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Sweet memories: epigenetic control in flowering

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Abstract

Many plants respond to winter with epigenetic factors that gradually dampen repression of flowering so that they can flower in spring. The study of this process was important for the identification of the plant Polycomb group (PcG) of proteins and their role in the epigenetic control of plant gene expression. Fittingly, these studies continue to illuminate our understanding of PcG function. We discuss recent advances, particularly the role of noncoding RNA in the recruitment of PcG to target genes, and the role of the PcG in regulating the stem cell pool in flowers.

Introduction and context

Many plants that grow in climates with a cold winter require growth for several months at low temperatures—a process called vernalization—to promote flowering in spring, when days lengthen and temperatures increase. Without this period of cold, plants would grow leaves in the spring, but would fail to flower. It was noticed that this phenomenon, familiar to every horticulturist, had distinctive features; something occurred during those cold months that left a mark, which, in effect, released a switch that permitted flowering in spring. Experimental manipulation of temperature showed that this mark was very stable and could persist for at least a year after a vernalization treatment [1]. Although stable during a plant's life, the marks were erased between generations, as the progeny of vernalized plants themselves required vernalization in order to flower. In recent years, the field has looked beyond the genome and tested whether epigenetic changes (i.e., ones that aren't based on DNA sequence alteration) were involved. One epigenetic system involves DNA methylation, and in plants this is often used for genome defense; for example, to inactivate invading DNA such as transposons, viruses, and transgenes.

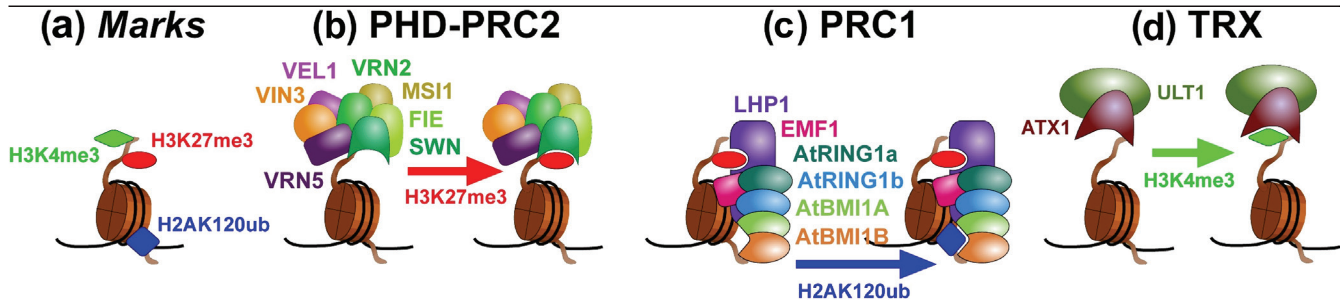
A second epigenetic system is implemented by two collections of genes known as the Polycomb group (PcG) and the trithorax group (trxG). Again, methylation is involved, but in this case it is not the DNA itself that is modified, it is the histone proteins that package DNA in the nucleus (Figure 1a). Unlike the changes wrought by DNA methylation, there is (so far) little clear-cut evidence that PcG- or trxG-mediated changes are passed between generations in plants. Rather, they are used to provide cells with memories of events that occur during the life of an organism. Typically this is a developmental memory, hence the PcG and trxG components were first identified on account of gross developmental abnormalities resulting from their mutation. However, they are also used to record transient environmental events that occur during the life of the plant. This can be used to predict and adapt to future environments and may be especially relevant in plants, which are sessile and so can't easily escape their circumstances. Here, we discuss important advances in our understanding of how PcG genes provide a memory of winter. Secondly, we describe the emerging role of PcG and trxG genes in controlling stem cell fate in flowers. This review is of necessity selective; for more comprehensive reviews see [2] or [3].

