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Genetic and Epigenetic Mechanisms Underlying Vernalization

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Plants have evolved a number of monitoring systems to sense their surroundings and to coordinate their growth and development accordingly. Vernalization is one example, in which flowering is promoted after plants have been exposed to a long-term cold temperature (i.e. winter). Vernalization results in the repression of floral repressor genes that inhibit the floral transition in many plant species. Here, we describe recent advances in our understanding of the vernalization-mediated promotion of flowering in Arabidopsis and other flowering plants. In Arabidopsis, the vernalization response includes the recruitment of chromatin-modifying complexes to floral repressors and thus results in the enrichment of repressive histone marks that ensure the stable repression of floral repressor genes. Changes in histone modifications at floral repressor loci are stably maintained after cold exposure, establishing the competence to flower the following spring. We also discuss similarities and differences in regulatory circuits in vernalization responses among Arabidopsis and other plants.

INTRODUCTION

The floral transition is a critical developmental change in plant life cycle. Plants ensure their reproductive success, in part, by flowering under favorable conditions. Plants undergo the floral transition only during certain seasons of the year through multiple regulatory networks that interpret environmental signals, such as day lengths and temperature fluctuations (Figure 1). In the model plant *Arabidopsis thaliana*, four major flowering pathways have been defined by classical genetic analyses: the photoperiod pathway, the autonomous pathway, the gibberellin (GA) pathway, and the vernalization pathway (Figure 1). In particular, many plant species in temperate climates need to be exposed to a certain period of winter cold to initiate the floral transition in following spring (Chouard, 1960; Lang, 1965; Bernier et al., 1981). This requirement for exposure to long-term cold for spring flowering is known as vernalization (Lang, 1965; Henderson and Dean, 2004; Sung and Amasino, 2006; Kim et al., 2009). The requirement for vernalization serves in part to prevent flowering in the fall season prior to winter, but permits flowering the following spring.

Plants integrate multiple internal and external flowering cues through gene expression changes. In Arabidopsis, a series of changes in the levels of floral gene expression in environmental and internal flowering pathways converge to regulate floral integrator genes, including *FLOWERING LOCUS T* (*FT*:At1g65480), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1:*At2g45660), and *AGAMOUS-LIKE 24* (*AGL24*:At4g24540) (Kim et al., 2009). To ensure optimal flowering time, plants use regulatory circuitries to control expression of floral integrator genes. Major components of these regulatory circuits are two major flowering time genes, *CONSTANS* (*CO*: At5g15840) and *FLOWERING LOCUS C* (*FLC*: At5g10140) (Figure 1). CO acts as a floral activator, whereas FLC acts as a floral repressor. CO is induced by inductive photoperiod (i.e. long days) and activates expressions of downstream floral integrators, thus promoting the floral transition. On the other hand, FLC acts to inhibit the floral transition through suppression of downstream floral integrators (Searle et al., 2006; Kim et al., 2009). Both the autonomous pathway and the vernalization pathway repress the floral repressor FLC. When *FLC* expression is high, flowering is inhibited even under inductive long days. Therefore, high levels of FLC are responsible for the vernalization requirement in Arabidopsis (Sheldon et al., 1999; Michaels and Amasino, 1999; Johanson et al., 2000). Over the past few decades, extensive molecular genetic studies have elucidated the molecular mechanisms governing the floral transition by both endogenous and environmental cues in Arabidopsis. Here, we describe our current understanding of vernalization-mediated flowering- time regulation.

VERNALIZATION AS A RESPONSE TO COLD TEMPERATURES

Cold temperature initiates a series of physiological and molecular responses in plants. Notably, a number of genes are rapidly induced by above-freezing cold temperatures to trigger cold acclimation in plants (Thomashow, 2001; Chinnusamy et al., 2007). Vernalization is similar to cold acclimation in that both responses are triggered by similar above-freezing temperatures (Lang, 1965; Bond et al., 2011; Zografos and Sung, 2012). However,

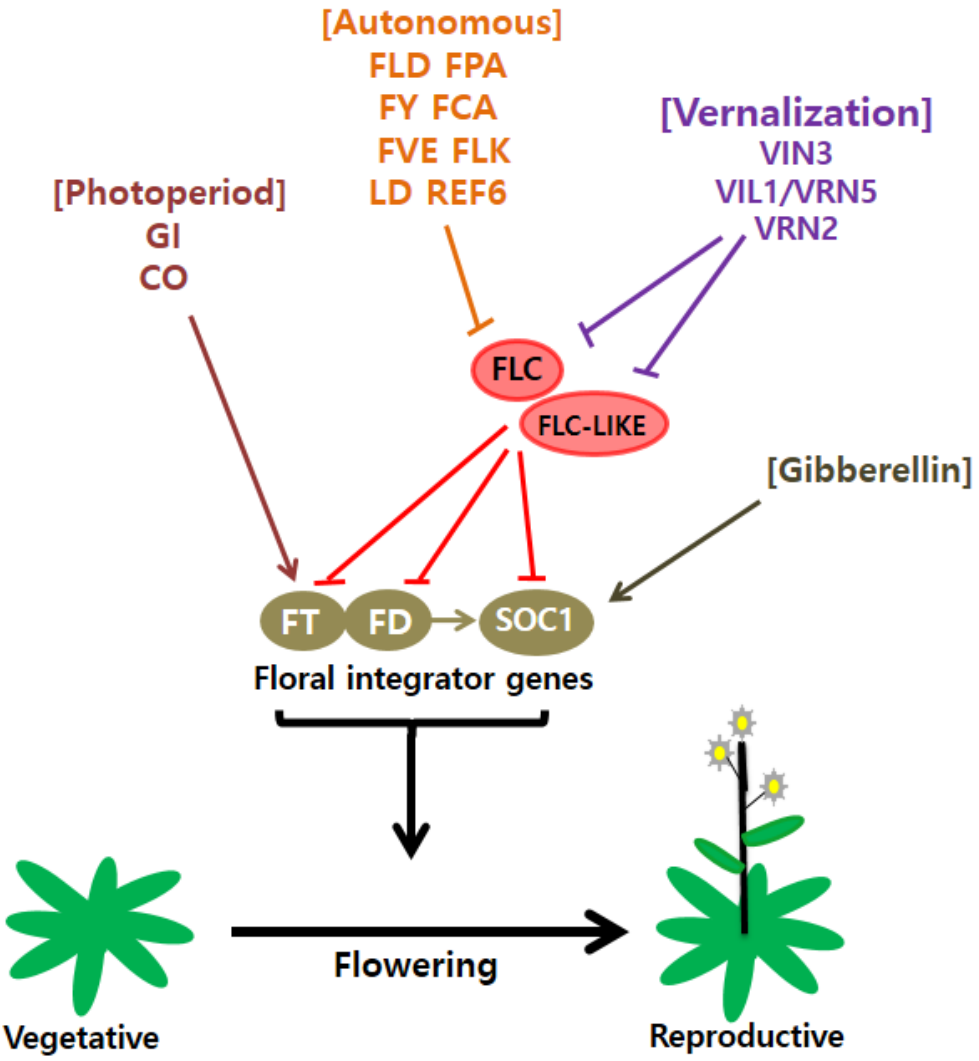

Figure 1. Overview on flowering pathways in Arabidopsis.

