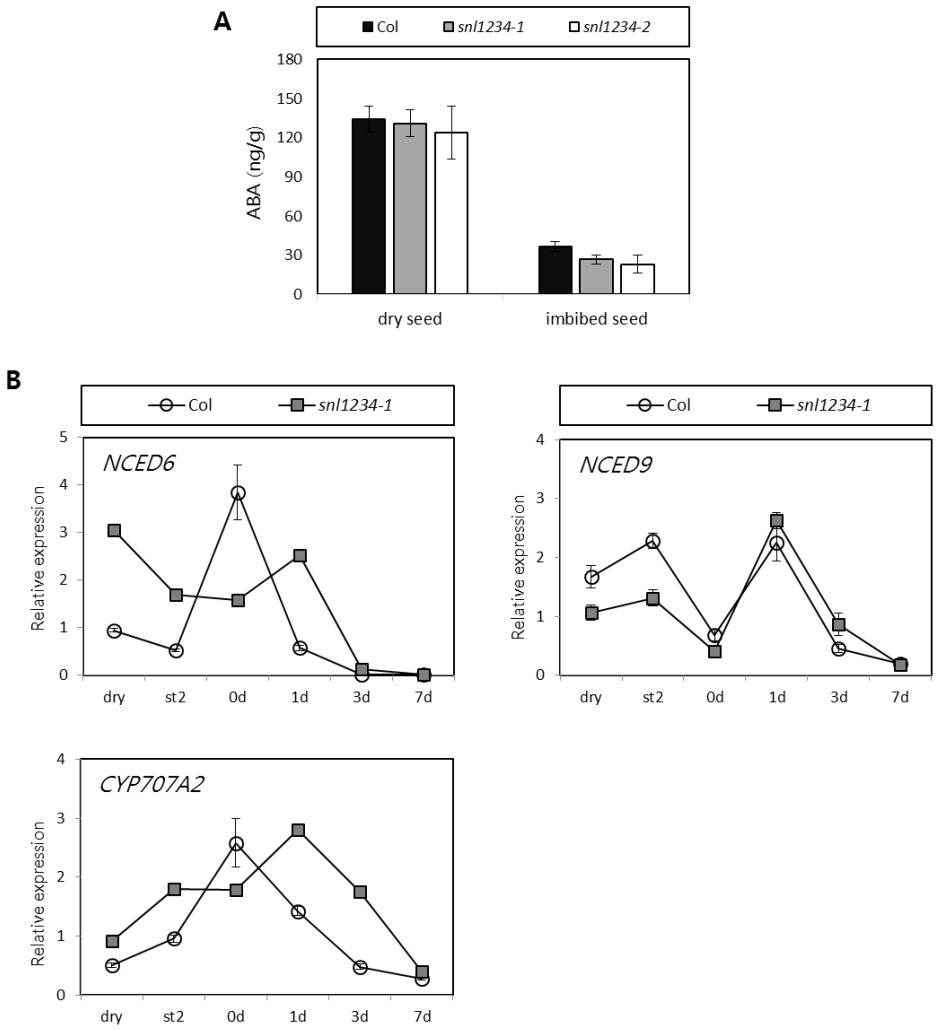

Figure 3.6 Effect of ABA in *snl1234* quadruple mutant

(A) ABA levels in Col, *snl1234-1*, and *snl1234-2* mutant seeds. Endogenous ABA concentration was measured in dried (left) or imbibed (right) seeds from wildtype and *snl1234* mutants. Imbibed seeds were supplied with water and stored at 4℃ for 24h dark condition. Black bars represent ABA concentration in wildtype. Gray or white bars are ABA concentration in *snl1234-1* or *snl1234-2* mutant,

respectively. The data is shown with ±SE of biological duplicates. **(B)** Relative transcript levels of ABA metabolic genes (*NCED6*, *NCED9*, and *CYP707A2*) in Col, *snl1234-1* mutant. All the conditions were the same as in Fig 5C. In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

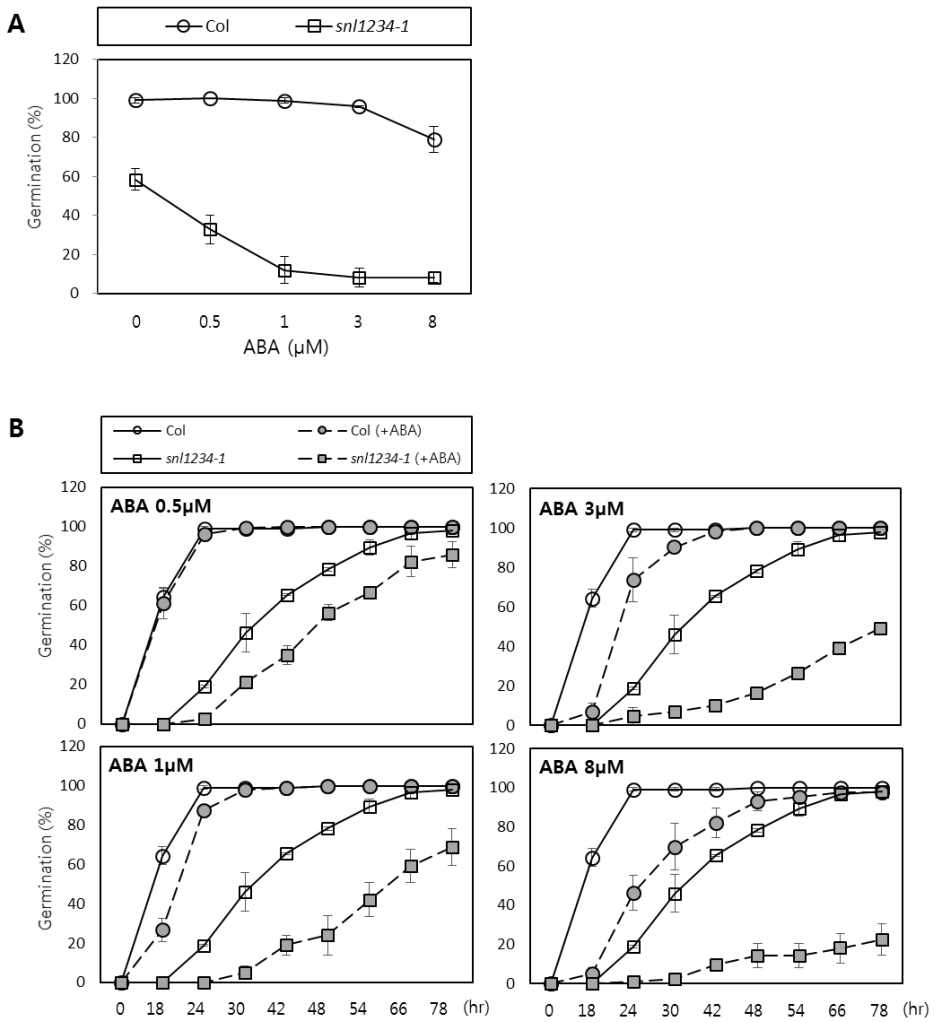

Figure 3.7 ABA hypersensitivity of *snl1234* in seed germination

(A) ABA response of Col and *snl1234-1* seeds. The germination was scored in half MS media containing 0, 0.5, 1, 3, or 8 μM ABA concentrations at 48h after sowing. The circle represents germination of wildtype seeds and the square is that of *snl1234-1* mutant seeds. Germination assay is performed in three replicates using about 100 seeds for each genotype and the average value is shown with ±SE. **(B)** Seed germination of Col and *snl1234-1* mutant in time course under various ABA

concentration conditions. Seed germination was scored at the indicated time under media containing 0, 0.5, 1, 3, or 8 μM ABA. Open circle or square represents germination of wildtype or *snl1234-1* mutant seeds on plain half MS media, respectively, while closed circle or square does that on ABA supplied condition. All germination assays are performed in three replicates using about 100 seeds for each genotype and the average value is shown with ±SE.

