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NATURAL VARIATION IN EPIGENETIC GENE REGULATION AND ITS EFFECTS ON PLANT DEVELOPMENTAL TRAITS

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In plants, epigenetic variation contributes to phenotypic differences in developmental traits. At the mechanistic level, this variation is conferred by DNA methylation and histone modifications. We describe several examples in which changes in gene expression caused by variation in DNA methylation lead to alterations in plant development. In these examples, the presence of repeated sequences or transposons within the promoters of the affected genes are associated with DNA methylation and gene inactivation. Small interfering RNAs expressed from these sequences recruit DNA methylation to the gene. Some of these methylated alleles are unstable giving rise to revertant sectors during mitosis and to progeny in which the methylated state is lost. However, others are stable for many generations and persist through speciation. These examples indicate that although DNA methylation influences gene expression, this is frequently dependent on classical changes to DNA sequence such as transposon insertions. By contrast, forms of histone methylation cause repression of gene expression that is stably inherited through mitosis but that can also be erased over time or during meiosis. A striking example involves the induction of flowering by exposure to low winter temperatures in *Arabidopsis thaliana* and its relatives. Histone methylation participates in repression of expression of an inhibitor of flowering during cold. In annual, semelparous species such as *A. thaliana*, this histone methylation is stably inherited through mitosis after return from cold to warm temperatures allowing the plant to flower continuously during spring and summer until it senesces. However, in perennial, iteroparous relatives the histone modification rapidly disappears when temperatures rise, allowing expression of the floral inhibitor to increase and limiting flowering to a short interval. In this case, epigenetic histone modifications control a key adaptive trait, and their pattern changes rapidly during evolution associated with life-history strategy. We discuss these examples of epigenetic developmental traits with emphasis on the underlying mechanisms, their stability, and adaptive value.

KEY WORDS: **Adaptation, genetic variation, molecular evolution, reproductive strategies.**

Epigenetic gene regulation can be defined as changes in gene activity states that are inherited across meiotic and/or mitotic cell divisions without an alteration in primary DNA sequence (Berger et al. 2009). Genetic analysis of such epigenetic states has a long history in plant biology (Brink 1956). The mechanisms underlying these states have been the subject of intensive study and here are broadly divided into two major types: DNA modification in which the DNA itself is covalently modified by methylation and histone modification in which the histone proteins that are components of the nucleosomes are covalently modified. As epigenetic states do not involve changes to the DNA sequence, their inheritance is associated with a higher possibility of reversion than classical genetic mutations, although this frequency varies depending on the underlying epigenetic mechanism. In this review, we discuss examples in plants in which epigenetic variation is linked to phenotypic alterations in developmental traits that are important for adaptation. We focus on those examples for which the mechanistic basis has been characterized in detail. Among these

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examples, the stability of gene inactivation by DNA methylation varies tremendously. An important conclusion to emerge from these case studies is that epigenetic variation is tightly linked to genetic variation because epialleles, those reduced in expression by DNA methylation, require the presence of genetically determined features that act *in cis* as *potentiators* for recruitment of methylation. However, epigenetic regulation by histone modification is often developmentally regulated, changing in response to environmental or developmental cues. We provide an example of how changes in the regulation of histone modification contribute to a change in life history between closely related taxa.

Mechanistic Aspects of Meiotically Inheritable Epigenetic Traits

A short introduction of the mechanisms underlying meiotically stable epigenetic variation is provided in the following paragraphs to provide a basis for better understanding the examples discussed subsequently. Methylation of DNA occurs on cytosine bases in the context of CG, CHG, or CHH sequences (where H is A, T, or C; Zhang et al. 2006; Zilberman et al. 2007). To stably influence gene expression, methylation of these bases must be faithfully inherited during cell division. The symmetric CG or CHG sequences allow the methylated base still present on the parental strand to direct methylation of the symmetric cytosine on the newly synthesized daughter strand. For example, in *Arabidopsis thaliana*, DNA-METHYLTRANSFERASE 1 (MET1) uses CG information present on one strand to methylate symmetric cytosines on the paired strand (Bartee and Bender 2001; Law and Jacobsen 2010). CHG methylation requires the activity of the plant-specific CHROMOMETHYLASE 3 (CMT3) for maintenance and setting (Lindroth et al. 2001; Cao et al. 2003). In contrast, information of nonsymmetric CHH methylation, which is found only in plants, is lost from one of the two sister chromatids during replication. The presence of 24 nucleotide short interfering RNAs (24nt siRNAs), which direct de novo DNA-methyltransferases to complementary sites is required for stable inheritance of nonsymmetric sites after replication (Teixeira et al. 2009). REARRANGED DOMAIN METHYLTRANSFERASE 2 (DRM2) is mainly responsible for de novo and maintenance methylation of CHH sites, but the enzyme seems capable of methylation also in the CG and CHG context (Cao and Jacobsen 2002). All three classes of DNA-methyltransferases are conserved in higher plants suggesting that their function is evolutionarily conserved (Pavlopoulou and Kossida 2007).

Pathways leading to DNA-methylation and epigenetic histone modifications are interconnected. In *A. thaliana* and rice, Histone H3 di-methylated at lysine residue 9 (H3K9me2), a mark associated with silent chromatin, recruits CMT3 to methylate cytosine in the CHG context (Du et al. 2012). This, in turn, provides a signal for the recruitment of the Histone methyltransferase KRYPTONITE (KYP) and its relatives SUVH5 and SUVH6, which target H3K9 for dimethylation (Johnson et al. 2007). As a result, H3K9me2 and DNA-methylation reinforce each other in a positive-positive feedback loop.

