Mechanisms guiding Polycomb activities during gene silencing in *Arabidopsis thaliana*

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Polycomb group (PcG) proteins act in an evolutionarily conserved epigenetic pathway that regulates chromatin structures in plants and animals, repressing many developmentally important genes by modifying histones. PcG proteins can form at least two multiprotein complexes: Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2, respectively). The functions of *Arabidopsis thaliana* PRCs have been characterized in multiple stages of development and have diverse roles in response to environmental stimuli. Recently, the mechanism that precisely regulates *Arabidopsis* PcG activity was extensively studied. In this review, we summarize recent discoveries in the regulations of PcG at the three different layers: the recruitment of PRCs to specific target loci, the polyubiquitination and degradation of PRC2, and the antagonism of PRC2 activity by the Trithorax group proteins. Current knowledge indicates that the powerful activity of the PcG pathway is strictly controlled for specific silencing of target genes during plant development and in response to environmental stimuli.

Keywords: *Arabidopsis*, epigenetics, histone modification, Polycomb group, Polycomb Repressive Complex, Trithorax group

INTRODUCTION TO POLYCOMB REPRESSIVE COMPLEXES IN

Multicellular eukaryotic organisms develop from a single cell called a zygote, which goes through cell division and differentiation to develop into multiple tissues and organs. Precise control of gene expression under the guidance of developmental cues/signals or environmental stimuli is strictly regulated to ensure proper development of organisms. Plants and animals have evolved to have multiple methods to regulate gene expression, among which epigenetic regulation is essential for correct genome-wide gene expression profiles (Russo et al., 1996; Liu et al., 2010a).

Polycomb group (PcG) proteins, one of the evolutionarily conserved epigenetic pathways, have critical roles in plant and animal development via regulation of gene expression levels (Whitcomb et al., 2007; Molitor and Shen, 2013). In the model plant *Arabidopsis thaliana*, PcG proteins are incorporated into two multi-protein complexes: Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2, respectively), which both have functions in the epigenetic repression of gene expression via histone modifications (Schatlowski et al., 2008; Molitor and Shen, 2013). The major function of PRC2 is to trimethylate lysine 27 on histone H3 (H3K27me3), while PRC1 recognizes the H3K27me3 marker and mono-ubiquitinates histone H2A (H2Aub; Schatlowski et al., 2008; Molitor and Shen, 2013).

The core component of *Arabidopsis* PRC2 is the SET-domain H3K27 methyltransferase CURLY LEAF (CLF; Goodrich et al., 1997; Schubert et al., 2006). In addition, SWINGER (SWN) has partially redundant functions with CLF (Chanvivattana et al., 2004), and MEDEA (MEA) functions as a methyltransferase in

gene loci that are modified by H3K27me3 in *Arabidopsis* seedlings (Zhang et al., 2007a). Such modifications are dynamic and the modification patterns differ among organs and tissues. Genome-wide identification of H3K27me3-modified loci revealed that this dynamic modification occurs during the shoot apical meristem (SAM)-to-leaf organ formation (Lafos et al., 2011), the embryo-to-seedling phase transition (Bouyer et al., 2011), in leaf-to-callus regeneration (He et al., 2012), and in the endosperm (Weinhofer et al., 2010). Therefore, PRC2-mediated H3K27me3 is precisely controlled during development.

PRC1 components have also been characterized in *Arabidopsis*. LIKE HETEROCHROMATIN PROTEIN1 (LHP1), also called TERMINAL FLOWER2 (TFL2), can recognize chromatin that is modified by H3K27me3, and its genome-wide binding sites show significant overlap with the H3K27me3 modification (Turck et al., 2007; Zhang et al., 2007b). There are two groups of RING-domain proteins in PRC1: two AtRING1 proteins and three AtBMI1 proteins (Xu and Shen, 2008; Bratzel et al., 2010; Chen et al., 2010). These RING-domain proteins function in the modification of H2Aub (Bratzel et al., 2010). Although the genome-wide identification of H2Aub loci has not been performed, it is possible that the role of PRC1 is also developmentally controlled (Kim et al., 2012).

RECRUITMENT OF PRCS TO SPECIFIC TARGET GENES
How PRCs specifically and dynamically recognize their targets during development and in different tissues or organs is a

key question for understanding the mechanism that controls the PcG pathway. Currently, there are two types of recruiters found in *Arabidopsis*: transcription factors and non-coding RNAs (ncRNAs).