FLC and other FLC-related proteins repress floral integrator genes, including *FT*, *FD* and *SOC1*, in Arabidopsis. Upon the activation of floral integrators, the floral transition ensues. *FT* is induced by the photoperiod pathway through the activation of CO. FT protein is a mobile flowering signal that physically interacts with FD protein at meristem to activate *SOC1* and other floral activators. Therefore, FLC and CO antagonistically determine proper timing of flowering in Arabidopsis. Two genetically independent pathways, vernalization and autonomous pathways, repress the transcription of *FLC*. The autonomous pathway is required for repression of *FLC* regardless of environment stimuli. The vernalization pathway triggers stable repression of *FLC*. Gibberellin, a phytohormone, independently promotes flowering through the activation of *SOC1* and other floral activator genes.

there are two major differences. Cold acclimation occurs in a wide variety of plant tissues, including mature leaves. On the other hand, vernalization is effective either at the shoot apical meristem (SAM) or young leaves, indicating that rapidly dividing cells are responsive to vernalizing cold (Wellensiek, 1964; Lang, 1965; Zeevaart, 1976). In addition, vernalization requires longer period of cold exposure than cold acclimation. For example, cold acclimation can be achieved within days of cold exposure whereas vernalization needs 4~6 weeks of cold exposure in Arabidopsis (Lang, 1965; Bond et al., 2011; Zografos and Sung, 2012). This is an adaptive feature of the vernalization response to ensure that plants respond to winter cold, but not to fluctuating temperatures.

THE VERNALIZATION REQUIREMENT

Variation in flowering time is commonly observed in many flowering plant species. One prominent variation in Arabidopsis accessions is the requirement of vernalization for accelerated flowering (Koornneef et al., 1991). Genetic studies on variations in the vernalization

requirement using natural accessions of Arabidopsis demonstrate that this requirement is mainly due to two dominant genes, *FRIGIDA* (*FRI*: At4g00650) and *FLC* (Lee et al., 1993; Clarke and Dean, 1994; Lee et al., 1994; Michaels and Amasino, 1999; Johanson et al., 2000; Le Corre, 2005; Strange et al., 2011). Naturally occurring mutations in *FRI* are responsible for early flowering without vernalization in many accessions of Arabidopsis (Johanson et al., 2000; Strange et al., 2011). In the absence of active *FRI* allele, the level of *FLC* expression is reduced and plants do not require vernalization for accelerated flowering. In addition, there is natural variation in *FLC* alleles, which can result in low expression of *FLC* (Michaels et al., 2003; Strange et al., 2011). Natural accessions that contain both active *FRI* and *FLC* alleles require vernalization treatment for them to flower early. Therefore, the vernalization requirement in Arabidopsis is largely due to the level of *FLC* expression. There are also induced mutants in which *FLC* expression is elevated even in the absence of an active *FRI* allele. These mutants are collectively known as autonomous pathway mutants (Koornneef et al., 1991; Michaels and Amasino, 2001). Mutations in autonomous pathway genes result in high level of *FLC* expression and thus confer the vernalization requirement for early flowering in Arabidopsis. *FLC* encodes a MADS-box DNA binding protein that acts as a transcriptional repressor. FLC directly binds to downstream floral integrators, including *FT*, *FD* (At4g35900), and *SOC1*, to inhibit their transcription (Helliwell et al., 2006; Searle et al., 2006). Vernalization triggers mitotically-stable repression of *FLC* and thus allows plants to flower under the inductive photoperiod. Next, we describe molecular bases of these two genetic determinants that require plants to be vernalized to flower early in Arabidopsis.

FLC activation by a FRIGIDA (FRI) complex

FRI mainly acts to up-regulate *FLC* transcription and thus contributes to the vernalization requirement in winter-annual Arabidopsis accessions (Michaels and Amasino, 2001). Forward genetic approaches have been used to characterize the molecular mechanisms of FRI in the activation of *FLC*. Suppressor screenings identified a series of components that are required for the FRI-dependent activation of *FLC*. These include *FRI-LIKE 1* (*FRL1*: At5g16320), *FRI-LIKE 2* (*FRL2*: At1g31814), *FRIGIDA ESSENTIAL 1* (*FES1*: At2g33835), *SUPPRESSOR OF FRIGIDA 4* (*SUF4*: At1g30970), *FLC EXPRESSOR* (*FLX*: At2g30120), *FLOWERING LOCUS C EXPRESSOR-LIKE 4* (*FLL4*: AT5g61920) (Michaels et al., 2004; Schmitz et al., 2005; Kim et al., 2006; Andersson et al., 2008; Ding et al., 2013; Lee and Amasino, 2013). Mutations in these genes commonly result in early flowering even in the presence of functional *FRI* allele, suggesting that these genes are required for the function of FRI. Interestingly, mutations in this group of genes have only a little effect on elevated levels of *FLC* expression caused by lesions in autonomous pathway genes. This suggests that *FLC* activation in mutations of the autonomous pathway genes is achieved independently of FRI. Biochemical purifications of FRI-containing complex (FRI-C) revealed that genetically identified components indeed form a large protein complex (Choi et al., 2011).

Among components of FRI-C, SUF4, a BED-type zinc finger protein, appears to recruit FRI-C to *FLC* through its binding to a 15 bp-sequence motif (-CCAAATTTTAAGTTT-) at the *FLC* promoter region (Choi et al., 2011). Leucine zipper-containing proteins, FLX and FLX4, shows transcriptional activator activity, which in part explains the transcriptional activation of *FLC* by FRI-C (Ding et al., 2013). However, the biochemical function of FRI or its related proteins, FRL1 and FRL2, are not known. FRI-C also shows strong association with components of chromatin remodeling complexes, including components of SWR1 complex: PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1: At3g12810), ACTIN-RELATED PROTEIN 6 (ARP6)/ SUPPRESSOR OF FRI 3 (SUF3)/ESD1 (At3g33520), and SERRATED LEAVES AND EARLY FLOWERING (SEF)/AtSWC6 (At5g37055) (Choi et al., 2011). Trithorax-like SET domain protein, EARLY FLOWERING IN SHORT DAYS (EFS: At1g77300*,* also known as SDG8, *ASHH2,* and *CCR1*) is also intimately associated with FRI-C, indicating that chromatin modifications play a role in the activation of *FLC* by FRI-C (Choi et al., 2011). Unlike core components of FRI-C, these components of chromatin remodeling complexes are also required for the activation of *FLC* in autonomous pathway mutants as well. Therefore, chromatin remodeling complexes are more generally required for transcriptional activation of *FLC*. We will discuss the role of chromatin remodeling complexes in the activation of *FLC* later in this review. Components of FRI-C are listed in Table 1.

FLC activation by mutations in autonomous pathway genes

Historically, late flowering mutants were classified into two groups according to their flowering behavior under different environmental conditions (Koornneef et al., 1991). Although autonomous pathway mutants flower more rapidly under inductive long days than non-inductive short days, their flowering is markedly delayed under both conditions. In contrast, photoperiod pathway mutants show delayed flowering only under inductive photoperiod, suggesting that photoperiod pathway mutants are blind to inductive photoperiod. In addition, the flowering of autonomous pathway mutants is accelerated by vernalization treatment whereas flowering of photoperiod pathway mutants is not (Koornneef et al., 1991). Because autonomous pathway mutants remain responsive to both photoperiod and vernalization, the promotion of flowering by the autonomous pathway is considered to be independent of environmental stimuli (photoperiod and temperature).