Effects of DNA Methylation on Developmental Gene States

In this section, we describe examples of DNA methylation affecting developmental traits. We focus on studies that illustrate the stability of epialleles as well as the underlying mechanisms. This analysis demonstrates the close relationship between DNA methylation of these genes and the presence of classical mutations caused by transposon insertions.

Pioneering work in *Linaria vulgaris* demonstrated that methylation state can give rise to phenotypic changes in natural populations and that such mutations can be unstable, frequently giving rise to mitotic and meiotic revertants (Cubas et al. 1999). *Linaria vulgaris* produces bilaterally symmetrical flowers that show asymmetry across a dorsal–ventral axis, where the dorsal side is closest to the stem. By contrast, mutants originally described by Linnaeus arise in natural populations and show radially symmetrical flowers (Linnaeus 1744). These mutant flowers are caused by loss of expression of a gene, *Lcyc*, which encodes a transcription factor homologous to the CYCLOIDEA protein of *Antirrhinum majus* that also confers asymmetry on the flowers (Luo et al. 1996; Cubas et al. 1999). In mutant plants, the *Lcyc* gene is heavily methylated. This methylation was shown to be the cause of the mutant phenotype because these plants frequently gives rise to spontaneous revertant branches on which wild-type flowers form, and in these branches the level of methylation of the *Lcyc* gene is greatly reduced (Cubas et al. 1999). The somatic revertant sectors that arise in these mutants also indicate that the mechanisms controlling methylation are likely to be largely cell autonomous, although why *Lcyc* is a target for such high levels of methylation is unclear (Cubas et al. 1999).

Studies of the *FLOWERING LOCUS WAGENINGEN* (*FWA*) gene in *A. thaliana* illustrated the importance of a mechanism that targets methylation to genes carrying transposon-based repeats in their promoters (Henderson and Jacobsen 2007). *FWA* influences the timing of the transition from vegetative to reproductive development in plants, which is a major determinant of reproductive success. In semelparous annual species such as *A. thaliana* flowering and reproduction occur only once during the life cycle, and therefore it is strictly regulated by endogenous signals and environmental cues to ensure that flowering occurs at the optimal time to maximize seed set and production

of progeny. Many induced mutations or naturally occurring alleles that alter flowering time have been described (Fornara et al. 2010; Andres and Coupland 2012). The *fwa-1* allele arose during a chemical mutagenesis experiment and *FWA* was shown to encode a homeobox transcription factor (Soppe et al. 2000). The wild-type *FWA* allele is not expressed in the vegetative tissues of Columbia, the laboratory accession of *A. thaliana*, but is specifically expressed in the endosperm (Kinoshita et al. 2004). However, dominant mutant alleles of *FWA* were recovered that caused late flowering due to ectopic expression of *FWA* in vegetative tissues (Soppe et al. 2000). Analysis of *FWA* in these mutants found no change in the DNA sequence but identified a reduction in DNA methylation in the upstream promoter region that was associated with increased transcription (Fig. 1A; Soppe et al. 2000). The methylated region of the *FWA* promoter of both the wild type and the mutant contains an insertion of a SINE transposon-like sequence that has been duplicated to generate a complex repeat structure (Lippman et al. 2004). When this repeat structure is demethylated, transcription initiates within it leading to expression of *FWA* in vegetative tissues and late flowering. Methylation is directed to these repeats by 24 nt siRNAs encoded at the locus, and these silencing RNAs are required but not sufficient to direct the methylation of the locus (Lippman et al. 2004; Chan et al. 2006). Interestingly, the SINE-like repeat structure found at *FWA* varies among *A. thaliana* accessions (Fujimoto et al. 2008). Analysis of the structure in 96 accessions revealed that three had only one of the two repeats found in the reference accession and two of these accessions exhibited higher *FWA* transcript levels in vegetative tissues than those with both repeats (Fujimoto et al. 2008). Whether these differences in expression correlate with alterations in flowering time among the accessions has not been reported. The SINE element is also found in the *FWA* promoters of related *Arabidopsis* species and is methylated, but the repeats either have different structures or are absent and this correlates with higher *FWA* expression levels (Fujimoto et al. 2008; Fujimoto et al. 2011). These observations led to the idea that the SINE element recruits methylation to the *FWA* promoter region and that subsequent duplication of parts of the SINE element increase the amount of methylation present and the extent of the repression of *FWA*. Consistent with this conclusion deletion of one of the repeats in a transgenic *FWA* copy prevented methylation (Chan et al. 2006). This result illustrates how the significance of DNA methylation in regulation of specific genes can be conserved during evolution and that the extent of methylation recruited by transposon insertions can act as a modifier of gene expression levels generating different levels of expression of a gene controlling a trait with adaptive significance.

The mechanistic observations made at *FWA* on the significance of transposon insertions in recruiting DNA methylation are likely to be more generally applicable. A related example was described in the case of sex determination in flowers of melon (Martin et al. 2009). Melon and cucumber belong to the genus *Cucumis*. Most varieties of these two species are monoecious with either unisexual flowers or both unisexual and hermaphroditic flowers (Sebastian et al. 2010). The development of unisexual flowers is believed to be adaptive, as it prevents self-fertilization and therefore promotes crossing between individuals leading to higher genetic diversity. In male flowers, the female reproductive tissues, carpels, are aborted and male reproductive organs, stamens, are formed. In melon, the production of male flowers requires the zinc finger transcription factor CmWIP1. In female flowers carrying a particular epiallele, *CmWIP1* is inactivated due to the spreading of methylation from an upstream transposon (Fig. 1B; Martin et al. 2009). Inactivation of *CmWIP1* allows carpels to develop and prevents the development of stamens. Therefore, the methylation state of *CmWIP1* contributes to the unisexual nature of melon flowers. In contrast to the behavior of induced mutants at the *CmWIP1* locus, plants carrying epialleles showed stochastic reversion of the suppression of stamen development, in particular in flowers developing on lateral branches (Martin et al. 2009).