Recent advances

Vernalization and flowering time

The mechanism of the vernalization response has been best studied in the model plant *Arabidopsis thaliana*, where it has been found to depend on the ability of the PcG system to silence the key gene mediating the

Figure 1. PcG and trxG proteins epigenetically control flowering and flower development



(a) Histone modifications 'mark' nucleosomes at specific genes. The nucleosome is an octamer containing two molecules each of histone H2A, H2B, H3, and H4. For simplicity, only one of each of the two H2A and H3 tails are shown in the figure. H2AK120 resides on an exposed surface of the nucleosome core. **(b,c,d)** Likely components and functions of *Arabidopsis* PcG and trxG protein complexes equivalent to animal PRC2, PRC1, and TRX complexes are shown. **(b)** During vernalization in *Arabidopsis*, the PHD-PRC2 complex catalyzes H3K27me3 methylation through the SWN histone methyltransferase subunit. VIN3, VEL1, and VRN5 are plant-specific, whereas the other four members are homologs of the animal PRC2 core components. Because three of the four core components of animal PRC2 have been duplicated in *Arabidopsis*, it is likely that several related complexes exist that differ in components and target gene specificities [2]. For example, in some complexes, CLF may replace SWN as the histone methyltransferase unit. **(c)** LHP1, a component of *Arabidopsis* PRC1, binds H3K27me3. PRC1 may catalyze H2AK120Ub ubiquitination via its E3 ligase components AtRING1a, AtRING1b, AtBMI1A, and AtBMI1B. EMF1 is likely another (plant-specific) component whose precise function is unclear. **(d)** ULT1 and ATX1 may be components of an *Arabidopsis* trxG complex that catalyses H3K4me3. ATX1, ARABIDOPSIS HOMOLOG OF TRITHORAX 1; CLF, CURLY LEAF; EMF1, EMBRYONIC FLOWER 1; FIE, FERTILIZATION INDEPENDENT ENDOSPERM; LHP1, LIKE HETEROCHROMATIN PROTEIN 1; MSI1, MULTICOPY SUPPRESSOR OF IRA 1; PcG, Polycomb group; PHD-PRC2, PLANT HOMEO DOMAIN–POLYCOMB REPRESSIVE COMPLEX 2; PRC1, POLYCOMB REPRESSIVE COMPLEX 1; SWN, SWINGER; trxG, trithorax group; ULT1, ULTRAPETALA1; VEL1, VERNALIZATION5/VIN3-LIKE 1; VIN3, VERNALIZATION INSENSITIVE 3; VRN2, VERNALIZATION2; VRN5, VERNALIZATION5.

vernalization response, *FLOWERING LOCUS C* (*FLC*). *FLC* is a potent repressor of flowering, most likely because its protein product binds and represses two genes, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which are central players in promoting flowering [4]. Thus in order for plants to become competent to respond to environmental factors promoting flowering (warm temperatures and long days), *FLC* must be switched off. During cold treatments, expression of *FLC* is progressively reduced and a vernalization-specific complex of PcG proteins (termed PHD-PRC2, which stands for 'Plant Homeo Domain–Polycomb Repressive Complex 2') (Figure 1b) is recruited to *FLC*. Biochemical purification of this complex [5] indicates that it contains the plant equivalents of the four core components of an animal PcG protein complex known as PRC2 (Polycomb repressive complex 2).

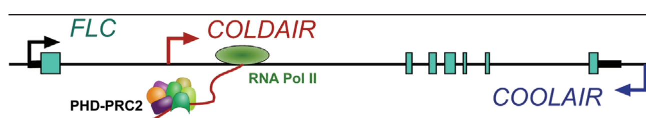
Recently, its function has become clearer. PRC2 acts as a histone methyltransferase, specifically catalyzing trimethylation of lysine 27 on the histone H3 tail (H3K27me3). In addition to the core PRC2 components, PHD-PRC2 contains several plant-specific components that are probably required to boost the histone methyltransferase activity of the complex. Consistent with this, H3K27me3 levels at *FLC* increase during cold treatments and persist afterwards when plants are moved to warm conditions. H3K27me3 is also necessary for maintenance of *FLC* silencing when plants are removed from the cold.