Even though the autonomous pathway is independent of the vernalization pathway, all autonomous pathway genes function to repress the expression of *FLC* (Michaels and Amasino, 2001), indicating that *FLC* is a common target for both autonomous and vernalization pathways. Autonomous pathway genes include *LUMINIDEPENDENS* (*LD*: At4g02560), *FCA* (At4g16280), *FPA* (At2g43410), *FY* (At5g13480), *FLOWERING LOCUS D* (*FLD*: At3g10390), *FVE* (At2g19520), *FLOWERING LOCUS K* (*FLK*: At3g04610) and *RELATIVE OF EARLY FLOWERING 6* (*REF6*: At3g48430) (Macknight et al., 1997; Schomburg et al., 2001; Lim et al., 2004; Noh et al., 2004; Simpson, 2004). Largely, two classes of proteins are notable among the autonomous pathway proteins. Some of the autonomous proteins are implicated in RNA processing. For example, FCA, FPA, and FLK contain RNA binding motifs and FY is a homolog of the yeast 3' processing factor Pfs2p (poly-

Table 1. Genes involved in activation or repression of ***FLC*** in Arabidopsis

Function	Complex	AGI number	Gene name	Domain
Activation	COMPASS complex	AT1G51450	ASH2R	SPRY domain
		AT1G66240	ATX1	SET domain (Trithorax-like)
		AT1G05830	ATX2	SET domain (Trithorax-like)
		AT5G42400	ATXR7/SDG25	SET domain (SET1-like)
		AT3G49660	AtWDR5a	WD40 domain
	PAF1 complex	AT3G22590	CDC73/PHP	CDC73-like
		AT1G79730	ELF7	PAF1-like
		AT2G06210	ELF8/VIP6	TPR domain
	FRI complex	AT2G33835	FES1	C3H zinc finger
		AT2G30120	FLX	Leucine zipper
		AT4G00650	FRIGIDA (FRI)	Coiled coil domain
		AT5G16320	FRIL1	Coiled coil domain
		AT1G31814	FRIL2	Coiled coil domain
		AT1G30970	SUF4	BED zinc finger
		AT1G77300	EFS/SDG8	SET domain
		AT5G61920	FLL4	FLX family
	RAD6-BRE1 complex	AT2G44950	HUB1	Ring finger
		AT1G55250	HUB2	Ring finger
		AT1G14400	UBC1	Ubiquitin conjugating
		AT2G02760	UBC2	Ubiquitin conjugating
		AT5G62540	UBC3	Ubiquitin conjugating
	SWR1 complex	AT5G37055	SEF/AtSW6C	HIT zinc finger
		AT3G33520	ARP6/SUF3/ESD1	Actin-related
Repression	PRC2 complex	AT2G23380	CLF	SET domain
		AT4G02020	SWN	SET domain
		AT4G16845	VRN2	C2H2 Zinc Finger
		AT3G20740	FIE	WD40 domain
		AT5G58230	MSI1	WD40 domain
		AT5G51230	EMF2	C2H2 Zinc Finger
		AT5G57380	VIN3	PHD domain
	PRC1-like	AT3G24440	VIL1/VRN5	PHD domain
		AT5G17690	LHP1	Chromodomain
		AT3G18990	VRN1	B3 domain
		AT5G11530	EMF1	Not predicted
		AT2G30580	AtBMI1A	Ring-finger
		AT1G06770	AtBMI1B	Ring-finger
		AT3G23060	AtBMI1C	Ring-finger
	Noncoding RNA	First intron AT5g10140	COLDAIR	CLF binding

adenylation factor 1 subunit 2). Other autonomous pathway proteins are chromatin-modifying enzymes. FLD and REF6, encode two different types of histone demethylases (He et al., 2003; Noh et al., 2004; Jiang et al., 2007). The predicted biochemical functions of these autonomous pathway proteins are consistent with a model in which autonomous pathway proteins function through a coupling of RNA 3' end processing and chromatin-modifying events (Kim et al., 2009; Michaels, 2009; Liu et al., 2010).

Coupling of RNA 3' end processing and chromatin modifications may happen during 3' end processing of a group of antisense RNAs (Liu et al., 2007; Hornyik et al., 2010; Liu et al., 2010). These antisense noncoding RNAs (ncRNAs; collectively known as COOLAIR) are transcribed from the 3' end of *FLC* locus. Although these ncRNAs may be responsible for the accumulation of siRNA that targets a downstream region of *FLC* 3' end (Swiezewski et al., 2007), overall transcription level of COOLAIR is not correlated with that of sense *FLC* mRNA. For example, a mutant in which COOLAIR is expressed higher than the wild type results in elevated level of *FLC* and thus late–flowering (Sun et al., 2013). Rather, a proposed model suggests that differential 3' end processing and polyadenylation result in repression of *FLC* (Liu et al., 2007; De Lucia and Dean, 2011). COOLAIR transcripts are preferentially processed at the proximal end in the presence of active FCA, FPA and FY. Given that FLD is required for the function of FCA in the repression of *FLC*, FCA-mediated 3' end processing of COOLAIR appears to be a part of mechanism in which FLD mediates the demethylation of methylated Histone H3 Lys 4 (H3K4) at *FLC* chromatin (Liu et al., 2007; De Lucia and Dean, 2011). However, how these coordinated processes are eventually merged to repress *FLC* transcription remains to be addressed.

FLC activation through chromatin modifications

Although there is a clear difference between the FRI-mediated activation of *FLC* and the activation of *FLC* in autonomous pathway mutants, a group of genes are commonly required for the activation of *FLC* in both cases. For example, EFS was isolated as a component of FRI-C (Choi et al., 2011). However, *EFS* is also required for the activation of *FLC* in autonomous pathway mutants (Kim et al., 2005). EFS encodes a histone methyltransferase that has a dual function for di- and tri-methylation of both histone H3 Lys 4 (H3K4) and H3 Lys 36 (H3K36) at *FLC* chromatin (Kim et al., 2005; Zhao et al., 2005; Xu et al., 2008; Ko et al., 2010). Methylations of H3K4 and H3K26 are hallmarks of actively transcribed chromatin and are required for the activation of *FLC* transcription. Similar to EFS, additional genes are required for transcriptional activation of *FLC*. This group of genes shares two interesting features. First, most of them function to modify and/or remodel chromatin. In addition, mutations in these genes cause pleiotropic effects on other developmental processes. For example, mutations in *EFS* result in not only early flowering but also other developmental defects, including reduced organ size and shoot branching, carotenoid accumulation, and seed fertility (Kim et al., 2005; Zhao et al., 2005; Dong et al., 2008; Xu et al., 2008; Cazzonelli et al., 2009; Grini et al., 2009).

H3K4me3 is a representative active histone mark in eukaryotes (Schneider et al., 2004) and is associated with high levels of *FLC* expression. Methylation of H3K4 is catalyzed by SET-domain containing proteins. In yeast, two SET-domain containing proteins, SET1 and SET2, are responsible for the methylation of H3K4 and H3K36, respectively. Yeast SET1 is an essential component of a complex named COMPASS (Complex proteins associated with SET1) (Krogan et al., 2003). Another protein complex, PAF1 (RNA polymerase II associated factor 1) complex, is also necessary for H3K4me3 enrichments in eukaryotes. COMPASS and PAF1 complexes are physically associated to coordinate transcriptional activation of target genes by linking H3K4 histone methylation and transcriptional activation (Krogan et al., 2003). PAF1 complex connects COMPASS to RNA polymerase II machinery and thus leads to active transcription of target genes (Betz et al., 2002).