Inactivation of a gene by methylation can be transferred to alleles on sister chromosomes or even to homologous genes on other chromsomes. Such processes can generate phenotypes that would not be caused by inactivation of a single gene copy. In paramutation, the methylated state is transferred from the allele present on one homologous chromosome to the allele present on the other chromosome (Chandler 2010). The *B1* locus that confers anthocyanin pigmentation in maize has been intensively used as a model of paramutation (Stam 2009). The methylated alleles of *B1* have many similarities to *FWA*, in that siRNAs are transcribed at repeated sequences originating from miniature inverted-repeat transposable elements (MITE) insertions in the promoter and these are required but not sufficient for methylation of the repeats through the RNA-directed DNA methylation pathway that leads to gene repression (Fig. 1C; Alleman et al. 2006; Arteaga-Vazquez et al. 2010). However, this methylated state at *B1* can be transferred from a methylated allele on one chromosome to a nonmethylated allele on the complementary chromosome whereas this does not occur at *FWA* (Soppe et al. 2000; Chan et al. 2006). Nevertheless, methylated alleles of *FWA* can transfer the methylated state to a copy of *FWA* introduced as a transgene during transformation, which occurs through the RNA-directed DNA methylation pathway and requires the presence of the repeats in the transgenic copy (Cao and Jacobsen 2002; Chan et al. 2004). Similarly, siRNAs expressed at one paralogue of the *A. thaliana FOLT1* gene induce methylation and inactivation of its paralogue on another chromosome (Durand et al. 2012). Such phenomena demonstrate communication between homologous genes in the genome apparently conferred by 24nt siRNAs,