Transcription factors usually bind to certain specific *cis* elements, termed Polycomb response elements (PREs), and recruit PRCs to their specific targets via direct interaction with PRCs (Schwartz and Pirrotta, 2008). AGAMOUS (AG), a MADS-box transcription factor that is essential for establishing floral organ identity and termination of floral meristem (FM), represses *WUSCHEL* (*WUS*) expression in FM (Bowman et al., 1989; Lenhard et al., 2001; Lohmann et al., 2001). AG binds to the *WUS* locus at CArG boxes and then recruits PRC2-mediated H3K27me3 and LHP1 to repress *WUS* (Liu et al., 2011). Mutation in *AG* results in the decreased H3K27me3 level and loss of LHP1 binding at the *WUS* locus. In this case, CArG boxes may serve as the PRE in *WUS* repression. However, it is not clear whether AG directly or indirectly recruits PcG proteins.

BREVIPEDICELLUS (*BP*) and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA2* (*KNAT2*) are members of the KNOX gene family and are expressed in the SAM but are silenced in leaves (Lincoln et al., 1994; Ori et al., 2000; Pautot et al., 2001). The MYB-domain transcription factor ASYMMETRIC LEAVES1 (AS1) and the LOB-domain transcription factor AS2 form a protein complex to repress *BP* and *KNAT2* expression in leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002; Sun et al., 2002; Lin et al., 2003; Xu et al., 2003). The AS1-AS2 complex targets the *cis* elements in the promoters of *BP* and *KNAT2* (Guo et al., 2008; Li et al., 2012). Recently, it was shown that AS1-AS2 interacts with PRC2 to recruit it to the *BP* and *KNAT2* loci (Lodha et al., 2013). Mutations in *AS1*, *AS2*, and the AS1-AS2-binding sites in *BP* and *KNAT2* promoters all result in decreased H3K27me3 levels at *BP* and *KNAT2* loci and ectopic expression of the two KNOX genes in leaves. In this case, the AS1-AS2-binding sites are likely to serve as PREs in PcG-mediated gene silencing.

Another possible PRE was found in the promoter of *LEAFY COTYLEDON2* (*LEC2*; Berger et al., 2011), a gene involved in embryo development, but which is silenced in the post-embryo stage (Stone et al., 2001). A *cis* element, called *Repressive LEC2 Element* (*RLE*), was identified to recruit PRC2 to trimethylate the *LEC2* locus (Berger et al., 2011). *RLE* is essential for *LEC2* repression in the post-embryo stage. PRC2 is unable to trimethylate H3K27 at the *LEC2* locus once *RLE* is mutated, leading to ectopic expression of *LEC2* in the post-embryo stage. An *RLE*-driven reporter gene could be repressed, accompanied by H3K27me3 modification at the transgene locus, suggesting that *RLE* is necessary and sufficient to recruit PRC2 for histone modification and gene silencing. It seems important in the future to identify the transcription factor that binds *RLE* and is able to interact with PRC2.

An analysis of *cis*-regulatory elements in the promoter of *FLOWERING LOCUS T* (*FT*), a key gene that controls flowering time (Kardailsky et al., 1999; Kobayashi et al., 1999), also indicated the existence of PRE within the promoter region (Adrian et al., 2010). However, the exact sequence that serves as the PRE and the transcription factor that binds the PRE of the *FT* promoter have not yet been identified.

The B3 domain proteins VP1/ABI3-LIKE1 (VAL1) and VAL2 are key regulators in the prevention of embryo traits in somatic tissues via repression of embryo specific genes. The loss-of-function double mutant *val1 val2* results in somatic embryogenesis, with ectopic expressions of embryo genes at the post-embryo stage (Suzuki et al., 2007). Somatic embryogenesis and ectopic expression of embryo genes were also observed in mutants corresponding to PRC1 components (Bratzel et al., 2010; Chen et al., 2010). A recent study showed that VAL proteins interact with PRC1 and recruit PRC1-mediated H2Aub to initiate repression of the embryonic genes after germination (Yang et al., 2013). The H2Aub modification at the embryo-gene loci is lost in *val1 val2* and *Atbmi1a Atbmi1b* mutants. Therefore, VALs may serve as a recruiter for PRC1.