How then does H3K27me3 cause gene silencing? Although it is necessary for silencing of *FLC*, it is not sufficient: in *vernalization1* (*vrn1*) mutants, *FLC* activity is restored after vernalization, despite the persistence of high H3K27 methylation levels [6]. H3K27me3 is not thought to directly cause chromatin compaction or inhibit transcription but is probably recognized by proteins that repress transcription of *FLC*. In animals, a second complex of PcG proteins, called PRC1, is also required for PcG-mediated silencing. Although the PRC1 complex is less well conserved than PRC2, an important recent finding is that several of its members are found in plants and play a similar role in PcG-mediated repression. One PRC1 member, POLYCOMB, binds H3K27me3, and in *Arabidopsis* the related LIKE HETEROCHROMATIN 1 (LHP1) protein has a similar function (Figure 1c) [7]. It is also likely that PRC2 itself binds H3K27me3, which may help in the stable propagation of this mark through cell division, by methylating newly synthesized (unmodified) histones as they are deposited into chromatin, for example [8,9]. Three other components of PRC1 in animals, RING1A, RING1B, and BMI1, form a ubiquitin ligase complex that monoubiquitinates histone H2A at lysine 119 (H2AK119Ub). The function of this modification is somewhat enigmatic. Although H2AK119Ub may help repress transcription directly by blocking RNA polymerase

II transcriptional elongation from the promoters of PcG target genes [10,11], its importance is challenged by the observation that PRC1 can cause chromatin compaction and transcriptional repression independently of its H2A ubiquitination activity [12]. Be this as it may, two groups have now identified *Arabidopsis* homologs of *RING1A*, *RING1B*, and *BMI1* (Figure 1c) and the similarity of their knockout phenotypes with those of mutants inactivating PRC2 is compelling evidence that they have a similar role to animal PRC1 [13,14]. In addition, the plant RING proteins can monoubiquitinate H2AK120 (the residue in plants that most closely corresponds to the animal H2AK119, although most of the 13 *Arabidopsis* H2A genes don't encode this residue) in in-vitro assays and are required for H2A ubiquitination in vivo in *Arabidopsis* [13]. Although so far only a handful of PcG targets have been shown to be misregulated in the *Arabidopsis atring/atbmi1* mutants, it will obviously be interesting to see if the *Arabidopsis ATRING/ATBMI* genes have any role in the vernalization-induced repression of *FLC*.