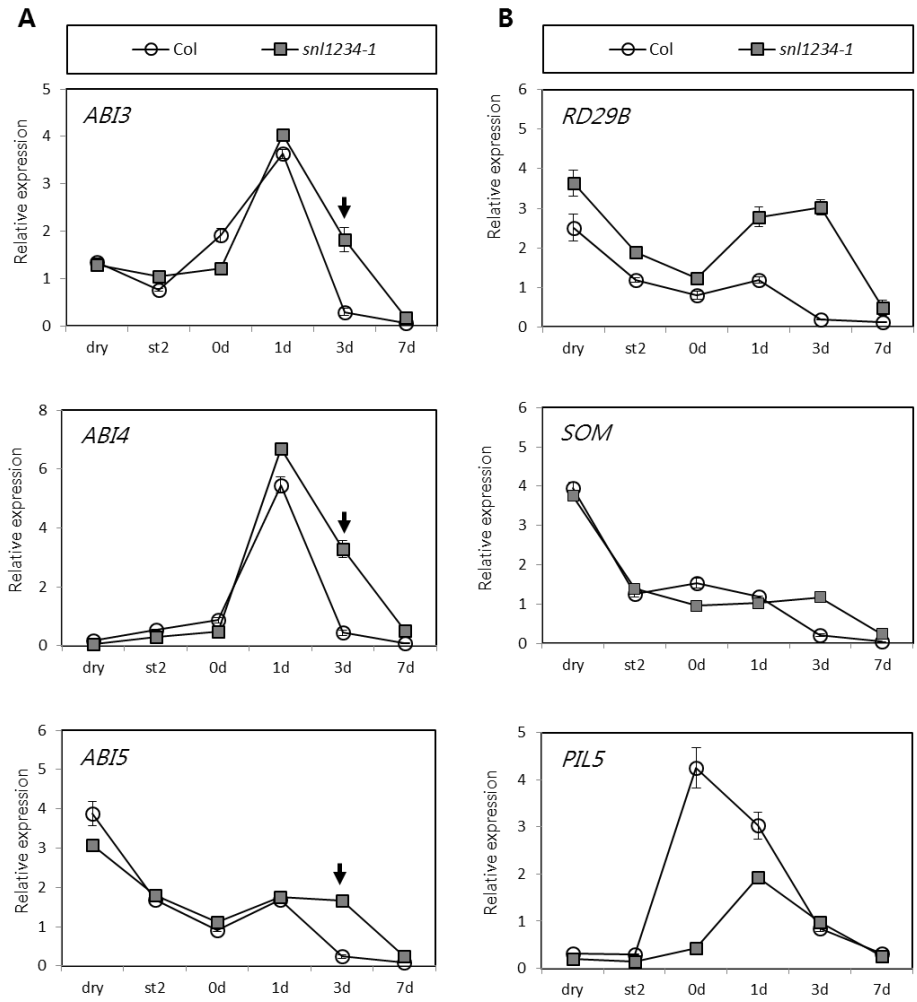

Figure 3.8 Expression of ABA signaling genes in *snl1234* quadruple mutant

(A) Relative expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in Col and *snl1234-1* mutant seeds during germination. Seeds were germinated and grown on half MS media without sucrose for 11 days (7 days under constant light condition after 4 days of stratification). Then seeds and seedlings were harvested at the indicated time for RNA extraction. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE from three independent biological

replicates. **(B)** RNA expression of *RD29B*, *SOM*, and *PIL5*, that may be altered by *ABI3* during seed germination. All the conditions are the same as (A). In the graphs, dry stands for after-ripened dry seed state, st2 for 2days of stratification (a period of moist and cold), 0d for immediately after 4 days of stratification, and 1d, 3d, and 7d means days in constant light condition after 4 days of stratification. Open circle represents transcript level in wildtype and closed square is that in *snl1234* mutant. *UBQ11* was used as an internal control. Experiments were repeated with three biological sets and the average value was represented with ±SE.

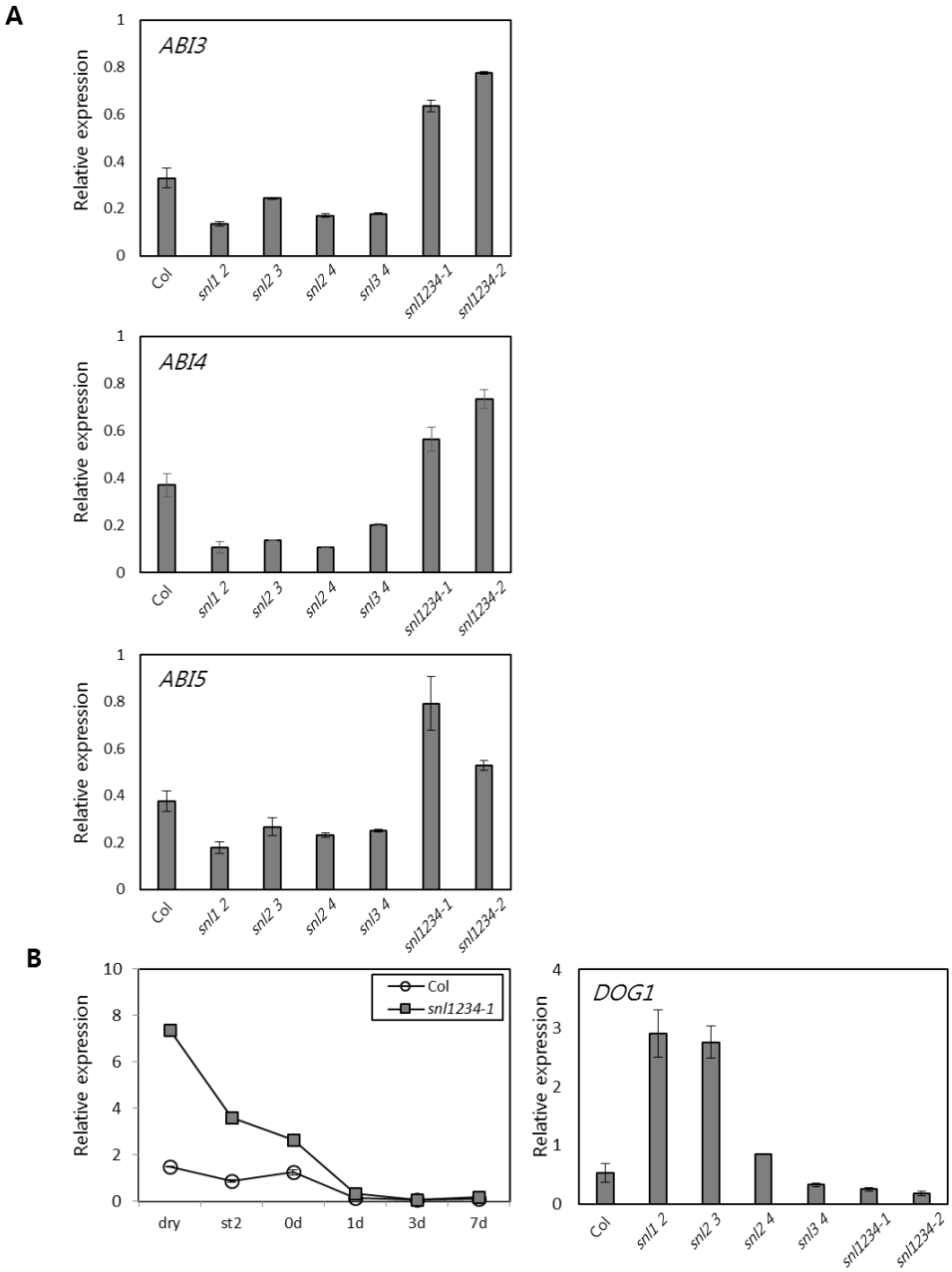

Figure 3.9 Expression of *ABI* genes in *snl* double mutants

(A) Relative expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in Col-0, different combinations of *snl* double and *snl* quadruple mutant seeds. Col, *snl double mutants* (*snl1snl2*, *snl2snl3*, *snl2snl4*, and *snl3snl4*) and *snl* quadruple mutants