Two Arabidopsis homologs of yeast PAF1 complex components, EARLY FLOWERING 7 (ELF7: At1g79730) and EARLY FLOWERING 8 (ELF8: At2g06210; also known as VERNALIZATION INDEPENDENCE 6, VIP6) were isolated as essential components for *FLC* activation. Both ELF7 and ELF8 are required for H3K4me3 enrichment at *FLC* chromatin and *ELF7* and *ELF8* encode homologs of yeast PAF1 and CTR9 (a component of yeast PAF1-complex), respectively (He et al., 2004). Other Arabidopsis homologs encoding members of yeast PAF1-COMPASS complexes have also been isolated as required components for the activation of *FLC*. These include *VERNALIZATION INDEPENDENCE 3* (*VIP3*: At4g29830: Arabidopsis homolog of human *hSki8*), *VERNALIZATION INDEPENDENCE 4* (*VIP4*: At5g61150: Arabidopsis homolog of yeast *Leo1*), *VERNALIZATION INDEPENDENCE 5* (*VIP5*: At1g61040: Arabidopsis homolog of yeast *Rtf1*), *ARABIDOPSIS TRITHORAX-LIKE 1* (*ATX1*: At2g31650) and *ARABIDOPSIS TRITHORAX LIKE 2* (*ATX2*: At1g05830) (Baumbusch et al., 2001; Zhang and van Nocker, 2002; Alvarez-Venegas et al., 2003; He et al., 2004; Oh et al., 2004; Pien et al., 2008; Saleh et al., 2008). Lesions in these PAF1-associated complex components commonly result in decreased H3K4me3 enrichment at *FLC* chromatin, and thus lead to early flowering in the presence of active *FRI* allele or autonomous pathway mutations.

A group of SET domain proteins, including ATX1, ATX2, ARABIDOPSIS TRITHORAX-RELATED 3 (ATXR3: At4g15180), and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7: At5g42400), are redundantly responsible for H3K4 methylations at *FLC* chromatin through their histone methyltransferase activities (Pien et al., 2008; Tamada et al., 2009; Berr et al., 2010; Guo et al., 2010; Yun et al., 2012). Therefore, the COMPASS-PAF1 complex establishes the vernalization requirement in Arabidopsis through the activation of *FLC*. Genetically characterized components of the COMPASS-PAF1 complex involved in *FLC* activation in Arabidopsis are listed in Table 1.

Mono-ubiquitination of histone H2B (H2Bub1), together with H3K4me3, is associated with gene activation in eukaryotes (Wood et al., 2003; Zhang, 2003). In yeast, RAD6 (E2-ubiquitin conjugating enzyme) and BRE1 (E3-ubiquitin ligase enzyme) are responsible for H2Bub1 for certain targets (Jentsch et al., 1987; Robzyk et al., 2000; Yamashita et al., 2004). Similar to COMPASS, PAF1 is necessary for RAD6-BRE1 containing complex-mediated H2Bub1. In addition, H2Bub1 is also required for proper H3K4me3 deposition (Wood et al., 2003). In Arabidopsis, there are three RAD6 homologs [UBUQUITIN-CONJUGATING ENZYME 1 (AtUBC1: At1g14400, AtUBC2: At2g02760, and AtUBC3:

At5g62540)] and two BRE1 homologs [HISTONE MONOUBIQUITINATION 1 (HUB1: At2g44950 and HUB2: AT1g55250)] (Cao et al., 2008). AtUBC1 and AtUBC2 are redundantly involved in flowering time regulation through enrichment of H2Bub1 at *FLC* chromatin, whereas AtUBC3 is dispensable for activation of *FLC* (Xu et al., 2009). Each *hub1* and *hub2* single mutant shows early flowering which results from defects in the enrichments of H2Bub1 and H3K4me3 at *FLC* chromatin (Cao et al., 2008), suggesting that HUB1 and HUB2 function non-redundantly at *FLC* chromatin. Known components of RAD6-BRE1 complex involved in *FLC* activation in Arabidopsis are listed in Table 1.

VERNALIZATION AS AN EPIGENETIC CHANGE

Unlike other biological responses, the vernalization response (accelerated flowering) is not immediately triggered by the stimulus (low temperatures). Rather, accelerated flowering happens when the original stimulus (low temperatures) is removed (warm temperatures in the following spring). This epigenetic nature of vernalization predicts that low temperatures during winter establish stable changes that last until the following spring to promote the floral transition (Lang, 1965). In Arabidopsis, a major stable change by vernalization is the stable repression of a floral repressor, *FLC*. Prior to vernalization, high levels of *FLC* block the floral transition in winter-annual strains of Arabidopsis (Figure 2). Prolonged exposure to low temperatures results in epigenetic repression of *FLC*. The repressed state of *FLC* triggered by low temperatures is stably maintained throughout subsequent mitotic cell divisions even when plants are returned to warm growth temperatures. Therefore, stable repression of *FLC* by vernalization allows the floral transition to occur when inductive day length activates the photoperiod pathway in the spring (Figure 1).

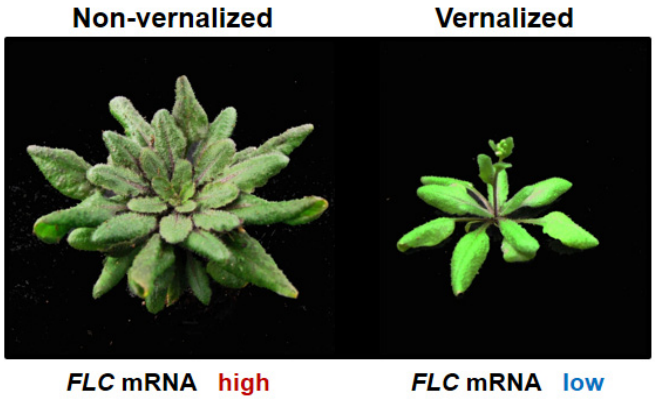

Figure 2. Vernalization-mediated acceleration of flowering.

Winter-annual strains of Arabidopsis flower late without vernalization (Left). Flowering of winter-annual strains of Arabidopsis is accelerated by vernalization (Right).

Genetic characterization of the vernalization pathway

To understand the molecular mechanism underlying vernalization-mediated *FLC* repression, genetic approaches have been used (Chandler et al., 1996; Gendall et al., 2001; Levy et al., 2002; Sung and Amasino, 2004; Greb et al., 2007). The first component characterized from genetic screens was *VERNALIZATION2* (*VRN2*: At4g16845) (Gendall et al., 2001). VRN2 is a homolog of the Polycomb-group zinc finger protein, SUPPRESSOR OF ZESTE-12 (Su(Z)12), and lesions in *VRN2* results in de-repression of *FLC*. Polycomb-group genes are classically known to be essential for gene repressions in higher eukaryotes (Ringrose and Paro, 2004). Biochemical purification and subsequent characterization revealed that Su(Z)12 is a component of a histone methyltransferase complex, Polycomb Repression Complex 2 (PRC2). PRC2 mediates methylation at H3K27 through one of its components, ENHANCER OF ZESTE (E(z)), a SET-domain containing methyltransferase (Cao et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Additional components of the vernalization pathway include *VERNALIZATION1* (*VRN1*: At3g18990), *VERNALIZATION INSENSITIVE 3* (*VIN3*: At5g57380) and *VIN3-LIKE 1* (*VIL1*)/*VERNALIZATION 5* (*VRN5*) (At3g24440), which are non-redundantly necessary for vernalization-mediated repression of *FLC* (Levy et al., 2002; Sung and Amasino, 2004; Sung et al., 2006a; Greb et al., 2007). VRN1 belongs to a small family of plant-specific B3 DNA-binding proteins and VIN3 and VIL1/VRN5 are plant-specific Plant Homeo Domain (PHD) motif-containing proteins. PHD motifs recognize and bind a wide variety of modified histones in eukaryotes (Musselman and Kutateladze, 2011). *VRN1*, *VRN2*, *VIL1/VRN5* are constitutively expressed regardless of vernalization. In contrast, *VIN3* is only induced when plants are kept under prolonged periods of cold temperature. When plants are returned to warm growth temperatures, transcription of *VIN3* quickly decreases (Sung and Amasino, 2004; Kim and Sung, 2013). Therefore, *VIN3* is a cold-specific component in the vernalization pathway in Arabidopsis.