Because the PcG components are mostly expressed fairly constitutively and seemingly lack specific DNA-binding activity, a major problem has been to explain how they are recruited to specific targets such as *FLC*. Numerous recent studies in animals have shown that noncoding RNA play a role in recruitment [3], and two noncoding RNAs have now been implicated in vernalization-induced recruitment of PHD-PRC2. One of these, termed COLDAIR, is a long (about 1 kb) noncoding sense RNA produced from sequences within the large first intron of *FLC* [15] (Figure 2). Expression of COLDAIR is cold-induced and several observations suggest that it is functionally important for *FLC* silencing. Firstly, two earlier studies using transgene reporters showed that sequences within the large first intron of *FLC* are needed for its stable silencing by cold [16,17]. In particular, transgenes with deletions that removed part or all of the COLDAIR gene showed fairly normal downregulation during cold treatments but regained activity when plants were returned to warm conditions. Secondly, several approaches showed that CURLY LEAF (CLF), the catalytic subunit of the PHD-PRC2 complex, binds COLDAIR RNA [15]. This may help recruit PHD-PRC2 to *FLC*; for example, if the COLDAIR transcript remains tethered to its site of transcription at *FLC* (Figure 2) or alternatively if the affinity of PHD-PRC2 for chromatin is altered by its binding COLDAIR. Consistent with this, downregulation of COLDAIR using RNA interference strongly reduces the recruitment of CLF to *FLC* and also impairs the maintenance of *FLC* silencing following cold treatments. A second noncoding RNA, termed COOLAIR, is also produced from *FLC* [18]. Like COLDAIR, COOLAIR expression is also strongly upregulated by cold temperatures. COOLAIR expression is driven by a promoter located downstream of the *FLC* coding region (Figure 2) and produces several antisense transcripts that differ according to their splicing or 3′ end. There is good evidence that COOLAIR also plays a role in regulating *FLC* expression. Two genes, *FPA* and *FCA*, promote flowering by downregulating *FLC* expression. Although *FPA* and *FCA* encode proteins that regulate mRNA processing, until recently it was problematic that they had no discernable effect on *FLC* mRNA processing. Two groups have now resolved this issue by demonstrating that they regulate the processing and relative amounts of the different COOLAIR transcripts [19,20]. However, *FPA* and *FCA* are not required for the vernalization response, and the function of COOLAIR in vernalization is less clear. Notably, *FLC* transgenes that lack sequences downstream of FLC that include the COOLAIR promoter retain a normal vernalization response [16]. Although this suggests that COOLAIR may not be necessary, interpretation is complicated because these plants retain an endogenous *FLC* gene that produces COOLAIR transcripts that could act in trans on the *FLC* transgene. It is also noteworthy that COLDAIR is not sufficient for a vernalization response; thus, the introduction of *FLC* intragenic sequences that contain COLDAIR into a heterologous reporter did not confer a vernalization response on the reporter [16]. One possibility, suggested by Heo and Sung [15], is that induction of COOLAIR by cold may reduce transcription from the normal promoter 5′ end of the *FLC* protein coding region and promote COLDAIR expression from its cryptic promoter within the first *FLC* intron.

Figure 2. The *FLC* locus produces at least two noncoding RNAs



The open reading frame of *FLC* is shown in turquoise, with exons depicted as green boxes and introns as black lines. The antisense *COOLAIR* RNA is upregulated early after cold treatment. However, only the *COLDAIR* transcript is thought to be bound by PHD-PRC2 and so may be more important in recruiting PHD-PRC2 to the *FLC* locus. FLC, FLOWERING LOCUS C; PHD-PRC2, PLANT HOMEO DOMAIN–POLYCOMB REPRESSIVE COMPLEX 2; RNA Pol II, RNA polymerase II.

Although many plants show a vernalization response with epigenetic features, the target genes and even the mechanisms may be different than those in *Arabidopsis*. In sugar beet, vernalization results in stable repression of a floral repressor, which, although it is unrelated to *FLC*, acts in a similar fashion to repress an *FT* homolog [21]. It is not clear yet if this is also PcG mediated. In cereals, vernalization causes the stable upregulation of *VRN1*,

which promotes flowering. Here, the epigenetic memory is of an active state, and is correlated with H3K4me3 methylation at *VRN1* [22]. It is likely, although not yet proven, that this will involve the trxG genes as these are known to catalyze H3K4me3 methylation and mediate stable gene activation.

Shaping floral architecture: stemming the stem cell pool

Once flower induction occurs, the indeterminate vegetative shoot apical meristem at the shoot apex changes its identity to become an inflorescence meristem, which makes floral meristems on its flanks. The floral meristem produces whorls of floral organ primordia, and so gives rise to the flowers. Like all meristems, the continued production of organ primordia requires the activity of a pool of stem cells in the centre of the meristem. Unlike the other meristems, the floral meristem is determinate so that organ production ceases once the carpels are initiated in the innermost whorl of the flower. Mutations in PcG and trxG genes are well known to cause changes in floral organ identity, reflecting their role in controlling floral homeotic gene activity. However, a second less well emphasized aspect of their mutant phenotype is an increase in floral organ number. This reflects an emerging role in controlling when the flower stem cell pool is terminated.