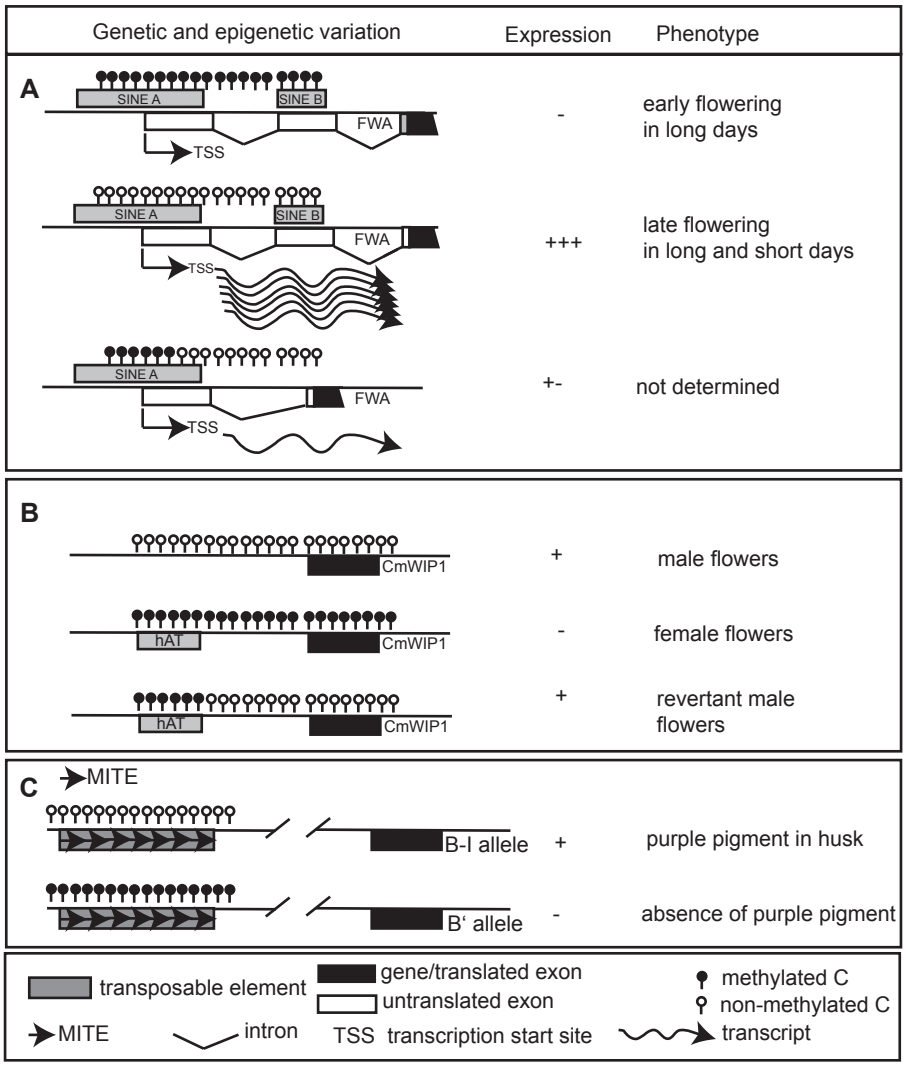

Figure 1. **Effects of methylation on genes controlling developmental traits. (A) Most *Arabidopsis thaliana* accessions carry an *FWA* locus containing two SINE transposable elements (top). The insertions overlap with the *FWA* promoter, transcriptional start site, and spliced UTR region and are methylated in all C contexts. *FWA* is expressed exclusively from the maternal copy in the endosperm where the locus is demethylated. Epigenetic mutants derived from a chemically induced screen express high levels of *FWA* mRNA in the sporophyte and are late flowering (middle). The activation of *FWA* is correlated with constitutive DNA-demethylation. Rare accessions carry a less complex locus containing a single SINE element that is expressed at low levels in the sporophyte (bottom). It has not yet been established whether expression of this *FWA* allele causes late flowering. (B) Activity of the *Cucumis melo CmWIP1* locus is required for the production of male flowers. In plants containing an active and nonmethylated *CmWIP1* gene, male flowers are produced (top). This gene is susceptible to methylation when a hAT transposable element is inserted in the promoter region (middle). In this case, *CmWIP1* is heavily methylated across the hAT element, the promoter, and genic region causing the gene to be inactivated and female flowers to be formed. In revertant male flowers, *CmWIP1* is expressed correlating with demethylation of promoter and genic regions (bottom). In contrast, the hAT element remains constitutively methylated. (C) The *Zea mays* B locus shows structural and epigenetic variation that leads to differences in husk pigmentation. Expression of B is controlled by a distal regulatory region containing a variable number of miniature inverted-repeat transposable elements (MITE) insertions. Genetic variants containing heptarepeats show epigenetic variation. The B-I allele is expressed in the husk causing intense pigmentation, whereas the B′ allele is weakly expressed causing a lack of pigmentation. In the B-I allele, the MITE repeats are not methylated (top), whereas in the B′ allele these repeats are methylated (bottom). In hybrids, B′ can convert B-I to B′, which becomes methylated and silenced. B loci with less than seven MITE repeats cannot be converted by B′.**

and this could be significant in stably maintaining the epigenetically silenced gene state in natural populations.

The phenotypes caused by methylated alleles are generally less stable than those due to classical mutations. The frequency of reversion of methylated DNA was recently measured genome-wide after taking plants through many generations of single seed descent (Becker et al. 2011; Schmitz et al. 2011). The frequency of differences in methylation at individual CG sites was much higher than had previously been observed in the same material for spontaneous changes in DNA sequence (Ossowski et al. 2010), although others were highly stable. Such stable alleles are consistent with the observations described above on *FWA*, where methylation of the promoter region has been maintained as an influence on gene expression levels for millions of years including the divergence of *Arabidopsis* species (Fujimoto et al. 2008). This stability may depend on the 24nt siRNAs encoded at the *FWA* locus (Chan et al. 2006), and in the single-seed descent experiments methylated sites encoding small RNAs did seem to be more stably methylated (Becker et al. 2011; Schmitz et al. 2011). In the *Linaria* and melon examples described above, occasional reversion events occur, and in the latter case might provide selective advantage. Instability of the methylated allele of *CmWIP1* would provide a selective advantage in dioecious varieties in the absence of male crossing partners. Such a scenario would provide an example of how the relatively high reversion rate of epialleles can provide a selective advantage.

The Role of Epigenetic Histone Modifications in Remembering or Forgetting Winter

Many plant species adapted to temperate climates must be vernalized by experiencing a prolonged period of cold before they can undergo the transition from vegetative to reproductive development. The response to vernalization requires a long-term memory that is realized by epigenetic gene regulation. For some plant species, the memory of winter must persist after a return to warmer ambient temperatures until other seasonal cues such as day-length may be perceived that trigger the reproductive transition. In contrast to the role of methylation described in the previous section, it is essential that the memory of vernalization is erased from one generation to the next thus allowing each generation to be responsive to winter. In the case of iteroparous perennial plants, which flower many times in their lives, the winter memory needs to be reset even within one generation to allow flowering to occur in response to winter each year (Albani and Coupland 2010). As discussed in this section, the epigenetic mechanisms behind the molecular memory of winter involve protein complexes that modify the tail of histone H3 by tri-methylating lysine 27 (H3K27me3). The complexes are structurally related to those involved in lysine 9 di-methylation, which were discussed in the previous section. Both histone modifications repress genes by causing a local compaction of associated chromatin. However, DNA-methylation plays no role in the more flexible epigenetic memory of winter and both, H3K9me2 and DNA-methylation, are indeed anti-correlated with the H3K27me3 modification on a genome-wide scale (Turck et al. 2007; Dong et al. 2012).

Key questions pertinent to the scope of this review are how plants have adapted their epigenetic memory of winter to accommodate differences between annual and perennial species and how, within a species, the memory adapts to differences in climate throughout the distribution range. We focus our attention on the comparison between closely related species within the Brassicacea family, where progress in understanding these issues was made recently.

COMPARING THE EXPRESSION OF *FLC* AND *PEP1*

In the annual species *A. thaliana*, vernalization leads to stable transcriptional repression of the gene encoding the MADS-domain transcription factor FLOWERING LOCUS C (FLC; Michaels and Amasino 1999; Sheldon et al. 1999). Transcriptional repression of *FLC* that occurs in cold is maintained after plants are returned to warm ambient temperatures (Fig. 2A). FLOWERING LOCUS C acts as a direct transcriptional repressor and prevents the activation of the flowering pathway integrator genes *FLOWERING LOCUS T* (*FT*) in the leaves and *SUPPRESSOR OF CONSTANS 1* (*SOC1*) in both leaves and the shoot apex (Helliwell et al. 2006; Searle et al. 2006). In the presence of high levels of FLC, *FT* and *SOC1* are insensitive to external and internal signals that normally lead to their transcriptional induction. The epigenetic state of *FLC* is reset during meiosis and seed development, so that the next generation is able to make their own experience of winter (Sheldon et al. 2008; Choi et al. 2009).