Changes in histone modifications at *FLC* by vernalization

Identification of VRN2 and VIN3 as essential components in vernalization-mediated *FLC* repression implicated that histone modifications play roles in the process. Indeed, the chromatin context of *FLC* undergoes a series of changes by vernalization. During and after vernalization, levels of histone modifications associated with gene activation are reduced (Bastow et al., 2004; Sung and Amasino, 2004). By contrast, repressive histone modifications (i.e. H3K9me2 and H3K27me3) are substantially increased at *FLC* chromatin by vernalization (Bastow et al., 2004; Sung and Amasino, 2004; Schubert et al., 2006; Sung et al., 2006a; Greb et al., 2007). VIN3 co-purifies with components of PRC2, including VRN2 (Wood et al., 2006; De Lucia et al., 2008). Both *VIN3* and *VRN2* are required for methylation of H3K27 at *FLC* chromatin by vernalization (Bastow et al., 2004; Sung and Amasino, 2004; Kim and Sung, 2013).

VIN3 is a member of a plant-specific small gene family together with *VIL1*/*VRN5*, *VIN3-LIKE 2* (*VIL2*)/*VRN5-LIKE 1* (*VEL1*) (At4g30200) and *VIN3-LIKE 3 (VIL3)* /*VRN5-LIKE 2* (*VEL2*) (At2g18880). VIL1 and VIL2 biochemically co-purify together with VIN3-containing PRC2 complex (De Lucia et al., 2008), suggesting that they share a common biochemical function in the vernal-

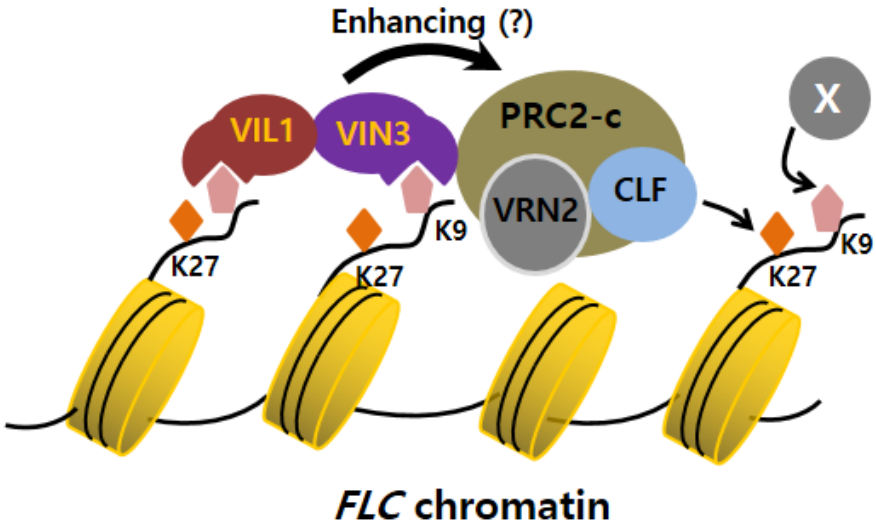

Figure 3. Cooperative activity of VIN3 and PRC2 for the repression of FLC by vernalization.

PRC2 mediates tri-methylation of H3K27 at *FLC* chromatin. H3K9me2 at *FLC* chromatin can be recognized by VIN3 and VIL1/VRN5 through their PHD motifs. VIN3 and VIL1/VRN5 physically associate with PRC2 and enhance the H3K27 methylation activity of PRC2.

ization response. Although *FLC* is a major target for repression by vernalization, other *FLC*-related genes are also repressed by vernalization (Kim and Sung, 2013). In the presence of functional *FRI* allele, *FLC* is the main contributor for the vernalization requirement. *FLC*-related genes include *FLOWERING LOCUS M* (*FLM*)/*MADS AFFECTING FLOWERING 1* (*MAF1*) (At1g77080), *MADS AFFECTING FLOWERING 2* (*MAF2*: At5g65050), *MADS AFFECTING FLOWERING 3* (*MAF3*: At5g65060), *MADS AFFECTING FLOWERING 4* (*MAF4*: At5g65070), and *MADS AFFECTING FLOWERING 5* (*MAF5*: At5g65080). They commonly act as floral repressors (Ratcliffe et al., 2001; Scortecci et al., 2001; Ratcliffe et al., 2003; Gu et al., 2013; Kim and Sung, 2013). The chromatin of all *FLC* and *FLC*-related loci become enriched with repressive histone marks (H3K9me2 and H3K27me3) as a result of vernalization (Kim and Sung, 2013).

Roles of VIN3 family of proteins in vernalization

Although all members of *VIN3* gene family function in vernalization, their contributions differ in "timing" during the course of vernalization. VIN3 and VIL2/VEL1 act during cold exposure, whereas VIL1/VRN5 and VIL3 are predominantly involved after cold (Sung et al., 2006a; Greb et al., 2007; De Lucia et al., 2008; Kim and Sung, 2013). Each member of VIN3 family of proteins is associated with certain *FLC* gene family chromatin to exert their repressive activities on their respective targets. VIN3 is required for repression of all members of *FLC* gene family, indicating that VIN3 is a master regulator for vernalization. Other members of the VIN3 family of proteins are necessary for a subset of *FLC*-related genes (Sung et al., 2006a; Kim and Sung, 2010, 2013). *VIL1/VRN5* is necessary for the repression of *FLC* and *FLM* whereas *VIL2/VEL1* is necessary for the repression of *MAF4* and *MAF5* by vernalization. VIL3/VEL2 is enriched at *MAF2* ~ *MAF5* chromatin and necessary for proper repression of *MAF2* ~ *MAF5*. Given that VIN3, VIL1 and VIL2 can be found together with PRC2 complex, it is likely that VIN3 family of proteins function through alternative complexes with the core components of PRC2 at their target chromatin (De Lucia et al., 2008; Kim and Sung, 2013). Although the VIN3 family of proteins can directly interact with one and another (i.e. direct interaction between VIN3 and VIL1 though their VID motifs) (Sung et al., 2006a; Greb et al., 2007), no direct interaction between members of the VIN3 family of proteins and core components of PRC2 is known.

All VIN3 family proteins contain a PHD finger, a motif known to bind a wide range of modified histones (Musselman and Kutateladze, 2011). Indeed, all VIN3 family proteins preferentially bind to H3K9me2 peptides *in vitro* through PHD motifs (Figure 3). H3K9me2 mark is enriched at *FLC* gene family chromatin by vernalization and the *VIN3* gene family is necessary for the enrichment of H3K9me2 at their respective target chromatin, supporting the biological significance of such binding activities by VIN3 family of proteins (Kim and Sung, 2013). Mutations in the *VIN3* gene family also impair vernalization-mediated enrichment of H3K27me3 at *FLC* gene family chromatin. Therefore, it appears that the preferential binding to H3K9me2 by VIN3 family proteins may reinforce the activity of PRC2, H3K27 methylation, at target chromatin (Figure 3) (Kim and Sung, 2013).

Polycomb-mediated *FLC* repression by vernalization

Polycomb group proteins maintain gene expression pattern in a wide variety of cells during development by regulating chromatin structure. Core components of PRC2 are well conserved in higher eukaryotes, including Arabidopsis (Hsieh et al., 2003). PRC2 core components consist of Su(z)12, E(z), EED and RbAp46/48

in mammals. Among Arabidopsis components of PRC2, two homologs of E(z), CURLY LEAF (CLF: At2g23380) and SWINGER (SWN: At4g02020) and a homolog of Su(z)12, VRN2, are involved in the repression of *FLC* (Chanvivattana et al., 2004). The enrichment of PRC2 increases at *FLC* chromatin by vernalization. PRC2 contributes to the repression of genes mainly through its H3K27 methyltranseferase activity (Cao et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Components of PRC2 complex involved in *FLC* repression in Arabidopsis are listed in Table 1.