In *Arabidopsis*, two ncRNAs, *COLD INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COOLAIR*) and *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*; Swiezewski et al., 2009; Heo and Sung, 2011; Ietswaart et al., 2012), regulate *FLOWERING LOCUS C* (*FLC*) expression. *FLC* is a flowering repressor that is essential for vernalization in response to cold treatment (Michaels and Amasino, 1999; Sheldon et al., 1999). *COOLAIR* is an antisense RNA that is transcribed in response to cold treatment (Swiezewski et al., 2009). *COOLAIR* is alternatively polyadenylated at the 3′ end, resulting in a proximal poly(A) site or a distal ploy(A) site (Liu et al., 2010b). The proximal poly(A) site stimulates the activity of FLD, a homolog of the human LYSINE SPECIFIC DEMETHYLASE1 (LSD1; Sanda and Amasino, 1996; Liu et al., 2007), to reduce the H3K4me2 level at the *FLC* locus, leading to a transition from an active chromatin state to a repressive state (Liu et al., 2010b). The reduction of H3K4me2 might benefit the H3K27me3 modification; thus, *COOLAIR* acts as an indirect recruiter of PRC2. However, how FLD is activated using the proximal site of *COOLAIR* remains unknown.

COLDAIR is a sense ncRNA that contains a 5′ cap, but no poly(A) tail (Heo and Sung, 2011). *COLDAIR* is induced by low temperature, and its transcription reaches a maximum level after 20 days of cold treatment, which is about 10 days later than *COOLAIR*. *COLDAIR* can directly interact with the CXC domain of the core PRC2 components CLF and SWN. In *COLDAIR* knockdown plants, CLF is not properly recruited to *FLC*, resulting in insufficient H3K27me3 modification at the *FLC* locus, consistent with the late flowering phenotype of these plants. Therefore, COLDAIR serves as a direct recruiter for PRC2 in *Arabidopsis*.

PRC2 DEGRADATION THROUGH POLYUBIQUITINATION

The *Arabidopsis* PRC2 is post-translationally regulated by the F-box protein UPWARD CURLY LEAF1 (UCL1; Jeong et al., 2011). UCL1 is a component of the SCF E3 ubiquitin ligase complex, which has a role in the degradation of proteins via polyubiquitination and the 26S proteasome pathway (Vierstra, 2003). Both activation tagging dominant mutant *ucl1-D*, and plants overexpressing *UCL1* under the control of the CaMV 35S promoter resulted in phenotypes that were similar to the *clf* mutant, with ectopic expression of some typical PRC2 targets, whose loci exhibit decreased H3K27me3 levels (Jeong et al., 2011). These results indicated that UCL1 inhibits PRC2 activity. Additionally, UCL1

directly interacts with CLF, but not with MEA, and overexpression of *UCL1* causes a reduced CLF protein level. Therefore, UCL1 was thought to polyubiquitinate CLF and to degrade CLF through the 26S proteasome pathway.

Interestingly, *UCL1* is expressed in the endosperm, where *CLF* and *MEA* transcripts are detectable. However, overexpression of *CLF* in the endosperm causes *mea*-like phenotypes, suggesting that the CLF protein level is strictly controlled in the endosperm. Jeong et al. suggested that UCL1 functions in the endosperm to specifically degrade CLF, and therefore to prevent CLF from competing with MEA during the formation of PRC2 (Jeong et al., 2011).

