The ASYMMETRIC LEAVES complex maintains repression of KNOX homeobox genes via direct recruitment of Polycomb-repressive complex 2

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Polycomb-repressive complexes (PRCs) ensure the correct spatiotemporal expression of numerous key developmental regulators. Despite their pivotal role, how PRCs are recruited to specific targets remains largely unsolved, particularly in plants. Here we show that the Arabidopsis ASYMMETRIC LEAVES complex physically interacts with PRC2 and recruits this complex to the homeobox genes BREVIPEDICELLUS and KNAT2 to stably silence these stem cell regulators in differentiating leaves. The recruitment mechanism resembles the Polycomb response element-based recruitment of PRC2 originally defined in flies and provides the first such example in plants. Combined with recent studies in mammals, our findings reveal a conserved paradigm to epigenetically regulate homeobox gene expression during development.

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Polycomb group (PcG) proteins are essential regulators of development in both plants and animals. PcG proteins form two functionally conserved complexes that maintain genes transcriptionally repressed by modulating chromatin structure. The Polycomb-repressive complex 2 (PRC2) catalyzes the trimethylation of histone H3 Lys27 [H3K27me3], and PRC1, which binds this chromatin mark, mediates the monoubiquitylation of histone H2A and generates a somatically heritable compacted chromatin state (Schuettengruber and Cavalli 2009; Köhler and Hennig 2010; Margueron and Reinberg 2011; Bemer and Grossniklaus 2012). Genome-wide studies predict that as many as 20% of Arabidopsis genes are marked with H3K27me3 and regulated by PRC2 [Zhang et al. 2007a; Bouyer et al. 2011; Roudier et al. 2011]. The precise spectrum of PRC2 targets varies depending on cell type and developmental stage and in response to external stimuli. How PRC2, which does not bind DNA specifically, recognizes defined targets is a major outstanding question.

Keywords: Polycomb proteins; homeobox genes; ASYMMETRIC LEAVES; stem cell; meristem; leaves

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and, more recently, in mammals (Sing et al. 2009; Woo et al. 2010), our findings show that species-specific DNA-binding proteins have evolved to interact with the highly conserved PRC2 core components to direct the epigenetic repression of homeobox genes during development.

Results and Discussion

Polycomb complexes maintain repression of BP and KNAT2 in differentiating leaves

To assess the contribution of PRCs to the stable repression of BP and KNAT2, we compared the levels of H3K27me3 deposited at these KNOX loci in wild-type leaves and leaves mutant for the PRC2 core component CURLY LEAF (CLF) (Goodrich et al. 1997). In wild type, an enrichment for H3K27me3 is detected at all regions examined across the BP and KNAT2 loci, with a localized peak of this repressive chromatin mark observed just downstream from the translation start site [Fig. 1]. In clf, the levels of H3K27me3 across both loci are strongly reduced. The residual H3K27me3 observed at BP and KNAT2 in clf mutants likely reflects redundancy with the H3K27 methyltransferase SWINGER [SWN], as seen for STM and other PRC2 targets in Arabidopsis (Schubert et al. 2006). Chromatin immunoprecipitation (ChIP) assays on chromatin isolated from seedling leaves expressing a functional LHP1-GFP fusion protein (Sung et al. 2006) showed that LHP1 also localizes to the promoter and coding region of both KNOX loci in leaves [Fig. 2]. In addition, perturbation of PRC1 or PRC2 function leads to ectopic expression of STM as well as BP and KNAT2 (Supplemental Fig. 2; Katz et al. 2004; Xu and Shen 2008; Bratzel et al. 2010). These observations indicate that, as for STM, stable repression of BP and KNAT2 in leaves requires PRC2 and PRC1.