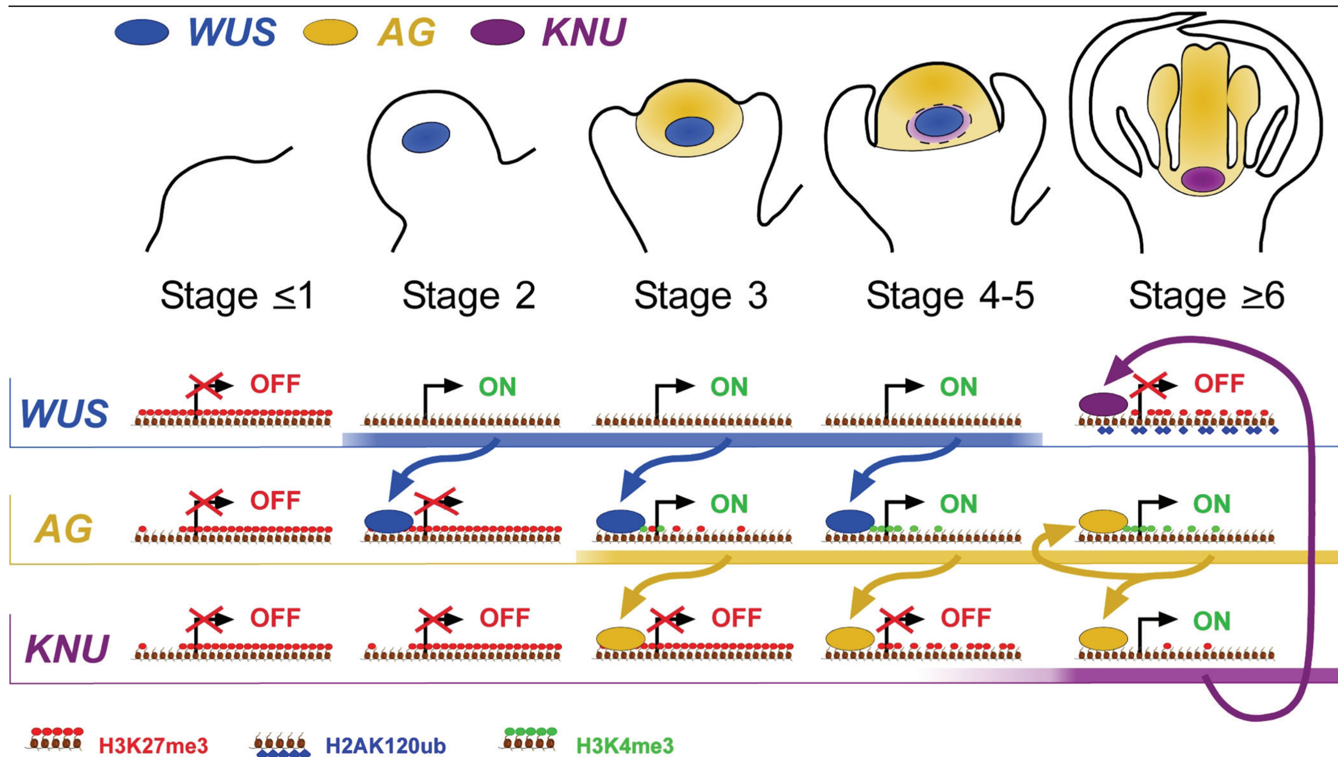
In *Arabidopsis* shoots, the stem cells at the top of the meristem secrete the CLAVATA3 (CLV3) signal peptide, which restricts the expression of the transcription factor WUSCHEL (WUS) in the organizing centre beneath the stem cells. In turn, *WUS* promotes stem cell fate and as a result also promotes *CLV3* expression non-cell-autonomously in cells above the organizing centre. This *CLV3*/*WUS* feedback loop mediates homeostasis of the stem cells via a rapid response to fluctuations in either *CLV3* or *WUS* expression [23-25].