PRC2 FUNCTIONS ARE ANTAGONIZED BY THE TRITHORAX GROUP PROTEINS

Trithorax group (TrxG) proteins were first identified in *Drosophila* and function in antagonism of PcG (Schuetten-gruber et al., 2011). The first identified plant TrxG protein was *Arabidopsis* HOMOLOG OF TRITHORAX1 (ATX1), which encodes a SET-domain protein that specifically trimethylates H3K4 (Alvarez-Venegas et al., 2003; Pien et al., 2008; Saleh et al., 2008). Two other SET-domain proteins were shown to participate in genome-wide control of histone methylation: SDG8 is responsible for H3K36me2/3 (Zhao et al., 2005; Xu et al., 2008), and SDG2 acts for H3K4me2/3 (Berr et al., 2010; Guo et al., 2010). The histone modifications H3K4me2/3 and H3K36me2/3 function in activation of gene expression, showing the opposite role of PcG-mediated H3K27me3 (Zhang et al., 2007a, 2009; Roudier et al., 2011). However, the molecular mechanism whereby H3K4me2/3 and H3K36me2/3 antagonize H3K27me3 is not clear in *Arabidopsis*. A recent study showed that the 35S enhancer decreases the H3K27me3 level but increases the H3K4me3 level at the insert locus (Chen et al., 2013). This suggests that *cis* enhancer sequences may play a role to recruit TrxG proteins to restrict the PRC2-mediated H3K27me3.

ULTRAPETALA1 (ULT1), a plant-specific SAND domain protein, is another TrxG protein (Carles and Fletcher, 2009). Overexpression of *ULT1* resulted in phenotypes similar to those of *clf*, and caused derepression of PcG target genes with decreased H3K27me3 levels. ULT1 is able to directly bind to ATX1 and guides it to the target genes.

RELATIVE OF EARLY FLOWERING6 (REF6) is a JmjC-domain protein (Noh et al., 2004) that specifically demethylates H3K27me2/3 *in vivo* and *in vitro* (Lu et al., 2011). Overexpression of *REF6* caused an *lhp1*-like phenotype (Lu et al., 2011). In addition, mutation of *REF6* could partially rescue the *clf* phenotype, and resulted in hypermethylation of H3K27me3 for hundreds of genes. Characterization of REF6 revealed that the H3K27me3 modification is a reversible process, and such regulation is critical to balance PcG activity (Lu et al., 2011).

The ATP-dependent chromatin remodeling factor PICKLE (PKL) is involved in antagonism of PcG (Aichinger et al., 2009, 2011), and the *pkl* mutant partially rescued developmental defects of the *clf* mutant, including the up-curled leaves and early flowering. Conversely, *pkl* enhanced the defects of the *clf swn* double mutant in the root. In roots, PKL

activates the expression of *EMF2*, *CLF*, and *SWN*, and this could explain why *pkl* enhances *clf swn*. In addition, PKL is very important for the activity of the root apical meristem (RAM). In the *pkl* mutant, a number of genes that respond to the activity of RAM were silenced because of the increased H3K27me3 level on these genes (Aichinger et al., 2011). However, several other studies suggested that PKL has a role in the promotion of PRC2-mediated H3K27me3 and that the PKL protein associates with H3K27me3-enriched loci (Zhang et al., 2008, 2012). Further studies to clarify the genome-wide function of PKL will improve our understanding of how the chromatin remodeling pathway coordinates with the PcG pathway to regulate their downstream targets. Overall, these studies revealed that chromatin structure, which is organized by chromatin remodeling factors using energy from ATP hydrolysis, is essential for regulation of histone modification states.
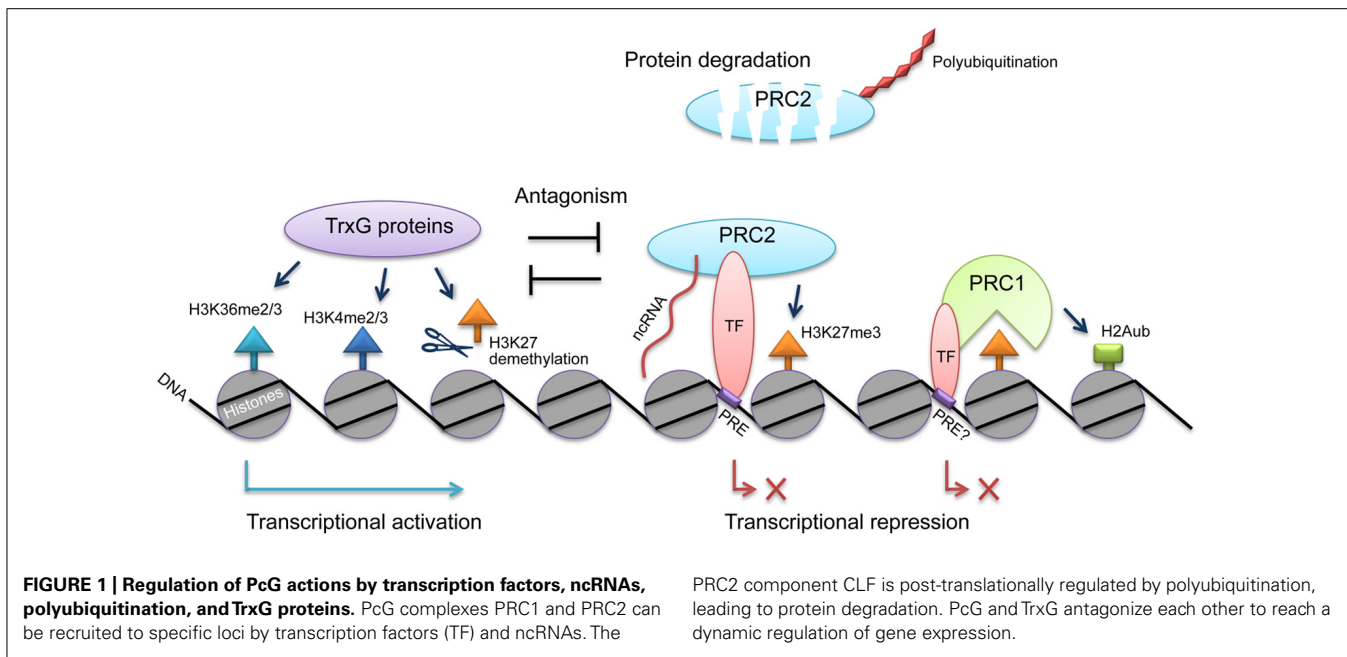
CONCLUSION AND PERSPECTIVES