In the related perennial species *Arabis alpina*, mutations in the *PERPETUAL FLOWERING 1* (*PEP1*) gene abolish both vernalization requirement for flowering and the coordinated cessation of flowering prior to the return to vegetative growth (Wang et al. 2009). *PERPETUAL FLOWERING 1* is the *A. alpina* orthologue of *FLC* (Wang et al. 2009; Albani et al. 2012). *PERPETUAL FLOWERING 1* expression is similar to that of *FLC* before and during vernalization, but in contrast to *FLC, PEP1* expression rises to prevernalization levels within the first weeks after a return to warm temperatures (Fig. 2A; Wang et al. 2009). *Arabis alpina* forms floral meristems during vernalization. As a consequence, the postvernalization increase of *PEP1* expression does not affect flowers that were already formed during the cold phase. By contrast, young axillary meristems exposed to the cold period remain vegetative and cannot develop into inflorescence meristems (Wang et al. 2011). After a return to warm ambient temperatures,

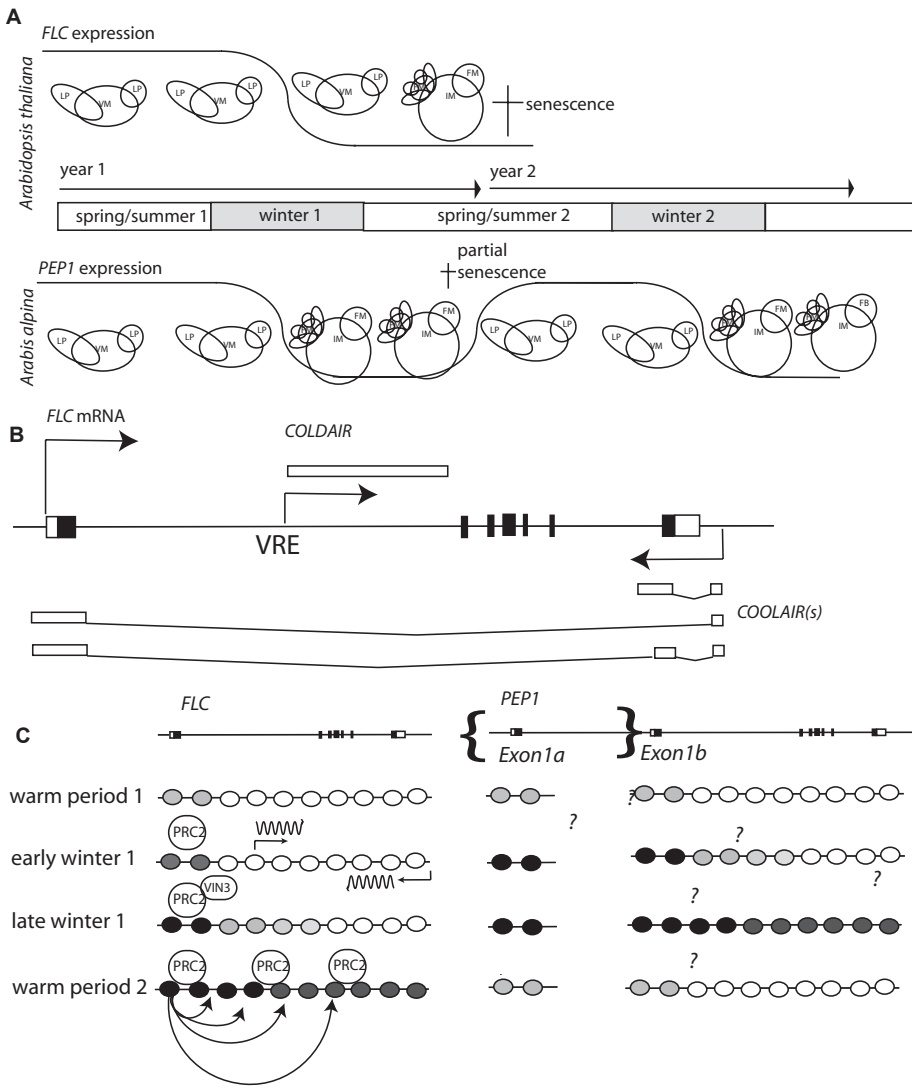

Figure 2. **The epigenetic memory of winter in *Arabidopsis thaliana* and *Arabidopsis alpina*. (A) The orthologous genes *FLC* and *PEP1* encode transcription factors that repress flowering. Their transcription is downregulated during a prolonged period of cold as experienced in winter. After the return of warmer temperatures in spring, *FLC* remains stably repressed in *A. thaliana* (top). This allows the annual plant to respond to other external cues, such as increasing day length, that promote the transition from a vegetative apical meristem (VM) to an inflorescence meristem (IM) that produces flower meristems (FM) instead of leaf primordia (LP). After completion of the reproductive phase, *A. thaliana* senesces allowing seeds to mature. In *A. alpina*, flower meristems develop during the vernalization period after the transcriptional repression of *PEP1* (bottom). These form on the shoot apical meristem and on a subset of axillary meristems. In spring, these floral meristems fully develop into flowers, set seeds, and the corresponding shoots eventually undergo senescence. Upon the return of warmer temperatures in spring, *PEP1* is upregulated, which prevents all newly formed axillary meristems from switching to the reproductive state. The nonflowering axillary shoots do not undergo senescence and support growth until the next period of cold. (B) Several noncoding transcripts are produced from the *FLC* locus. Transcripts produced from the *FLC* locus are depicted as boxes with nontranslated exonic (white boxes) and translated exonic (black boxes) regions. Transcription start sites of the *FLC* mRNA encoding the protein and the noncoding transcripts are indicated by arrows. *FLC* and *COLDAIR* are encoded by the same strand, *COOLAIR* is transcribed in antisense. (C) The kinetics of the trimethylation of histone 3 (H3K27me3) chromatin modification at *FLC* and *PEP1* during a growing cycle. Nucleosomes modified by H3K27me3 are indicated as circles, the rate of modification is indicated by a color gradient from weak (white) to medium (gray) to strong (black). The production of *COLDAIR* and *COOLAIR* in early winter is indicated (zigzagged line). Recruitment of PRC2 complexes and associated proteins (VIN3) to *FLC* (labeled circles) and the positive feedback of the nucleation region across the locus are indicated (arrows). For *PEP1*, only the dynamics of H3K27me3 but not the presence or absence of noncoding RNAs have been described (indicated by ?). *PEP1* is transcribed from two independent promoters producing two mostly identical versions of Exon1. The chromatin state cannot be resolved between Exon1a and Exon1b for regions which the highest sequence identity (indicated by ?).**