(*snl1234-1* and *snl1234-2*) seeds were germinated and grown on half MS media without sucrose for 6 days (2 days under constant light condition after 4 days of stratification). Then germinated seeds were harvested for RNA extraction. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates. **(B)** Relative expression levels of DOG1 in wildtype and *snl1234* mutant seeds was measured during 11 days of germination (left). Relative mRNA of *DOG1* in Col and various *snl* double and *snl1234* quadruple mutant seeds at day 2 under constant light condition after stratification (right). All the conditions were the same as Fig 8A and 9A, respectively. The circle represents transcript levels in wildtype and the dark square does those in *snl1234* mutant. The transcript levels of each genes were normalized relative to *UBQ11*. Error bars represent ±SE of two independent biological replicates.

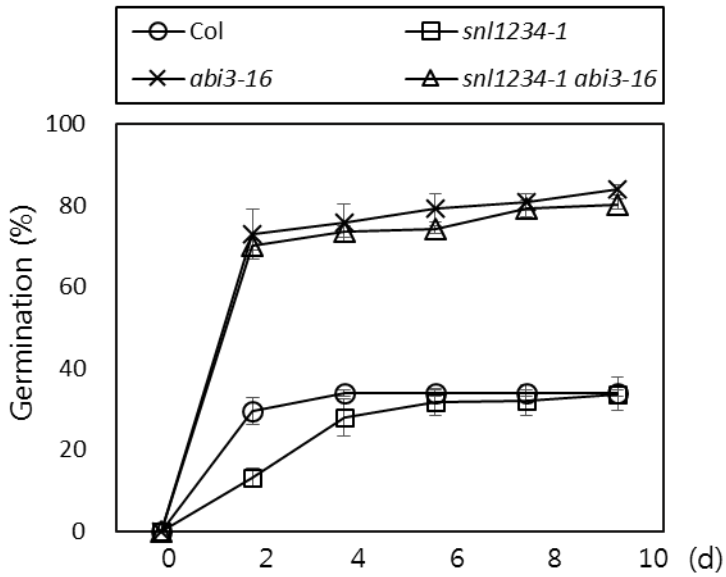

Figure 3.10 Suppression of the delayed germination phenotype of *snl1234* by the *abi3-16* mutation

Seed germination of Col, *abi3-16*, *snl1234-1*, and *snl1234-1 abi3-16* quintuple mutant. Seeds were freshly harvested and stratified for 7 days to break the seed dormancy. Dormancy-released seeds were used for germination assay on half MS medium without sucrose. Germination were scored every 2 days for 10 days. Opened circles represent wildtype seeds; square do *snl1234-1*; Xs do *abi3-16*; and triangles do represents *snl1234-2* mutant seeds. All germination assays were performed in triplicates using at least 100 seeds for each genotype and the average value is shown with ±SE.

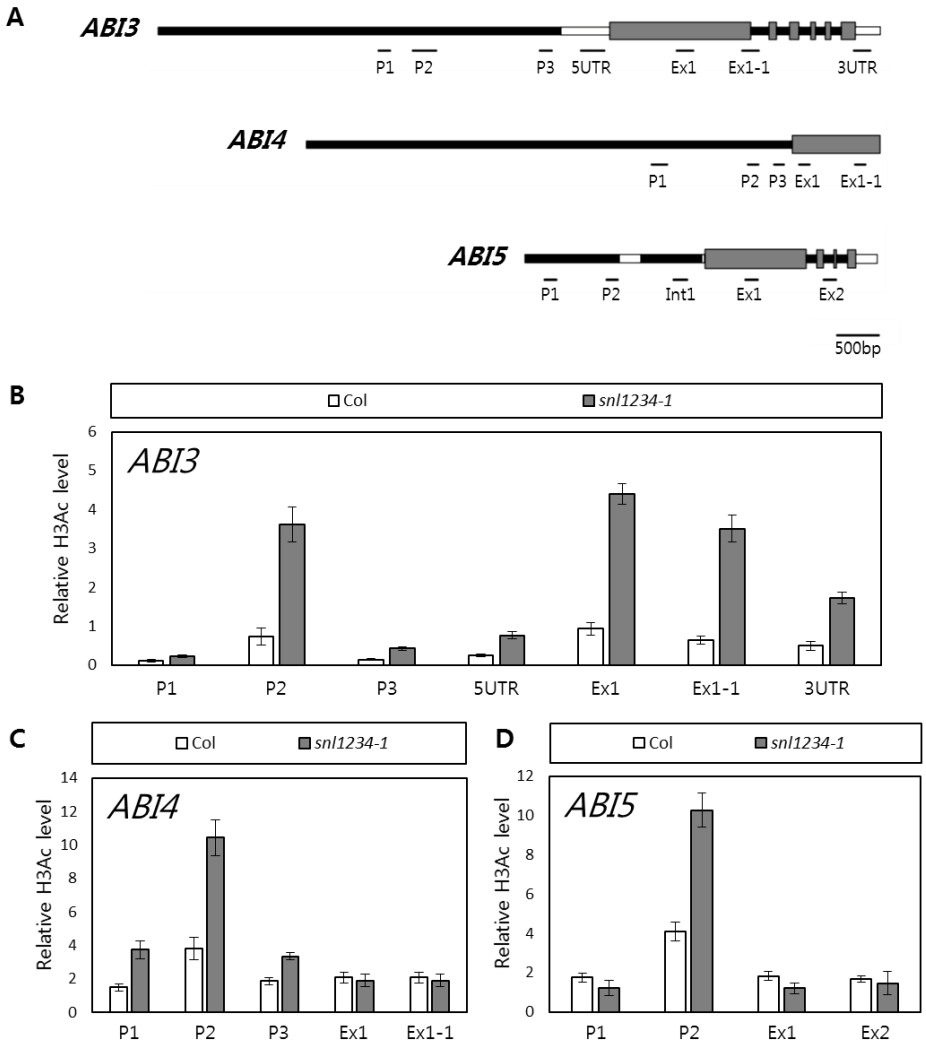

Figure 3.11 Increased Acetylation of histone 3 at *ABI3, 4,* and *5* locus by *snl1234* mutations

(A) Schematic representation of *ABI3*, *ABI4*, and *ABI5* locus showing regions amplified by the primers used for ChIP analysis. White boxes indicate 5' and 3' UTRs. Gray boxes indicate exons, black boxes indicate introns and intergenic regions. ChIP-qPCR anslyses of *ABI3* **(B)**, *ABI4* **(C)**, and *ABI5* **(D)** chromatin with

an antibody against H3Ac. Seeds of each genotype were germinated and grown under constant light for 3 days after stratification, and then harvested for ChIP assay. White bar represents H3Ac levels on each gene locus in wildtype and gray bar is that in *snl1234* mutant. The values of immunoprecipitated chromatins were normalized with that of inputs then by the internal control *UBQ11*. 'Input' indicates chromatin before immunoprecipitation. Shown are the means ±SE of three (B) and two (C and D) biological replicates.