Repressed KNOX loci lack heterochromatic marks

In animals, Polycomb-mediated silencing of pluripotency factors, such as the homeobox genes Oct4 and Nanog, is likewise required to allow cellular differentiation [Margueron and Reinberg 2011; Orkin and Hochedlinger 2011]. This initial repressed state is subsequently reinforced by methylation of H3K9 and DNA [Vire et al. 2006; Mikkelsen et al. 2008; Bhutani et al. 2010]. To determine whether KNOX gene silencing in Arabidopsis leaves is similarly reinforced by establishment of a local heterochromatic state, we analyzed the distribution of H3K9me2 and cytosine methylation across BP and KNAT2. Digestion with McrBC, which cleaves methylated DNA, did not significantly reduce the levels of amplifiable DNA, whether from the promoter or gene body of BP and KNAT2 [Supplemental Fig. 3A]. This result is consistent with data from genome-wide methyl-cytosine profiling (Cokus et al. 2008) and suggests that both KNOX loci are largely unmethylated in leaves. Likewise, no significant enrichment of H3K9me2 was detected at either locus [Supplemental Fig. 3B]. These data argue against a prominent role for heterochromatin-based gene silencing mechanisms in the stable repression of KNOX genes during leaf development, a finding consistent with observations that differentiated leaf cells are relatively easily reprogrammed to form new shoots or somatic embryos.

The ASYMMETRIC LEAVES complex acts upstream of PRC2 in KNOX gene silencing

Given that the stable repression of BP and KNAT2 in leaves involves the activities of the AS1–AS2 complex as well as PRC2, we asked whether these complexes function in a hierarchical manner. Toward this end, we compared the H3K27me3 profiles at BP and KNAT2 in wild-type, as1, and as2 leaves. As BP and KNAT2 misexpression in these mutants is most prominent in young leaves and at the base of expanded leaves [Supplemental Fig. 4; Guo et al. 2008], we compared H3K27me3 levels specifically in these tissues. Moreover, to correct for potential differences in nucleosome density across BP and KNAT2 in wild type versus either mutant, H3K27me3 levels were normalized relative to histone H3 occupancy. In agreement with the data from whole seedling leaves [Fig. 1], enrichment for H3K27me3 was observed at all sites tested across both KNOX loci in wild type [Fig. 2A]. The levels of H3K27me3 peak just downstream from the proximal AS1–AS2-binding site, which, at BP, coincides with the location of enhancer elements that drive expression in developing leaves (Guo et al. 2008). In as1 and as2, H3K27me3 levels are significantly reduced at nearly all regions tested [Fig. 2A; Supplemental Fig. 1]. Loss of this PRC2 signature is correlated with reduced PRC1 occupancy, as the enrichment levels of LHP1-GFP at BP and KNAT2 are significantly lower in as2 compared with wild type [Fig. 2B]. Depletion of H3K27me3 in as1 is further correlated with increases in the levels of H3K4me3 [Fig. 2C], a chromatin mark deposited by Trithorax group proteins that act antagonistically to PcG complexes and promote gene expression (Carles and Fletcher 2009; Berr et al. 2010; Guo et al. 2010).

These experiments indicate that AS1 and AS2 act upstream of the Polycomb complexes in forming a repressive chromatin state at BP and KNAT2 during leaf development. The observation that BP and KNAT2 transcript levels in as1, clf, and the as1 clf double mutant are comparable [Supplemental Fig. 2D] is
The AS1–AS2 complex mediates the recruitment of PRC2 to these target loci. Together, these observations support a model in which the AS1–AS2 complex physically interacts with PRC2 and recruits this complex onto the KNOX promoters. Deposition of H3K27me3 at regions away from the PRC2 recruitment sites might then reflect spreading of PRC2 away from the proximal AS1–AS2 complex-binding site. Importantly, CLF occupancy at BP and KNAT2 is dramatically reduced in as1 (Fig. 3A), consistent with an AS1–AS2 complex-dependent recruitment of PRC2 to these target loci.

The possibility that the AS1–AS2 complex recruits PRC2 was substantiated by immunoprecipitation assays, which revealed an in vivo interaction between these complexes. In plants carrying an inducible YFP-tagged copy of AS2 (pOlexA:AS2-YFP), MYC-CLF is present in anti-GFP precipitates specifically following induction of AS2-YFP expression (Fig. 3B). Likewise, AS2-YFP is pulled-down in immunoprecipitation assays with anti-MYC but not using a nonspecific IgG antibody. An in vivo interaction between the AS1–AS2 and PRC2 complexes was also observed when pOlexA:AS2-YFP is coexpressed with an HA-tagged version of FERTILIZATION INDEPENDENT ENDOSPERM (FIE), the Arabidopsis ortholog of the Drosophila PRC2 component Extra sex combs (Esc) (pFIE-FIE-HA) (Köhler and Hennig 2010). Bemer and Grossniklaus (2012). FIE-HA is present in anti-GFP precipitates only following induction, and AS2-YFP is specifically pulled down from total protein extracts in immunoprecipitation assays with anti-HA antibody (Fig. 3B).