However, flowers are determinate structures and as a consequence, a second feedback loop terminates the stem cell pool of floral meristems. In contrast to the fast and continuously operating *CLV3*/*WUS* system, this feedback loop, built by WUS and the MADS-box transcription factor AGAMOUS (AG), occurs just once. Early in flower development (stage 3), WUS and another transcription factor, LEAFY, together activate expression of *AG* in the centre of the floral meristem. In turn, AG represses *WUS* expression but there is a delay of about two days before *WUS* expression terminates at stage 6 [26,27]. In addition, the first step (the activation of *AG* expression by WUS) also exhibits a delay: *WUS* expression occurs during floral stages 2-5. By contrast, *AG* is not detectable in floral meristem until stage 3. The reason for these delays has been unclear but two recent publications [28,29] reveal the relevance of epigenetic regulation by the PcG and trxG (Figure 3).

Carles and Fletcher [28] investigated whether the activation of *AG* is dependent on the *Arabidopsis* SAND (Sp100, AIRE-1, NucP41/75, DEAF-1) domain protein ULTRAPETALA1 (ULT1) and furthermore, whether *ULT1* is involved in trxG function. The authors were inspired by the gain-of-function phenotypes of *35S::ULT1* transgenic plants, which resemble *clf* mutants in showing increased *AG* expression in leaves and inflorescences [28,30]. Furthermore, *ult1* mutants suppress the *clf* mutant phenotype in *ult1 clf* double mutants and show reduced *AG* expression [28]. This fits with idea that ULT1 is a trxG factor and works antagonistically to the PcG to promote *AG* activity, and may also explain why *WUS* is misexpressed in *ult1* mutants [30]. Consistent with this antagonism, *35S::ULT1* seedlings, similar to *clf* mutants, have reduced levels of the repressive mark H3K27me3 at *AG*, whereas *ult1* mutants have increased H3K27me3 levels. The structure of ULT1 makes it unlikely that it is itself an H3K4me3 methyltransferase, rather it may interact with such an enzyme as part of a trxG complex or promote trxG recruitment to targets. Consistent with this, ULT1 interacts physically with the trxG protein ARABIDOPSIS HOMOLOG OF TRITHORAX 1 (ATX1), which is responsible for local H3K4me3 deposition (Figure 1d) [28,32]. ATX1 cannot be the only factor for *ULT1*-dependent H3K4me3 at the *AG* locus because *atx1* mutants, unlike *ult1* mutants, do not show indeterminacy of floral meristems [28] and also retain 85% of global H3K4me3 levels [33]. Furthermore, although the *ULT1*-dependent activation of *AG* by a slow increase of H3K4me3 may provide a time buffer that the transcription factor WUS on its own may not provide, it remains unclear whether *ULT1* and *WUS* work in parallel or in the same pathway.

Intriguingly, the delay in the second step of the *AG*/*WUS* feedback loop—the final suppression of *WUS* by *AG*—may also involve epigenetic control. Sun et al. [29] identified *KNUCKELS* (*KNU*), which encodes a C2H2-type zinc finger transcription factor, as a direct target of *AG* and a key intermediate in the suppression of *WUS* expression. Genetic analysis and overexpression experiments imply that *KNU* is activated by *AG* and represses *WUS* expression [29]. Curiously, although *AG* binds the *KNU* promoter directly, there is a two day lag between the activation of *AG* and that of *KNU*. This time, buffering may be caused by PcG regulation of *KNU*. Several observations indicate that *KNU* is a PcG target: first, early in floral meristem development the *KNU* locus is strongly decorated with H3K27me3; second, in various PcG mutants *KNU* is misexpressed. Using an elegant system for synchronizing

Figure 3. Mechanistic model for transcriptional and epigenetic control of the floral meristem stem cell pool