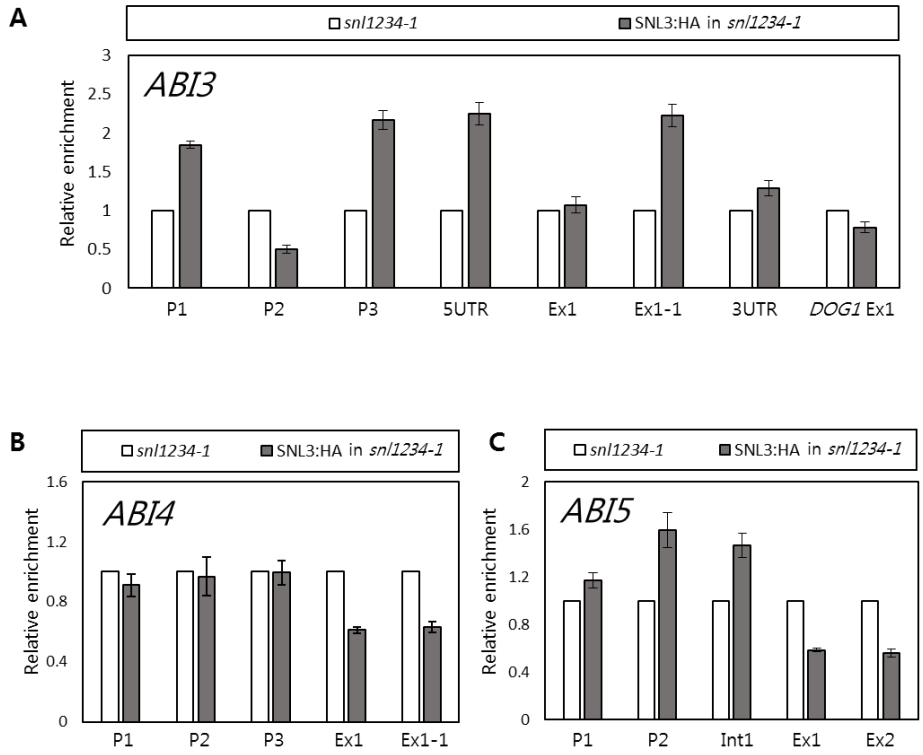

Figure 3.12 Direct association of SNL3 with *ABI3* chromatin

ChIP-qPCR analyses of relative SNL3:HA enrichment on the *ABI* genes using an anti-HA antibody. *snl1234-1* and SNL3:HA in *snl1234-1* seeds were germinated under constant light for 2 days after stratification and harvested for ChIP assay. Amplified regions of *ABI3* **(A)**, *ABI4* **(B)**, and *ABI5* **(C)** locus for the ChIP-qPCR analysis are shown in Figure 11A. Dark bar represents relative enrichment levels of SNL3 on each gene locus from SNL3:HA *snl1234* transgenic seeds. White bar stands for *snl1234-1*. The enrichment levels from Col were set to 1 after normalization by input and the internal control *UBQ11*. The ChIP-qPCR value of *DOG1* was demonstrated as a negative controls. Shown are the means ±SE of three (A) and two (B and C) biological replicates.

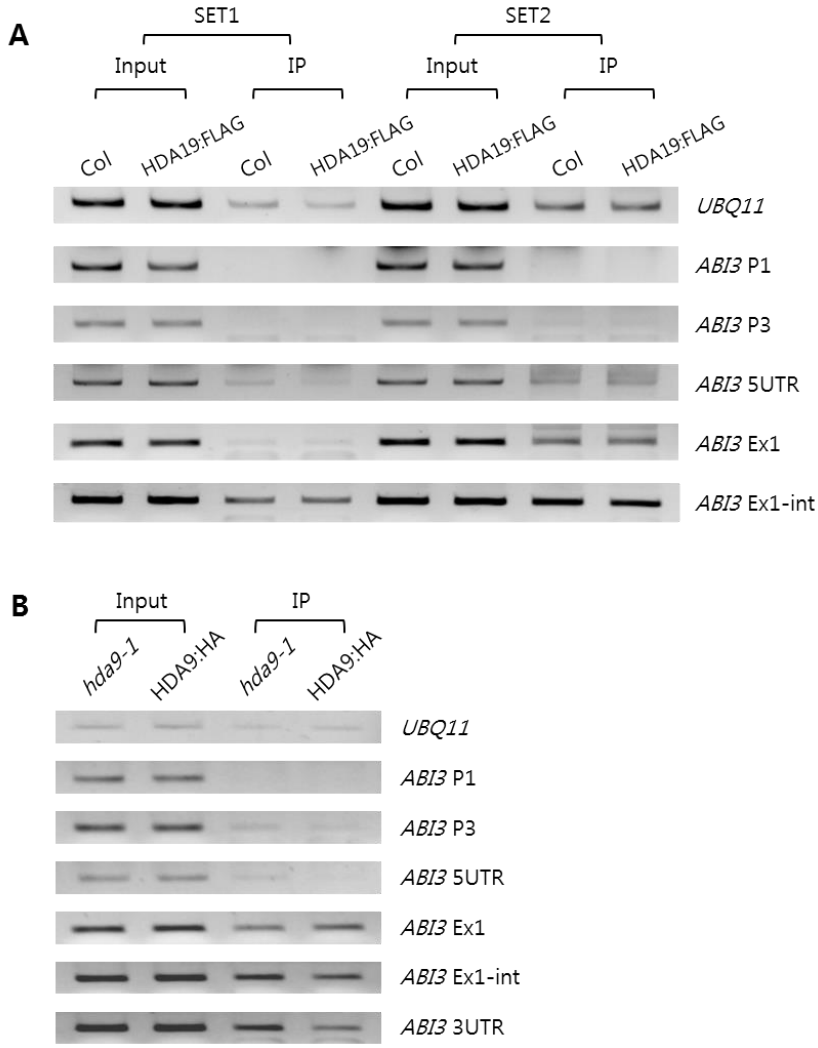

Figure 3.13 Enrichment of HDAC19 and HDA9 on *ABI3*

(A) ChIP-qPCR analysis of HDAC19 enrichment. Seeds of wildtype and *HDA19:FLAG* construct-harboring transgenic plants were germinated under constant light for 2 days after stratification and harvested. ChIP assay was performed with an antibody against FLAG. *UBQ11* was used as an internal control. Data represent two biological repeats. **(B)** ChIP-qPCR analysis of HDA9. Seeds of wildtype and *HDA9:HA* construct-harboring thransgenic plants were germinated

under constant light for 2 days after stratification and harvested. ChIP assay was performed with antibody against HA. ‘Input’ indicates chromatins before immunoprecipitation. *UBQ11* was used as an internal control. Data represent two biological repeats. Schematic view of amplified *ABI3* locus by the ChIP analysis is shown in Figure 11A.