they are prevented from switching to the reproductive state by the presence of PEP1. These meristems support subsequent vegetative growth until the next round of vernalization is completed (Fig. 2A).

Thus, the irreversible *FLC* repression contributes to the annual life cycle, whereas the reversible *PEP1* repression is associated with a perennial life cycle. What are the regulatory components controlling *FLC* and *PEP1* expression and which of these have diverged during evolution to confer distinctive molecular memories to both genes? To suggest answers to this question, we must first consider molecular details known in the genetic model *A. thaliana* and integrate knowledge from studies of related processes in animals, assuming that the general principles of the underlying epigenetic pathway are conserved between the kingdoms.

THE MOLECULAR MECHANISM OF THE EPIGENETIC MEMORY OF WINTER

The molecular response of *FLC* to prolonged cold can be subdivided into different physiological phases that have distinct genetic requirements. After transfer of seedlings to cold, *FLC* expression is gradually downregulated over a period of weeks (Michaels and Amasino 1999; Sheldon et al. 1999). If the period of cold is not sufficiently long to confer full vernalization, repression reverses once plants are returned to warm temperatures (Angel et al. 2011). In contrast, once vernalization is completed, *FLC* repression is maintained throughout the remaining life of the plant in all meristems and newly developing organs. During the initial downregulation, two classes of noncoding RNAs are transcribed from the locus in transient consecutive waves (Fig. 2B). First, several splice variants of antisense RNA initiated from a promoter located in the 3'-flanking region of the *FLC* locus are produced, collectively called *COOLAIR* transcripts (Swiezewski et al. 2009). Their functional requirement for *FLC* downregulation is still unclear because mutants in which *COOLAIR* is not expressed retain the ability to repress *FLC* under laboratory conditions (Swiezewski et al. 2009; Helliwell et al. 2011). Following *COOLAIR* expression, the next wave of noncoding RNA expression produces *COLDAIR,* a noncoding RNA transcribed from the second half of the first *FLC* intron (Heo and Sung 2011). *COLDAIR* associates with Polycomb repressive complexes 2 (PRC2) in vitro and could thus participate in targeting this complex, which catalyzes the H3K27me3 modification, to the *FLC* locus (Heo and Sung 2011). Activity of a PRC2 complex containing the VERNALIZATION 2 protein (VRN2-PRC2) is required to reach a point of irreversible *FLC* repression (Gendall et al. 2001). In addition, several members of the VIN3-like (VIL) protein family, which interact with PRC2 complexes to increase their activity to form H3K27me3, are required to reach a fully memorized *FLC* repression (Sung and Amasino 2004; Wood et al. 2006; Greb et al. 2007; De Lucia et al. 2008). Of these partially redundant *VIL*s, *VIN3* has the distinguishing attribute of being transcriptionally upregulated late during the cold phase, which corresponds to the time when *FLC* repression becomes irreversible (Sung and Amasino 2004; Greb et al. 2007). Once plants are returned to warmer ambient temperatures, *VIN3* transcripts rapidly decrease to prevernalization levels (Sung and Amasino 2004).

Interestingly, the increase in H3K27me3 levels at *FLC* during the cold is relatively small and first restricted to a nucleation region located around the transcriptional start site (Fig. 2C). Prolonged vernalization leads to a slight increase in the abundance of the modification across the locus. However, the most general increase in H3K27me3 across the entire transcribed region is observed after a return to warm temperatures once plants have been fully vernalized (Finnegan and Dennis 2007; Angel et al. 2011). During this phase, plant growth resumes rapidly resulting in increased rates of cell division. The increase in the H3K27me3 level is somewhat counter intuitive because histone modifications are thought to be diluted during DNA replication, similar to the DNA-methylation discussed earlier in this review. This signifies that an active mechanism of maintenance must be in place to circumvent loss of chromatin-encoded information. Strong activity of such maintenance mechanisms at the *FLC* locus may explain a net increase in chromatin modification and spreading of modifications from their initial nucleation site (Barrero et al. 2007; Hyun et al. 2013).

For *FLC*, the postvernalization spreading of H3K27me3 is strongly correlated with stable repression (Sheldon et al. 2008; Angel et al. 2011). In contrast, such a correlation is not observed for the *PEP1* locus of *A. alpina*. At the *PEP1* locus, H3K27me3 levels increase detectably across the entire locus both in leaves and apical–meristem enriched tissue (Wang et al. 2009). It is not quite clear if this difference reflects an important difference in H3K27me3 nucleation or is explained by experimental differences because H3K27me3 distribution was detected at the end of the required vernalization period, which lasts longer in *A. alpina* than in *A. thaliana*. In fact, *A. thaliana FLC* also shows an increase in histone methylation across the locus and spreading of H3K27me3 if exposed to cold for over 4 weeks (Angel et al. 2011). However, the differences between the species are obvious after a return to warm temperatures. At this stage, H3K27me3 levels decrease across the *PEP1* locus, correlated with the rise in *PEP1* expression, whereas H3K27me3 levels increase at *FLC*, which remains stably repressed (Fig. 2C; Wang et al. 2009).