Another major Polycomb group complex, POLYCOMB REPRESSIVE COMPLEX 1 (PRC1), exerts another histone modification activity, mono-ubiquitination of histone H2A (H2Aub1, a repressive histone mark) (Margueron and Reinberg, 2011). PRC1 is also involved in chromatin compaction through the binding to H3K27me3 mark by Polycomb protein in Drosophila and mammals. In Arabidopsis, LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1: At5g17690), binds to H3K27me3 in vitro (Zhang et al., 2007) and accumulates at *FLC* chromatin by vernalization (Mylne et al., 2006; Sung et al., 2006b; Turck et al., 2007). In *lhp1* mutants, vernalization-mediated repression of *FLC* is not stable, indicating its essential role in the maintenance of repressed chromatin. *EMBRYONIC FLOWERING 1* (*EMF1*: At5g11530) encodes a plant-specific protein with motifs found in transcriptional regulators and may function as a component of PRC1-like complex in Arabidopsis (Aubert et al., 2001; Calonje et al., 2008; Kim et al., 2010; Kim et al., 2012). In mammals, PRC1 stabilizes repressed state of H3K27me3-enriched target chromatin through its H2A mono-ubiquitination activity (Margueron and Reinberg, 2011). Similarly, two Arabidopsis RING-finger proteins, AtBMI1A (At2g30580) and AtBMI1B (At1g06770), function to mediate the formation of H2Aub1 at chromatin in Arabidopsis (Bratzel et al., 2010; Yang et al., 2013). *atbmi1a/atbmi1b* double mutants result in de-repression of Polycomb target genes and thus display similar phenotypes to those of PRC2 component mutants. AtBMI1A and AtBMI1B show H2Aub1 activity *in vitro* and interact with other Arabidopsis PRC1-like components, LHP1 and EMF1. Another Arabidopsis RING finger protein AtBMI1C (At3g23060) was also reported to be involved in repression of *FLC* through its H2Aub1 activity (Li et al., 2011). Therefore, Arabidopsis PRC1-like complex consists of at least LHP1, EMF1, AtBMI1A, AtBMI1B, and AtBMI1C and contributes to Polycomb-mediated repression of *FLC* (Figure 4).

Physical alteration of higher order structure is closely correlated with gene expression in eukaryotes (Fraser and Bickmore, 2007; Hubner et al., 2013). Conformational changes of higher order chromatin structure are also reported for Polycomb-mediated silenced loci in Drosophila and mammals, known as polycomb bodies within nucleus (Lanzuolo et al., 2007; Bantignies and Cavalli, 2011; Nora et al., 2012). Similarly in plants, it has been observed that *FLC* chromatin is repositioned in nucleus by vernalization (Rosa et al., 2013). Therefore, Polycomb-mediated silencing of *FLC* by vernalization may also involve physical repositioning of chromatin.

Role of noncoding RNAs in vernalization

Polycomb group complexes regulate a wide range of genes in eukaryotes. However, how these complexes are recruited to certain target genes is not well understood. Recent studies show that noncoding RNAs (ncRNAs) are becoming recognized as a part of PRC2 recruitment machinery. In mammals, several long ncRNAs, such as *ANRIL*, *HOTAIR*, *Xist* and *Kcnq1ot1*, physically interact with components of PRC2 and direct PRC2 to target chromatin (Pandey et al., 2008; Zhao et al., 2008; Tsai et al., 2010; Kotake et al., 2011). The involvement of ncRNAs is not restricted to PRC2. A number of ncRNAs have been co-purified with various types of chromatin modifying complexes, indicating that ncRNAs function in various gene expression regulations in eukaryotes (Tsai et al., 2010; Guttman et al., 2011; Spitale et al., 2011; Guttman and Rinn, 2012). In the regulation of *FLC*, two different types of long ncRNAs, COOLAIR and COLDAIR, appear to have regulatory function (Liu et al., 2007; Swiezewski et al., 2009; Hornyik et al., 2010; Liu et al., 2010; Heo and Sung, 2011).

First, a group of antisense transcripts are detectable from *FLC* locus. These antisense ncRNAs are largely grouped into two classes based on different polyadenylation sites, proximal and distal. A total of 6 splicing variants also exist among these two classes of antisense ncRNAs (Hornyik et al., 2010). It has been proposed that the proximal polyadenylation by components of autonomous pathway, including FCA, FY and FPA, triggers the repression of *FLC* through the recruitment of FLD (De Lucia and Dean, 2011). Transcriptional activity of these antisense RNAs transiently increases during the course of vernalization. Therefore, these antisense ncRNAs, known as COOLAIR, are also implicated in the vernalization-mediated *FLC* repression (Swiezewski et al., 2009). Both proximally and distally polyadenylated antisense transcripts increase during early time periods (~ 2 weeks) of vernalization treatment, but eventually decrease to the basal level at later periods of vernalization treatment (Swiezewski et al., 2009). Alternative polyadenylation of COOLAIR does not play a role in vernalization since all known autonomous pathway mutants are responsive to vernalization treatment. Several T-DNA insertion lines, in which COOLAIR transcription is largely impaired, are responsive to vernalization (Helliwell et al., 2011). In addition, a mutant in which COOLAIR is up-regulated exhibits de-repression of *FLC* (Sun et al., 2013). Therefore, increased levels of COOLAIR transcripts do not trigger *FLC* repression. An alternative model has been proposed to have a “co-transcriptional” regulation circuitry (De Lucia and Dean, 2011). In this model, the antisense transcription “read-through” may interfere with sense transcription of *FLC*, therefore contributes to initial transcriptional repression of *FLC* during the course of vernalization. Mechanistic details of this model remain to be elucidated.

Another long ncRNA, known as COLDAIR (COLD ASSISTED INTRONIC NON-CODING RNA), is also transcribed from the *FLC* locus. COLDAIR is transcribed from the first intron of *FLC* in a sense direction compared to the *FLC* transcript (Heo and Sung, 2011). Similar to COOLAIR, COLDAIR is also transiently induced by vernalization. COLDAIR transcripts (about 1.1 kb long) physically interact with CLF, one of PRC2 components. Reduced expression of COLDAIR using RNAi impairs the vernalization-mediated enrichment of PRC2 at *FLC* chromatin. Reduced enrichment of PRC2 at *FLC* chromatin results in decreased enrichment of H3K27me3 (Heo and Sung, 2011). Taken together, COLDAIR is a part of the machinery that recruits PRC2 to *FLC* chromatin by vernalization. Biochemical properties of COLDAIR are similar to those of PRC2 associated ncRNAs in mammals (Rinn et al., 2007; Tsai et al., 2010; Guttman et al., 2011; Spitale et al., 2011; Guttman and Rinn, 2012). Therefore, ncRNA-mediated recruitment of PRC2 may be an evolutionally conserved mechanism in