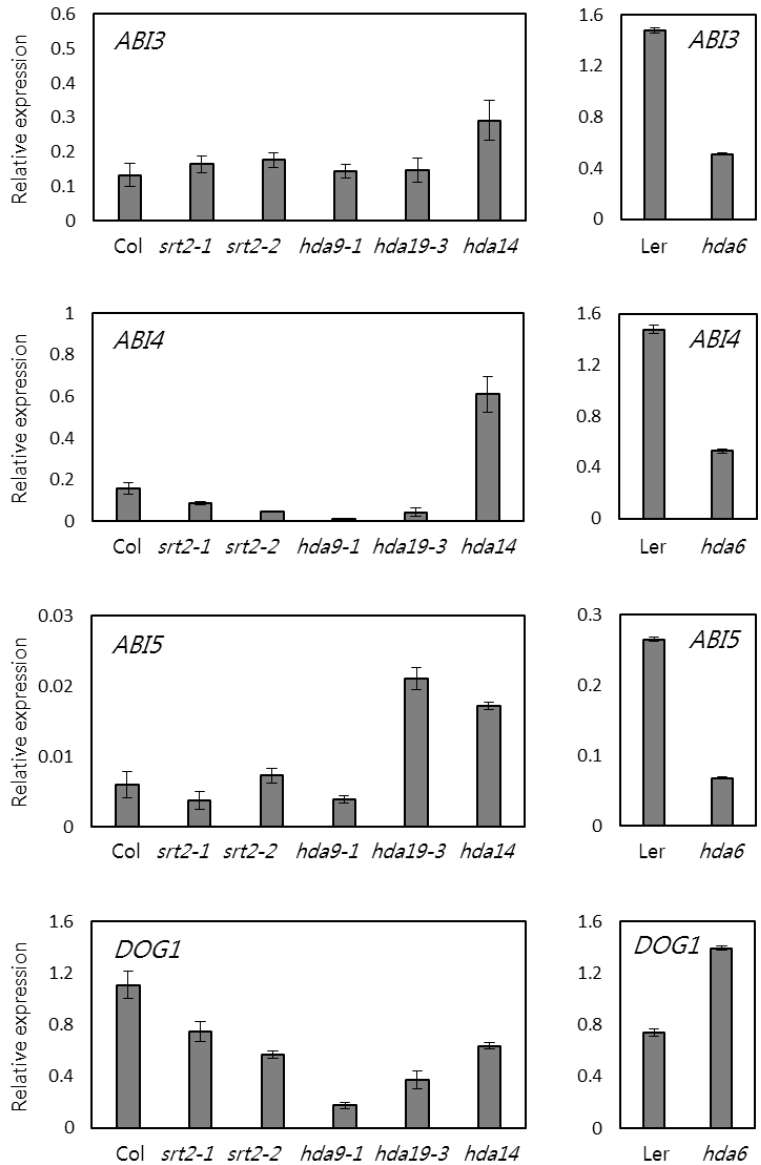

Figure 3.14 Expression of *ABI* genes in various *hdac* mutants

mRNA expression of *ABI* genes (*ABI3*, *ABI4*, and *ABI5*) in wildtype and various *hdac* mutant seeds grown for 3 days under constant light condition. The seeds were germinated and grown on half MS media without sucrose for 7 days (3 days under

constant light condition after 4 days of stratification), and then germinated seeds were harvested for RNA extraction. Transcription levels in Columbia ecotype background mutants, *srt2-1*, *srt2-1*, *hda9-1*, *hda19-3*, and *hda14* are shown on the left, and those in Ler-background are shown on the right. The transcript levels of each genes were represented relative to *UBQ11*. Error bars represent ±SE of three independent biological replicates.

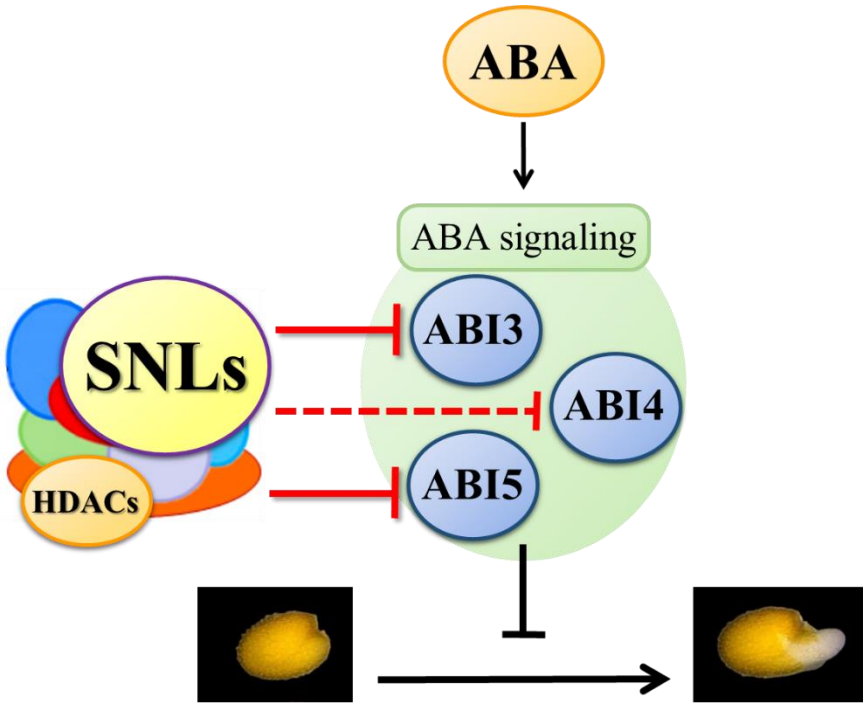

Figure 3.15 Proposed model for the role of SNLs in pathways regulating seed germination in Arabidopsis

Histone deacetylation mediated by SNLs negatively regulates the ABA signaling pathway, which inhibits seed germination.

Table 3.1. Oligonucleotides used for T-DNA flanking sequence analysis

Name	Sequence
SALKLB1	5’-GCAAACCAGCGTGGACCGCTTGCTGCAACT-3’
SNL1-1 F	5'-GAGAGCTTGTTCAGATGCGAAGA -3'
SNL1-1 R	5'-CAGGATAAAGGAACTACTTGAATA-3'
SNL2-1 F	5'-ACTCAGAGCAGCAATGAAGCGAATTAG-3'
SNL2-1 R	5'-CAAGATGAAGAATTGTTCCCAGAGGCT-3'
SNL3-1 F	5'-CTAGGTTTTGATTTGTAGATGAACATGAAG-3'
SNL3-1 R	5'-AACCTAAACATAACAAGGAGAAG-3'
SNL3-2 F	5'-GAAGATGACTGCCGAGCG-3'
SNL3-2 R	5'-TCTGCGATCCTCCCTTGC-3'
SNL4-1 F	5'-CCTTTCTTATCAACCTAATGC-3'
SNL4-1 R	5'-TCATGTGCGAGGGGTAAACCGC-3'
SNL4-2 F	5'-CTTAGAGACATCACTGGTTTGGATATTG-3'
SNL4-2 R	5'-CTGGTTGGCTGATTATCTCC-3'