Despite the apparent stability of the repressed state at *FLC*, it is important to realize that repression is the outcome of a dynamic equilibrium between antagonistic activities that promote either a repressed or a more open transcriptionally competent state.

In addition to the dilution of modifications during cell divisions, mentioned above, a continuous high turnover rate of nucleosomes results in a local exchange of those carrying modified histones with freshly synthesized nucleosomes that are unmodified (Deal et al. 2010). This turnover creates gaps in the repressive armament of H3K37me3 target loci, which can provide stochastic windows of opportunity for the binding of transcriptional activators. To maintain a repressed state, H3K27me3 is engaged in a positive feedback loop by directly recruiting PRC2 complexes (Hansen and Helin 2009). The H3K27me3 modification has its nemesis in so-called active chromatin marks that are correlated with gene expression. These histone modifications can passively prevent the deposition of repressive histone marks by interfering with the catalytic activity of the PRC2 (Schmitges et al. 2011). In addition, active chromatin marks could recruit enzymes capable of removing the H3K27me3 modification such as REPRESSOR OF FLOWERING 6 (REF6), a jumonji-domain protein capable of demethylating H3K27me3 that is involved in *FLC* regulation (Lu et al. 2011).

Mathematical modeling helps to understand how the dynamics of positive and negative feedback may contribute to either stable or unstable memory (Dodd et al. 2007; Angel et al. 2011; Satake and Iwasa 2012). Modeling simplifies the complicated interplay of histone modifications to three possible states corresponding to repressive, active, or neutral modifications for each nucleosome. To reach bistability in simulations, each repressive or active nucleosome must be allowed to interact without spatial constraint with all other nucleosomes within the repressed region. The cross-talk is modeled as positive feedback between histone marks of the same nature and negative feedback loops to the opposing state. The mathematical approaches show that to be bistable, the system must have a minimal size in the model, which in practical terms corresponds to a minimal gene length. Modeling also confirmed that a local perturbation of the equilibrium corresponding to the small region of increased H3K27me3 occurrence during the cold at *FLC* is sufficient to eventually flip the epigenetic state of the entire locus under the condition that its increased modification rate persist after the return to warmer temperatures (Angel et al. 2011). A second modeling approach simulated how differences in the strength of positive and negative feedback can explain the differences in epigenetic stability for *FLC* and *PEP1* (Satake and Iwasa 2012).

Taken together, available data argue that differences between *FLC* and *PEP1* are less likely to concern their mode of repression during the cold, which is rather similar, but rather molecular events that take place after vernalization. Thus, the two noncoding RNAs described in *A. thaliana*, which are thought to be involved in the early downregulation of *FLC* but not in the maintenance of its repression, are unlikely candidates for the observed differences in epigenetic stability. Strong candidates for relevant changes are mutations resulting in an altered activity of protein complexes that either maintain or antagonize H3K27me3 levels during cell divisions. Modifications of these activities would fit both possible scenarios where either the more stable allele is derived from the unstable or *vice versa*. The presence of a transcriptional activator required for reversing the epigenetic repression in *A. alpina* and its absence in *A. thaliana* could also explain the observed epigenetic differences. The mathematical models suggest that a reduction of gene length or the insertion of sequences that prohibit free cross-talk across the locus may destabilize the epigenetic memory whereas an increase in gene length may have the opposite effect.

STRUCTURAL DIFFERENCES BETWEEN *FLC* AND *PEP1*

Interestingly, the *PEP1* locus shows potential for greater regulatory complexity than *FLC* due to a partial tandem duplication of the gene. This duplication resulted in the formation of two independent promoters from which two versions of *PEP1* exon1 are transcribed (Fig. 2C). Either first exon can be combined with downstream exons and both resulting transcripts encode functional PEP1 protein (Albani et al. 2012). Extensive structural variation is observed among *A. alpina* accessions that possess a partially duplicated *PEP1* and some accessions lack the duplicated exon 1. This high diversification is indicative of a dynamic rate of evolution but it is as yet unclear whether this is due to adaptive selection or neutral evolution in particular because the characterized alleles originated from *A. alpina* accessions that have lost their obligate vernalization requirement because they produce nonfunctional PEP1 proteins (Albani et al. 2012).

Arguments for a duplication event being a relevant step in creating annual/perennial diversity are the presence of similar tandem arrays of *FLC* orthologs in *Arabidopsis arenosa* and *Arabidopsis lyrata* both species being perennials and closely related to *A. thaliana* (Nah and Chen 2010). In such a scenario, an ancestral duplication event at the base of Brassicaceae associated with perennial life history could have been modified by partial deletions that contributed to local adaptation by fine-tuning the molecular memory. However, the absence of single nucleotide polymorphisms in the coding region and the proximal part of both copies of intron 1 of *A. alpina* argues for a relatively young duplication event. Notably, tandem duplications occur relatively frequently as has been shown by comparing the evolutionary distance of gene paralogs in several plant species. They seem to be the most frequent causes of gene birth but are also frequently purged from the genome (Haberer et al. 2004; Moore and Purugganan 2005). Interestingly, tandem duplicated genes are more likely targets of the H3K27me3 modification than single copy or segmentally duplicated genes, which suggests that there might be a connection between the epigenetic pathway and genome evolution (Turck et al. 2007).