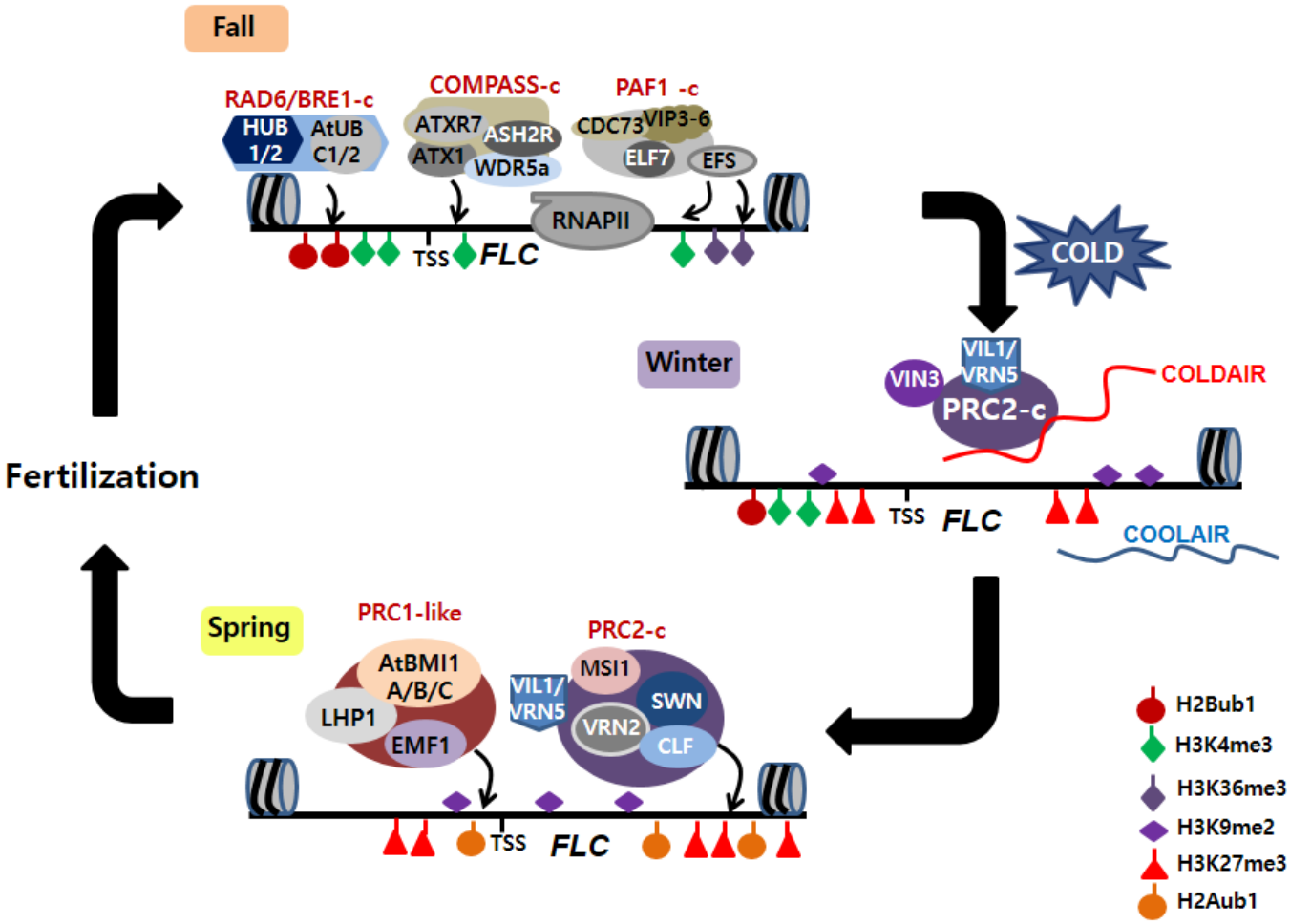

Figure 4. Schematic representation of mechanisms underlying FLC activation and repression.

A) Prior to vernalization (fall), *FLC* is highly expressed by activation chromatin-remodeling complexes, PAF1-C, COMPASS-C and RAD6-BRE1-C.
B) During winter, a long ncRNA, COLDAIR, is transcribed from the first intron of *FLC* and functions to recruit PRC2. COOLAIR and *VIN3* is also transiently induced by cold and PRC2 together with PHD finger proteins, VIN3 and VIL1/VRN5, becomes associated with *FLC* chromatin. Level of *FLC* mRNA decreases during cold exposure.
C) After cold (Spring), the repressed state of *FLC* is stably maintained through combinatorial activities of PRC2 and PRC1-like complex.

eukaryotes. Transiently increased transcription of COLDAIR indicates that COLDAIR may function in initial recruitment of PRC2 to *FLC* by vernalization. However, it is not yet clear whether the same mechanism also plays a role in the maintenance of PRC2 recruitment to *FLC* after the cold treatment. PRC2 recruitment by HOTAIR includes a conserved sequence motif of HOTAIR appears to be necessary for the recruitment of PRC2, perhaps through RNA-DNA sequence recognition (Chu et al., 2011). However, it remains to be addressed how the COLDAIR targets PRC2 to *FLC* locus.

Re-activation of *FLC* in the next generation

Prior to vernalization, *FLC* is highly expressed and its chromatin is enriched with H3K4me3, an active histone mark. By vernalization, active histone marks at *FLC* chromatin decrease. The reduction of active histone marks at *FLC* chromatin is accompanied by decreased enrichment of active chromatin modifying complex components, including ATXR7 and EFS (Kim and Sung, 2013). Instead, repressive chromatin modifying complexes, including PRC2, become predominantly associated with *FLC* chromatin, which result in the enrichment of repressive histone marks, such as H3K27me3. Therefore, vernalization triggers changes in chromatin landscape at *FLC* (Figure 4). This repression of *FLC* is stable even after the cold exposure. However, this repression is only stable throughout mitosis and *FLC* is re-activated in the next generation. This is an adaptive feature of the vernalization response to ensure that each generation of Arabidopsis plants re-achieve the vernalization requirement. *FLC* appears to be re-activated during the gametogenesis and early embryogenesis after fertilization (Sheldon et al., 2008; Choi et al., 2009). At this stage, *FLC* chromatin must undergo reprogramming of chromatin

context from repressive to active states. Active chromatin modifying complex components, particularly components of FRI-C and PAF1, are necessary for the reactivation of *FLC* (Yun et al., 2011). Mechanisms of how these active chromatin modifying complexes are recruited to *FLC* chromatin and how repressive chromatin modifying complexes are excluded from *FLC* chromatin are not known.

VERNALIZATION IN OTHER FLOWERING PLANTS

Although Arabidopsis has served as an excellent model system to understand molecular mechanism of the vernalization response, other vernalization-required species use different gene regulatory circuitries. Here, we briefly describe current understanding on molecular circuitries of the vernalization response in other flowering species.

Arabis alpina

Arabis alpina, a perennial relative of Arabidopsis, is distinctive in the vernalization response compared to annual/biennial Arabidopsis accessions (Koch et al., 2006; Ansell et al., 2008). Annual plants initiate the floral transition in all apical meristems at the same time during their life cycle, known as monocarpy. In contrast, perennial plants bloom in spring and summer seasons but arrest flowering later. Perennial plants resume vegetative growth in fall and repeatedly undergo vernalization. Therefore, perennial plants flower and set seeds many times in their life cycle (known as polycarpy). Arabis plants repeat the cycle of vegetative and reproductive growth phases. Similar to Arabidopsis, an ortholog of *FLC* (*PERPETUAL FLOWERING 1* (*PEP1)) acts as a major floral repressor* in Arabis (Wang et al., 2009)*. PEP1 is repressed by vernalizing cold and thus allow plants to bloom. Unlike Arabidopsis, however, PEP1 is re-activated* when plants are returned to warm growth temperature. This transient nature of the repression of *PEP1* confers polycarpic flowering behavior in Arabis. Consistent with fluctuating expressions of *PEP1*, a repressive histone mark, H3K27me3, is accumulated at *PEP1* chromatin during the cold exposure, but depleted when plants are returned to warm temperature.