Table 3.2. Oligonucleotides used for constructs

Name	Sequence
SNL1-promoter F	5'-ACTgtcgacGATGAGGATGAGGATTAAG-3'
SNL1-promoter R	5'-TTCGCTggatccCTGCTCTGAGTAAAACAAAC-3'
SNL2-promoter F	5'-ACGgtcgacTACAATCGTTGCCTTTC-3'
SNL2-promoter R	5'-CATCTCggatccGCTTCATCACAAG-3'
SNL3-fullength F	5'-CACCTATGTTCCGTTTTTATGGTT-3'
SNL3-fullength R	5'-CAAGAAGTTATTTTTGTAATATTGTAACCTC-3'
SNL3-promoter F	5'-TCGgtcgacCGGAAAGATAGTAATG-3'
SNL3-promoter R	5'-CTATTCAggatccCATAAGACCAAAGAACG-3'
SNL4-promoter F	5'-TTTgtcgacCATCCAAACTGCATCAATG-3'
SNL4-promoter R	5'-TCCAAggatccTTTATATTTTGCCAGAGCTTC-3'

Restriction sites used for cloning are in small letters and underlined**.**

Table 3.3. Oligonucleotides used for RT-PCR analysis

Gene	Name	Sequence
Ubiquitin11	UBQ11 F	5’-GATCTTCGCCGGAAAGCAACTT-3’
	UBQ11 R	5’-CCACGGAGACGGAGGACC-3’
LEC1	LEC1 F	5'-GAGACAAACCTATGGAGGAAATGG-3'
	LEC1 R	5'-CCAACACTGGATTCATCTTGACC-3'
LEC2	LEC2 F	5'-TCCTAACAACAATCGCTCGC-3'
	LEC2 R	5'-TGAGGATAACACTCCGATAAGTAAACC-3'
FUS3	FUS3 F	5'-CTCCGACGTATGATACTCCCGAAG-3'
	FUS3 R	5'-CGCCTGTGTTTTCTAGCACGTACATT-3'
CRC	CRC F	5'-ACAACCTAGATGTTCTCCAAGCCACC-3'
	CRC R	5'-ACTCTTCCGCTGATACCCGTTC-3'
RAB18	RAB18 F	5'-CGTCTTACCAGAACCGTCCAGG-3'
	RAB18 R	5'-TCCGTATCCTTGGCCACCTG-3'
GA3ox1	GA3ox1 F	5'-CCGAAGGTTTCACCATCACT-3'
	GA3ox1 R	5'-CCCCAAAGGAATGCTACAGA-3'
GA3ox2	GA3ox2 F	5'-TAGATCGCATCCCATTCACA-3'
	GA3ox2 R	5'-TGGATAACTGCTTGGGTTCC-3'
GA20ox2	GA20ox2 F	5'-TCCCGTTCATCGATCTCTCAAGC-3'
	GA20ox2 R	5'-CTTTCCATCAAACGGTGAGCATCC-3'
RGA1	RGA1 F	5'-TACATCGACTTCGACGGGTA-3'
	RGA1 R	5'-GTTGTCGTCACCGTCGTTC-3'
RGL1	RGL1 F	5'-CAAGCATGTTGTTGGCACTT-3'
	RGL1 R	5'-GCAACAAACAACCTTCATTCTCT-3'
RGL2	RGL2 F	5'-TCAGAACATGGGCGTTGAAT-3'
	RGL2 R	5'-AAGGTTTCAGATTCGGGTCG-3'
NCED6	NCED6 F	5'-ACCGGGTCGGATATAAATTGGGTTG-3'
	NCED6 R	5'-CCCGGGTTGGTTCTCCTGATTC-3'
NCED9	NCED9 F	5'-CTGTCCCAAGATGCTCATCACTC-3'
	NCED9 R	5'-TGAAGTTGAGAAAGTTCGGTCGAGG-3'
CYP707A2	CYP707A2 F	5'-TGGTGGTTGCACTGGAAAGAGC-3'

	CYP707A2 R	5'-TTGGCGAGTGGCGAAGAAGG-3'
ABI3	ABI3 F	5'-AAGCTGAGACACACTTGCCG-3'
	ABI3 R	5'-CCAAAACCTGTAGCGCATGT-3'
ABI4	ABI4 F	5'-ATCCTCAATCCGATTCCACC-3'
	ABI4 R	5'-ATTTGCCCCAGCTTCTTTGT-3'
ABI5	ABI5 F	5'-GGTGAGACTGCGGCTAGACA-3'
	ABI5 R	5'-GTTTTGGTTCGGGTTTGGAT-3'
RD29B	RD29B F	5'-AGCAAGCAGAAGAACCAATCAG-3'
	RD29B R	5'-TGCTCGTCATACTCATCATCATC-3'
SOM	SOM F	5'-ATGGATGTCGTTTGTACGGAACATCAA-3'
	SOM R	5'-TCAAGTCAAGAGATCATTGACCCATCC-3'
PIL5	PIL5 F	5’-ATGATTTCTGCTCAGATCTTCTCTTCT-3’
	PIL5 R	5’-AGATTCACCACCTCTACCGTTATTAAA-3’
DOG1	DOG1 F	5'-AAGAAAGTCTCAAGCCTAC-3'
	DOG1 R	5'-CGAGGATCTTCGCTAAAG-3'

Table 3.4. Oligonucleotides used for ChIP assay

Gene	Region	Name	Sequence
ABI3	P1	ABI3 P1 F	5'-ACGCATAGTAAAACAAAGTTCACATG-3'
		ABI3 P1 R	5'-GCATTGATGTATATATCAGTACTAGTCG-3'
	P2	ABI3 P2 F	5'-TCACCATCGTATCCACAAACATTATCG-3'
		ABI3 P2 R	5'-CTTGTACGTCGAGATGGCATGT-3'
	P3	ABI3 P3 F	5'-GCTGCAAAGAGAAAGAGAATAACTTAAACCC-3'
		ABI3 P3 R	5'-GAGCCCATGTGTTCCAGTTTGTTCCAT-3'
	5UTR	ABI3 5UTR F	5'-ATTGGTCTTTGTTCATCTGAAGTTGGAG-3'
		ABI3 5UTR R	5'-CTAGATTGGTGGAGAGAGAAAAGTTAGGG-3'
	Ex1	ABI3 EX1 F	5'-CTAATCCCACCGTCCGAC-3'
		ABI3 EX1 R	5'-TCTGGCTGTGGCGGATAG-3'
	Ex1-1	ABI3 Ex1-1 F	5'-AGTGATGGAGACTCAGTTACCTACC-3'
		ABI3 Ex1-1 R	5'-GCTTCTTCATCAAACCAAACGAGTG-3'
	3UTR	ABI3 3UTR F	5'-TCGCTTCACCAACTTCTCAAACTG-3'
		ABI3 3UTR R	5'-GACCAAACAGCTTTAATCATGACCCTCC-3'
ABI4	P1	ABI4 P1 F	5'-ACGTGTTGTACCAGATGTTTTTCCTCC-3'
		ABI4 P1 R	5'-GAGAAAAATTTAAGCTGTTGGGAAATCACC-3'
	P2	ABI4 P2 F	5'- GAATCCTCTGAAATCTGAATGCCTTGG-3'
		ABI4 P2 R	5'-GGGTAACTATAGCAAATCATGAGCGA-3'
	P3	ABI4 P3 F	5'-AAGAAGTGAGTGAGAAGAGAGTGTAAG-3'
		ABI4 P3 R	5'-GGAGAGGACGAATCAAGAAGGAAGG-3'
	Ex1	ABI4 Ex1 F	5'-ATCCTCAATCCGATTCCACC-3'
		ABI4 Ex1 R	5'-ATTTGCCCCAGCTTCTTTGT-3'