CAN VARIATION IN THE *FLC* RESPONSE TO VERNALIZATION EXPLAIN THE TRANSITION FROM LONG- TO SHORT-TERM MEMORY?

Only *A. thaliana* accessions that contain an active *FRIGIDA (FRI)* allele express high levels of *FLC* (Michaels and Amasino 1999; Johanson et al. 2000). Mutations leading to loss of *FRI* or *FLC* function have occurred several times independently in *A. thaliana* accessions, indicating that the loss of vernalization requirement confers an adaptive advantage for particular habitats (el-Assal et al. 2004; Le Corre 2005; Salome et al. 2011; Strange et al. 2011). These mutations are caused by changes in DNA sequence caused either by conventional single nucleotide polymorphisms, small indels, or disruptive transposable element insertions. Natural variation of the vernalization response has also been reported in *A. thaliana* accessions that require vernalization for both the minimum length of the vernalization period for full irreversibility of *FLC* repression and the effective temperature optimum for vernalization (Shindo et al. 2006; Wollenberg and Amasino 2012). These differences are likely to correspond to adaptive changes that optimize the vernalization response to differences in local climate.

Of particular interest is a comparative study between the reference Columbia and the Lov-1 accession from northern Sweden, which require 4 and 9 weeks of exposure to cold for full vernalization, respectively. For these two accessions, the causal differences were mapped to a region roughly corresponding to the H3K27me3 nucleation site (Coustham et al. 2012). In Lov-1 *FLC*, the H3K27me3 levels across the locus were reduced before vernalization and the increase of H3K27me3 was delayed during the exposure to cold. The region that determines the differences between Col and Lov-1 corresponds to the region duplicated in the *PEP1* locus, which shows H3K27me3 dynamics somewhat similar to Lov-1 (Fig. 2C; Wang et al. 2009; Albani et al. 2012). However, although this confirms that the nucleation region of *FLC* and *PEP1* is crucial for epigenetic regulation, it also underscores the fact that a more dynamic memory can be achieved without a duplication of this important region.

EVOLUTION OF EPIGENETIC REGULATION OF VERNALIZATION RESPONSE

Within the Brassicaceae family, a transition from an ancestral, perennial to a derived annual behavior has occurred several times in independent phylogenetically related clades (Nasrallah 2000; Clauss and Koch 2006; Koch et al. 2006; Karl et al. 2012). Thus, changes in epigenetic regulation of *FLC* orthologues must have occurred frequently in short evolutionary times, which suggests that either the same underlying changes occur with high frequency or that several alternative routes achieve the same phenotypic distinction between annuals and perennials. Given the number of candidate genes that may affect the stability of epigenetic gene repression in *trans*, it is possible that several independent changes have had a similar effect within the Brassicaceae family. In addition, alterations of the *FLC* and *PEP1* gene structure in *cis* may also affect the epigenetic memory. For such structural changes, the H3K27me3 nucleation region emerges as the strongest candidate for causative changes in the epigenetic memory.

Perspectives: Significance of Epigenetic Regulation of Developmental Traits in Adaptation

Since the discovery of epigenetic gene regulation, the possibility of partially reversible "soft" inheritance has inspired several hypotheses for how the underlying mechanisms could be involved in the evolution of adaptive traits (Kalisz and Purugganan 2004; Jablonka and Lamb 2005; Feinberg and Irizarry 2010). Important attributes of epigenetic variation that are frequently discussed are its partial reversibility, greater diversity of gene expression levels and accumulation of hidden variation that could be released in particular environmental conditions. However, documented cases of variation in development under epigenetic control are still relatively rare and in no case has a rigorous study of the adaptive value of the epigenetic trait been conducted. Distinguishing whether a developmental change is persistently maintained as an epigenetic trait because the reversibility per se represents an adaptive advantage or whether epigenetically controlled gene inactivation precedes genetic fixation by mutation will be required.

Indeed, most major effect epialleles seem stable and variation is only revealed in mutant backgrounds or after extreme stress, which appears to argue against the idea that stochastic reversibility contributes to greater phenotypic variation within a population. Possibly, closer examination will reveal more quantitative reversibility of epigenetic traits or instability may be more evident over longer, evolutionary time scales. Similarly, performing more studies outside standardized laboratory conditions might reveal the higher variability associated with epigenetic modifications (Roux et al. 2011). Even in greenhouse conditions, epigenetic variation at anonymous sites exists both among populations and among sibling offspring within the same population, but so far no phenotypic developmental variation has been linked to it (Becker et al. 2011; Schmitz et al. 2011). Nevertheless, variation in DNA methylation clearly causes differences in gene expression in natural populations, and there are indications that this may be more prevalent at genes that recently originated de novo (Bortlini Silveira et al. 2013).

Plants carrying mutations that impair major epigenetic pathways may increase the effect of epigenetic variation on plant phenotypes. Arabidopsis mutants affected in the maintenance of DNA methylation produced progeny exhibiting many diverse

potentially adaptive developmental phenotypes such as altered flowering time or leaf shape (Kakutani 1997; Johannes et al. 2009; Roux et al. 2011; Zhang et al. 2013; Wang et al. 2013). However, in most cases, these were shown to be genetically linked to de novo transposable element insertions that were promoted in the mutants (Ito et al. 2011; Paszkowski and Grossniklaus 2011; Weigel and Colot 2012). Thus, a striking effect of defects in epigenetic regulation is to increase the frequency of classical Mendelian mutations, indicating an indirect mechanism by which epigenetic changes could produce adaptive variation in developmental phenotypes.

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