An APETALA2-type transcription factor, *PERPETUAL FLOWERING 2* (*PEP2*, an Arabis ortholog of Arabidopsis AP2) also function to repress flowering in Arabis (Bergonzi et al., 2013). PEP2 acts to up-regulate *PEP1* to prevent flowering prior to vernalization. Interestingly, Arabis plants respond vernalization only when plants reach to a certain mature age. This age-dependent response to vernalization is achieved via a microRNA, miRNA156. When plants are young, miRNA156 is abundant and prevents flowering through blocking expression of floral activators *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*). However, as plants age, miRNA156 levels decline, resulting in an increase of floral activator, SPL. Therefore, it is likely that PEP2-PEP1 and miRNA156 act in parallel to ensure that Arabis plants become competent to flower only when they have reached appropriate vegetative stage and have been exposed to vernalization (Figure 5).

Cereals (wheat and barley)

In temperate cereals, such as wheat and barley, genetic analysis between winter and spring cultivars of the crops has identified several genes involved in the vernalization requirement (Trevaskis et al., 2007; Distelfeld et al., 2009). Three loci, *VERNALIZATION1* (*VRN1*), *VRN2*, and *VRN3* (also known as *HvFT1* in barley) are important in determining vernalization requirement and flowering time regulation in temperate cereals (Danyluk et al., 2003; Trevaskis et al., 2003; Yan et al., 2003; Yan et al., 2004a; Yan et al., 2004b). *VRN1* and *VRN2* from cereals are not related to Arabidopsis *VRN1* and *VRN2*. *VRN1* encodes a MADS-box transcription factor that promotes flowering in cereals, while *VRN2* encodes a CCT-domain protein and acts as a floral repressor by blocking *VRN3* expression (Yan et al., 2004b; Yan et al., 2006). There is no apparent homolog of Arabidopsis *FLC* in cereals. Instead, a floral repressor, VRN2, acts as a floral repressor in cereals, similar to *FLC* in Arabidopsis. For example, vernalization results in stable repression of VRN2 (Figure 5). In winter cultivars, *VRN1* is induced by vernalizing cold treatment and is required for the repression of *VRN2*. It is interesting to note that *VRN1* chromatin context is subjected to histone modification change by vernalization. Vernalization results in decreased level of enrichment of H3K27me3 and increased level of enrichment of H3K4me3 at VRN1 chromatin. On the other hand, there are no significant changes in histone modifications at *VRN2* and *VRN3* chromatins (Oliver et al., 2009). Taken together, changes of chromatin structure at *VRN1* locus appear to take part in the epigenetic mechanism of the vernalization response in cereals.

Sugar beet (*Beta vulgaris*)

In sugar beet, a pair of *FT* homologs (*BvFT1* and *BvFT2*), which encode phosphatidylethanolamine-binding protein, acts antagonistically in the floral transition. *BvFT1* acts as a floral repressor whereas and *BvFT2* promotes flowering (Pin et al., 2010). In addition, vernalization results in down-regulation of *BvFT1*. Vernalization-induced repression of Bv*FT1* is stably maintained even after plants are returned to warm growth temperatures, indicating that *BvFT1* functions similarly to *FLC*. *Vernalization requirement in sugar beet is mainly conferred by a dominant allele named BvBTC1 through its regulation of BvFT1 and BvFT2 (Pin et al., 2012). In annual sugar beet, expression of a dominant BvBTC1 allele is increased by long days. This results in the floral transition through the repression of BvFT1 and the activation of BvFT2 under long day conditions. Therefore, annual sugar beet plants with a dominant BvBTC1 allele do not need vernalization for early flowering. In contrast, biennial sugar beet plants* carry a partial loss-of-function allele of *Bvbtc1*. *Bvbtc1* is not significantly induced even under long days without vernalization treatment. *Bvbtc1 allele can be gradually activated by vernalization treatment to the level sufficient to repress BvFT1 and activate BvFT2 (Figure 5).*

Divergent regulatory circuitries of vernalization pathway in flowering plants suggest that plants have independently evolved systems to mediate the vernalization response. Despite clear differences in components of flowering regulatory circuits, one basic theme is conserved; vernalization commonly results in competence to flower through ‘repression of floral repressor’ (Figure 5).

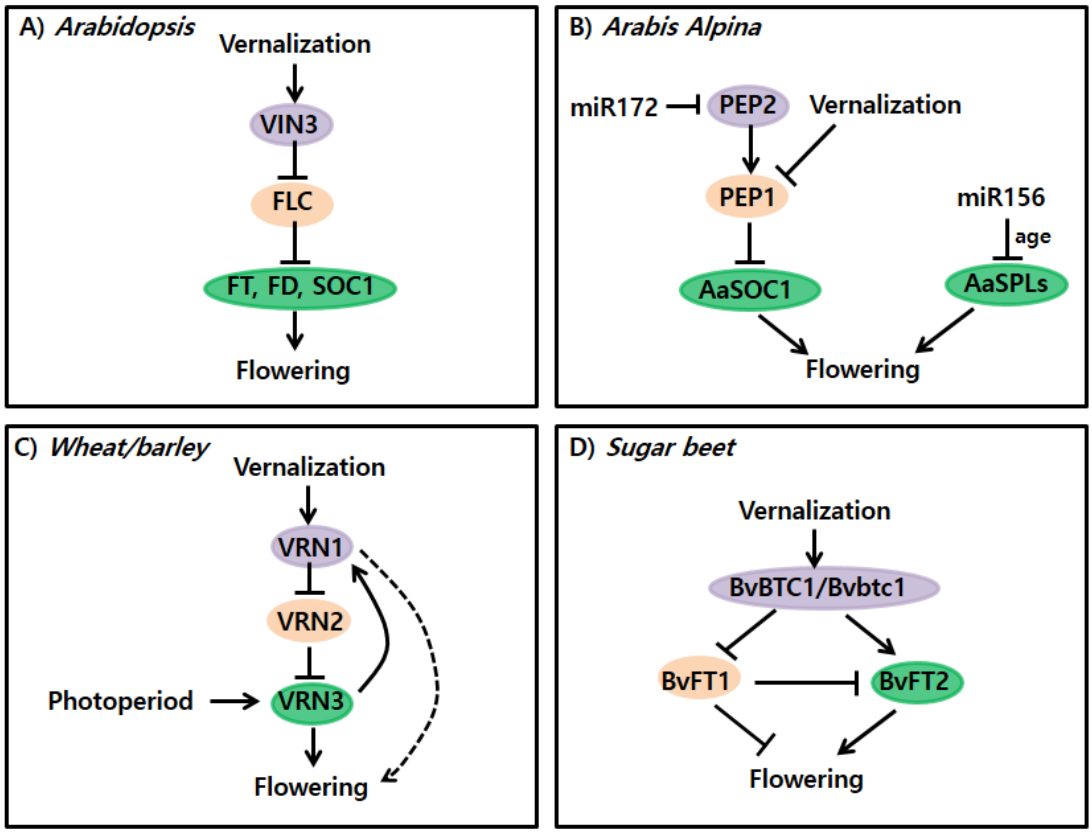

Figure 5. Models of flowering time regulation by vernalization in various flowering plants.

Green: floral activator, Pink: floral repressor, Violet: upstream repressor of floral repressor.

CONCLUSION

Studies using Arabidopsis shed light on our understanding on molecular mechanisms of the vernalization response. Mechanisms underlying vernalization involves various modes of gene expression regulation, from histone modifications to noncoding RNAs. Therefore, what we learn from vernalization studies contributes to our understanding of gene expression. The inducible nature of gene expression makes vernalization one of the best model systems to study mechanistic details of gene expression changes by environmental stimuli in eukaryotes. Combined with a rich genetic resource and recent technological advances, vernalization study using Arabidopsis and other flowering plants continue to provide insights on our understanding of gene regulation in eukaryotes.

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