	Ex1-1	ABI4 Ex1-1 F	5'-GCTCACTGATGTTCCGGTAACTAATTCG-3'
		ABI4 Ex1-1 R	5'-TGATAGACTCGAACCCACCGAACC-3'
ABI5	P1	ABI5 P1 F	5'-AGTTGCTGTAATCTTTAGGTCGCTGG-3'
		ABI5 P1 R	5'-CACGTGGACTATTCACTGCATAAGG-3'
	P2	ABI5 P2 F	5'-TGTCTCTGATCATGGGCCTGG-3'
		ABI5 P2 R	5'-GCGCGTGGGGTCTAAGAAG-3'
	Int1	ABI5 Int1 F	5'-TTTGTCGCTGTCACGATGTGGACC-3'
		ABI5 Int1 R	5'-ACTTGTCCCTGTTCAGCTATTCACG-3'
	Ex1	ABI5 Ex1 F	5'-GGTGAGACTGCGGCTAGACA-3'
		ABI5 Ex1R	5'-GTTTTGGTTCGGGTTTGGAT-3'
	Ex2	ABI5 Ex2 F	5'-AGTTGAAAGAAGAGAATGCGCAGC-3'
		ABI5 Ex2 R	5'-TGCTTCCTCTTCCTCTCCAACTCC-3'
DOG1		DOG1 F	5'-AAGAAAGTCTCAAGCCTAC-3'
		DOG1 R	5'-CGAGGATCTTCGCTAAAG-3'
UBQ11		UBQ11 F	5’-TCAGTATATGTCTCGCAGCAAACTATC-3’
		UBQ11 R	5’-GACGACTCGGTCGGTCACG-3’

3.6 Discussion

Breaking seed dormancy and seed germination are often viewed as a simultaneous phenomenon that initiates plant life. However, these two processes actually occur sequentially and somewhat distinctively. Generally, seed dormancy is acquired during embryo developmental stages, built up until the completion of embryo maturation, and then released as seeds undergo desiccation-accompanied ripening. After ripening, when seeds are in non-dormant state, germination is then finally in action depending on environmental cues. Radicle expansion and hypocotyl elongation required for early seedling establishment are triggered when seeds have gained the ability to readily germinate. Therefore, seed germination and early seedling establishment process, as one of the critical transition phases in plant's life cycle, has been closely examined and pinpointed for the underlying mechanisms.

Since the early 90's, the role of ABA has been described in association with seed development and germination (Meurs et al., 1992). It has been established that ABA deficiency or insensitivity results in aberrant seed development and precocious germination. More recently, defects in late embryo development have been explained by ABI-mediated pathways via genetic analyses (Nambara et al., 2000; Nakashima et al., 2006; To et al., 2006). Together with *FUS3*, *LEC1*, and *LEC2*, *ABI3* has been known to control a particular set of genes that are involved in most aspects of seed maturation. Thus, ABI3 has been considered as a paradigm for late embryogenesis and seed-dormancy establishment. However, regulation mechanisms for the master controller, ABI3, have remained abstruse.

About a decade ago, it was reported that PKL is involved in the repression of *ABI3* and *ABI5* expression (Perruc et al., 2007). Perruc et al. concluded that PKL-influence was to limit the transcriptional potential of the embryogenic genes. Later on, PKL was found to repress the expression of the seed-associated genes, such as *FUS3*, *LEC1*, and *LEC2*, through H3K27me3 enrichment during germination (Zhang et al., 2008). Despite the efforts, contradictory influences of PKL on *ABI* genes with low H3K27me3 levels compared to those on other embryonic genes with high H3K27me3 levels and low H3Ac levels could not be explained. In our results, expression patterns of embryonic genes, such as *LEC1*, *LEC2, FUS3*, and others, could not be explained in the aspect of the delayed germination phenotype of *snl1234* and the predicted function of SNLs (**Figure 3.4**). Thus, we believe that the alteration of seed-associated gene expressions, except for *ABI3*, is controlled in rather collaborative but distinct manner from *ABI*-specific regulations.

Lately, it was reported that SNL1 and SLN2 have a redundant role in mediating the antagonism between ABA and ethylene during the establishment of seed dormancy (Wang et al., 2013). To confirm whether the altered dormancy by the mutations of *SNL1* and *SNL2* may also disrupt seed germination, we carefully scrutinized the germination phenotypes of the *snl12* double mutant and other combinations of *snl* mutant seeds. However, such mutant seeds at dormancy released stage did not show altered germination phenotypes (**Figure 3.1**). On the other hand, expression levels of a dormancy marker gene, *DOG1*, were remained elevated in *snl12* double and *snl23* triple mutants compared to wt or *snl1234* quadruple mutants (**Figure 3.9B**). The inconsistency between the germination

phenotype and the *DOG1* expression level could be explained by following suppositions. First, the higher level of *DOG1* expression in the double and triple mutants was not sufficient for conferring dormancy in dormancy released AR seeds. As shown in **Figure 3.9B**, *DOG1* expression continuously decreases from FH to AR stages and further during stratification and germination processes both in wt and *snl1234* seeds. Considering that the seed samples were harvested at two days after stratification, it is possible that the residual *DOG1* expression might be insufficient to induce delayed germination. Second, although *DOG1* might be an excellent dormancy marker in FH seeds, its expression level in AR seeds might be less meaningful in representing dormancy level. Lastly but more likely, results in **Figure 3.9B** might suggest that there might be a more powerful factor that can overcome the effect of DOG1 in conferring germination. In sum, our results indicate that only the *snl1234* quadruple but none of the double and triple *snl* mutants is defective in germination, and this germination phenotype cannot be explained by *DOG1* expression level. Thus, it is strongly endorsed that seed dormancy is not exactly coincided with seed germination efficiency and that all the four SNL proteins (SNL1, SNL2, SNL3, and SNL4) share a redundant role in seed germination that is not observed in seed dormancy.

In our study, *snl1234* seeds showed delayed germination phenotype and ABA hypersensitivity. And this was correlated with the altered expression patterns of ABA signaling genes during seed germination and early seedling establishment. During the transition process, transcript levels of the key components, *ABI3, ABI4*, and *ABI5,* in the ABA-signaling pathway were more slowly decreased in *snl1234* mutant compared to wt. This indicates that the mutations of those four *SNL* genes

result in the failure of precise transcriptional repression of the *ABI* genes. To prove the involvement of the SNLs in the ABA-signaling mechanism in depth, we provided genetic evidence on the relationship between *SNLs* and *ABI* genes (**Figure 3.10**). Germination test using the *snl1234 abi3* quintuple mutant seeds demonstrated wt level germination unlike the *snl1234* mutant seeds. It indicates that *abi3* mutation can completely block the effect of *snl1234* in seed germination. In other words, *ABI3* is epistatic to *SNLs* and, therefore, it opened a possibility that *ABI3* might be the factor that powerfully controls seed germination as a target of the SNL complex.