Serial floral stages with the expression of *WUS*, *AG*, and *KNU* are shown. Underneath, the genomic regions of these genes are shown schematically with likely binding of transcription factors, expression status, and histone modifications. At the initial stage of flower development (stage ≤1), the *KNU* locus is covered by the repressive mark H3K27me3. *WUS* is activated at stage 2, but the transcriptional activation of *AG* by WUS is inhibited by H3K27me3. At stage 3, the *AG* locus undergoes activation by progressive ULT1/ATX1-dependent H3K4me3 and likely demethylation of H3K27. WUS binds in the large second intron of *AG. AG* expression is maintained by autoactivation as well as by H3K4me3. *WUS* expression terminates at stage 6. The activation of *KNU* by AG is initially inhibited by H3K27me3 at the *KNU* locus between stages 3 to 5. The level of H3K27me3 at the *KNU* locus is gradually depleted, perhaps by dilution via successive rounds of cell division, and at stage 6 *KNU* is expressed, causing *WUS* repression. The punctual termination of *WUS* expression needs additional PRC1 activity, which suggests that the *WUS* locus accumulates H3K27me3 and H2AK120Ub. AG, AGAMOUS; ATX1, ARABIDOPSIS HOMOLOG OF TRITHORAX 1; KNU, KNUCKELS; ULT1, ULTRAPETALA1; WUS, WUSCHEL.

floral development, Sun et al. show that between floral development stage 3, when *AG* is activated, and stage 6, when *KNU* is activated and *WUS* expression terminated, H3K27me3 is gradually removed from the *KNU* locus. But how can the MADS domain transcription factor AG cause histone demethylation? One possibility is that *AG* recruits H3K27me3 demethylases, which have been identified in animals but not yet in plants. A simple alternative is that *AG* somehow inhibits the binding of PRC1 and PRC2 complexes to the *KNU* locus and the H3K27me3 mark is gradually diluted as unmodified histones are incorporated onto replicated DNA during cell division [34]. Either way, the H3K27me3 at *KNU* is not sufficient to prevent *KNU* activation by *AG* but does delay it. Similarly, two other recent studies that looked at the activation of another PcG target, in this case by cold, also conclude that H3K27me3 is important for slowing the kinetics of induction [35,36]. Although the tardy induction of *KNU* in flowers suggests a mechanism for the delayed suppression of *WUS* by *AG*, it cannot be the only factor that terminates *WUS* expression. Thus, *knu* mutants have much weaker effects on floral determinacy than *ag* mutants, leaving space for other *AG*-dependent mechanisms. As *WUS* is itself a target of H3K27me3 [37], it is also possible that its repression by KNU is just the initial step that facilitates a long-term epigenetic repression by PRC1. Supporting this thesis, *WUS* is misexpressed in mutants of components of PRC1, which bear extra floral organs [13,38].

Future directions

It is likely that whole genome profiling studies will soon clarify how generally noncoding RNAs are involved in PcG recruitment; for example, by sequencing the RNAs that are associated with PRC2 components, as has recently been done in animals [39]. It will be important to test whether these RNAs can act in trans, that is, to recruit PRC2 to loci

that are not adjacent to the site of their production. If so, the mechanism of recruitment is an issue, as it is unlikely to be simply by sequence homology between the RNA and the target gene. Whether or not PRC2 binds specific RNA sequences or secondary structures is also an interesting question as, at least in in-vitro assays, components such as CLF bind RNA and single-strand DNA quite generally [40]. It will be important to know whether the AtRING1/AtBMI1 proteins and their putative mark (H2AK120Ub) colocalize with PRC2 targets, or rather are restricted to a subset of targets, and if the latter, whether these targets show more stable silencing. Lastly, it will be interesting to learn whether the PcG and trxG play a more general role in recording transient environmental events than simply the vernalization response.

Abbreviations

AG, AGAMOUS; ATX1, ARABIDOPSIS HOMOLOG OF TRITHORAX 1; CLF, CURLY LEAF; CLV3, CLAVATA3; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; KNU, KNUCKELS; PcG, Polycomb group; PHD-PRC2, PLANT HOMEO DOMAIN–POLYCOMB REPRESSIVE COMPLEX 2; PRC2, Polycomb repressive complex 2; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; trxG, trithorax-group; ULT1, ULTRAPETALA1; VRN1, VERNALIZATION1; WUS, WUSCHEL.

Competing interests

The authors declare that they have no competing interests.

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