Controls for the PcG action at different levels are essential to ensure its specificity and activity (see the model in **Figure 1**). However, many questions still remain to be answered. First, although several candidates that recruit PRC1 and PRC2 to the target genes have been characterized, the common rule of the recruitment remains unclear. For example, how do these transcription factors bind PRCs? If PRCs are recruited generally by transcription factors, common domains or motifs might exist in the PcG proteins and transcription factors, similar to the binding of the TOPLESS corepressor and transcription factors (Causier et al., 2012). On the other hand, if ncRNAs are the common factors that associate with PRCs, the RNA-binding domain of PcG proteins may contain various ncRNAs. Detection of these ncRNAs might be helpful in studying the specificity of PcG. In addition, how the ncRNAs are transcribed in response to developmental or environmental cues is not clear. Recently, it was proposed that the chromatin structure and its modification status also affect the recruitment of PRCs to target loci in the "chromatin sampling" model (Klose et al., 2013), and it was also shown that PRC2 binding sites contain GAGA factor binding sequences (Deng et al., 2013).

Second, the mechanism guiding the balance between PcG and TrxG activities in regulating developmental processes in plants is currently unknown. In animals, several transcription factors can regulate both PcG and TrxG activity, providing a dynamic and reversible epigenetic state (Schwartz and Pirrotta, 2008). Therefore, comparison of the regulation mechanisms of PcG and TrxG may be helpful to understand how a balance is established between PcG-mediated gene silencing and TrxG-mediated gene activation.

Third, some novel proteins were identified in plant PRCs, for example, the CLF interacting protein BLISTER (BLI; Schatlowski et al., 2010) and LHP1-INTERACTING FACTOR2 (LIF2; Latrasse et al., 2011). It will be of interest to test whether these proteins act as regulators of PRCs. LIF2 is an RNA-binding protein, suggesting that PRC1 may also be subject to regulation from RNAs.

Finally, recent studies revealed that PRC1 and PRC2 physically interact in *Arabidopsis* (Derkacheva et al., 2013), indicating the possibility that the two PcG complexes have a crosstalk in silencing common targets. Further studies on mechanisms that regulate PcG

FIGURE 1 | Regulation of PcG actions by transcription factors, ncRNAs, polyubiquitination, and TrxG proteins. PcG complexes PRC1 and PRC2 can be recruited to specific loci by transcription factors (TF) and ncRNAs. The PRC2 component CLF is post-translationally regulated by polyubiquitination, leading to protein degradation. PcG and TrxG antagonize each other to reach a dynamic regulation of gene expression.

activity would be helpful to understand the epigenetic regulation of plant development.

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