Dormancy, which is achieved by ABA metabolism and signaling, can be expanded to primary and secondary dormancy. The primary dormancy is established during embryo maturation whereas the secondary dormancy is programmed, when unfavorable condition for seed germination is unexpectedly given, to overcome the already-released primary dormancy and to inhibit seed germination (Hilhorst et al., 1998). However, the regulation of secondary dormancy is largely unknown and only established upon environmental cues. This very year, it was reported that, for the secondary dormancy, seeds require changes in GA contents and sensitivity (Ibarra et al., 2015). Although the secondary dormancy also requires ABA, Ibarra et al. (2015) could not observe significant alterations in either endogenous ABA level or sensitivity. Although altered *DELLA* expression was suggested to promote *ABI5* expression, the relationship between *DELLA* and *ABI5* was not clearly addressed. Furthermore, a genome-wide study demonstrated that global HDAC enrichment is strongly observed where enhanced HAT enrichment is observed (Wang et al., 2009). According to this report, HDAC

enrichment overlaps with genomic loci where histone acetylation can be readily induced. Thus, repressive role of histone deacetylation is more important in curbing active genes in need of repression than in maintaining constitutive heterochromatin. Applying this idea to our results, repression of *ABI* chromatin by SNLs might be important and prominent for the suppression of *ABI* expression during seed germination. Given that ABA, especially through the ABI-mediated signaling mechanism, is prerequisite for primary and secondary dormancy acquisition, ABI should be readily activated and tightly regulated during the first critical transition period in plant development. Therefore, we suggest that SNLs might provide a master scaffold on which HDACs can directly regulate all of the three main *ABI* genes during seed germination and early seedling establishment.

Identification of an HDAC(s) that partners with SNLs for *ABI* regulation is yet to be achieved. Although, it has been reported that HDA19, HDA9, and HDA6 are involved in seed germination or dormancy via *ABI3*-mediated pathway (Chen and Wu, 2010; Song et al., 2005; Kazan, 2006), our ChIP assays indicated that there might be no direct interaction between HDA19 or HDA9 with *ABI3* chromatin (**Figure 3.13**). Moreover, under our experimental conditions, there was no significant changes of *ABI3, ABI4,* and *ABI5* transcripts were induced, with the exception of *ABI5* transcript in *hda19,* by both *hda19* and *hda9* mutations (**Figure 3.14**). In fact, role of HDA19 on *ABI* genes was described in association with brassinolide (BR) signaling (Ryu et al., 2014). Upon BR signaling, BES1-TPL-HDA19 complex is assembled and regulates *ABI3-ABI5* module (Wang et al., 2013). Moreover, it was reported that HDA19 and SNL1 have a direct interaction *in vivo* and *in planta*. Considering our results indicating that HDA19 might not

directly target *ABI3* locus, we suspect that the SNL1-HDA19 complex might primarily act on *ABI5* and then, altered *ABI5* expression might subsequently affect *ABI3* expression.

Lastly but not less importantly, our results demonstrated that SNL3 is not only enriched at *ABI5* but also at *ABI3* loci, whereas no direct targeting of HDA19 to *ABI3* chromatin was detected. Taken together, we concluded that role of HDA19 is insufficient to explain the delayed germination of *snl1234*. As our results in **Figure 3.14** showed relatively high level of all the three *ABI* genes in *hda14* mutants, HDA14 might be one of the strongest candidates to act in concert with SNLs. Until this moment, HDA14 is left as an unclassified subfamily member of the *Arabidopsis* RPD3-type HDACs. Therefore, further study is required to see if HDA14 might form a complex with SNLs to directly modulate all *ABI* genes during seed germination.

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국문초록 (Abstract in Korean)

식물의 생활사는 발달에 중요한 여러 번의 전이과정을 포함한다. 전이과정은 내부적 신호(호르몬, 생체 나이 등)와 외부의 환경적 신호(광주기, 온도, 영양 등)에 의하여 조절되며, 수많은 유전자들의 발현 변화를 동반한다. 최근 연구들을 통하여 특정 유전자들의 에피유전학적 조절이 전이과정에 관여하는 하나의 중요한 메커니즘으로 대두되고 있다. 에피유전학적 조절은 DNA 메틸화, 히스톤 변형, RNA 간섭 등에 의하여 염색질 구조변형을 유발하므로 유전자의 전사활성에 영향을 주는 것을 의미한다. 개화와 종자발아는 에피유전학적 조절을 받는 가장 잘 알려진 전이과정이다. 그러므로, 본 연구자는 개화와 종자발아 과정에서 에피유전학적 조절에 대한 보다 깊은 이해를 얻고자 하였다.

개화는 영양생장에서 생식생장으로 전환되는 중요한 전이과정이다. 특정 유전자들의 발현이 균형을 이루어 식물의 개화를 조절한다. 그 중 FT는 개화를 유도하는 신호 단백질로서 개화과정 중 그 역할이 매우 중요하다. *FT*에서 유전자 발현 억제를 유발하는 히스톤 변형 마커인 히스톤 H3 리신 27 메틸화 축적이 이미 보고된 바 있다. 그러나, 유전자 발현 활성을 유발하는 히스톤 변형 마커인 히스톤 H3 리신 4 메틸화 혹은 이에 관여된 조절요소들은 명확하게 연구되지 않은 상태이다. *Arabidopsis thaliana Jumonji 4* (*AtJmj4*)와 *EARLY FLOWERING 6* (*ELF6*) 유전자의 기능이 상실된 애기장대 돌연변이체에서 조기 개화

표현형을 보았으며, 이는 이들의 2중 돌연변이체에서 더욱 극대화되었다. 또한 *atjmj4*와 *elf6* 돌연변이체에서 *FT* 전사체 발현의 증가와 더불어 히스톤 H3 리신 4 메틸화 레벨이 높아져 있었다. Atjmj4는 히스톤 H3 리신 4를 특이적으로 탈메틸화 시킬 수 있는 능력을 지니고 있는 효소로써 *FT* 유전자의 전사 시작부위에 직접적으로 결합함을 확인하였다. ELF6도 *FT*로의 결합이 유사하게 관찰되었으며, 그 결합부위는 이들 유전자들의 돌연변이체에서 메틸화된 히스톤 H3 리신 4의 변화를 보였던 부분과 일치 하였다. 그러므로 본 학위논문의 제 2장에서는 식물의 개화과정에서 탈메틸화 효소로서 *FT* 발현 억제에 직접적으로 관여하는 AtJmj4와 ELF6의 기능을 밝혔다.

종자발아는 배아에서 후배발달과정으로 전환되는 식물의 또 다른 중요한 전이과정이다. 종자발아는 식물호르몬인 gibberellic acid (GA)와 abscisic acid (ABA)의 상호작용에 의하여 조절된다. ABA는 ABI 단백질을 통한 신호전달에 의해 종자의 발아를 억제하는 기능을 하지만, 이에 관한 분자생물학적 이해는 아직 부족하다. 애기장대 SIN3-LIKE (SNL) 단백질들은 탈아세틸화효소와 함께 유전자들의 발현 억제 기작에 관여한다. 이에 본 학위논문의 제 3장에서는 종자발아 과정 동안의 SNL단백질들의 역할을 규명하고자 하였다. SNL1, SNL2, SNL3, SNL4 유전자 모두에 돌연변이를 일으킨 4중 돌연변이체인 *snl1234* 종자에서 발아가 지연되는 표현형을 보았다. 이 표현형은 ABA 처리 시 야생종과 돌연변이체 사이에서 더욱 큰 차이로 관찰되었다. 종자발아 과

정 중에 야생종에 비하여 *snl1234*에서 *ABI3*, *ABI4*, *ABI5* 유전자들의 아세틸화 증가와 함께 발현 증가가 관찰되었다. 더불어 SNL3 단백질이 *ABI3*와 *ABI5*에 직접적으로 결합하였다. 이를 통하여, 본 연구자는 식물의 종자발아 과정에서 SNL 단백질이 *ABI* 유전자들의 억제자인 것을 밝혔다.

주요어: 에피유전학, 히스톤 변형, 개화, 종자발아

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