Together, these observations support a model in which the AS1–AS2 complex physically interacts with PRC2, recruits PRC2 to regions within the KNOX promoters, and recruits PRC2 to regions within the KNOX promoters, and recruits PRC2 to regions within the KNOX promoters, and recruits PRC2 to regions within the KNOX promoters.
Mark is predominantly deposited by a PRC2 complex that, in addition to the Enhancer of zeste [E(z)] homologs CLF or SWN, contains the Esc homolog FIE, the Suppressor of zeste 12 [Su(z)12] homolog EMBRYONIC FLOWER2 (EMF2), and the P55 homolog MULTICOPY SUPPRESSOR IRA1 (MSI1) (Hennig and Derkacheva 2009). Bimolecular fluorescence complementation (BiFC) assays show that AS1 can physically interact with CLF and FIE, whereas AS2 interacts specifically with EMF2 (Fig. 3C; Supplemental Table 1).

Recruitment of PRC2 to BP and KNAT2 involves a PRE-based mechanism

Together, these data indicate that recruitment of PRC2 to BP and KNAT2 is mediated by direct interactions with the AS1–AS2 complex, which in turn binds specific sequence motifs in the promoters of these homeobox genes (Guo et al. 2008). This recruitment mechanism is reminiscent of the PRE-based recruitment of PRC2 originally defined in flies (Schwartz et al. 2006; Schuettengruber and Cavalli 2009). However, a defining property of PREs is that these DNA elements are sufficient for the Polycomb-dependent repression of flanking genes at ectopic integration sites. To verify that intact AS1–AS2-binding sites are required for PRC2-mediated gene silencing and test whether a promoter element containing these sites has PRE-like activity, we generated multiple independent transgenic lines carrying a GFP-GUS reporter gene driven by a chimeric promoter containing a BP promoter fragment with wild-type or mutated AS1–AS2-binding sites upstream of the 35S minimal promoter (Fig. 4). Leaf tissues from three independent transgenic lines containing either wild-type or mutated AS1–AS2-binding sites in the promoter were pooled, and two pools for each were analyzed by ChIP. Using this strategy, H3K27me3 deposition at multiple independent loci could be evaluated simultaneously, and any effects of integration sites would be averaged. H3K27me3 levels are enriched at the GFP-GUS reporter in lines where the transgene contains intact AS1–AS2-binding sites in the promoter, and this enrichment is significantly reduced when the AS1–AS2-binding sites are mutated (Fig. 4; Supplemental Fig. 7). The AS1–AS2-binding sites are thus required and, in this context, also sufficient for the recruitment of PRC2 activity.

Considering these properties, we conclude that the AS1–AS2 complex recruits PRC2 to BP and KNAT2 via a PRE-based mechanism, the first such example in plants.

Figure 3. The AS1–AS2 complex physically interacts with PRC2 and directly recruits this complex to BP and KNAT2. [(A)] ChIP analyses showing CLF occupancy at specific regions of BP and KNAT2. CLF occupancy at these sites is significantly reduced in as1 compared with wild type. [*] \( P < 0.05; [**] P < 0.01 \). Values (mean ± SE; \( n \geq 3 \)) are relative to the enrichment of CLF at AG. [(B)] In plants carrying an inducible AS2-YFP fusion (pOlexA:AS2-YFP), MYC-CLF (top panel) and FIE-HA (bottom panel) communoprecipitate with AS2-YFP specifically upon induction. AS2-YFP also communoprecipitates with MYC-CLF (top panel) and FIE-HA (bottom panel) in communoprecipitation assays with anti-MYC and anti-HA antibody, respectively, but not in control communoprecipitation assays with IgG. Antibodies used in communoprecipitation assays are listed at the top, and proteins detected by Western are at the right. (C) Bimolecular fluorescence complementation assays reveal direct physical interactions between AS1 and the PRC2 core components FIE and CLF and between AS2 and EMF2. Cobombardment of functional AS2 and CLF fusion constructs (Supplemental Table 2) yields no fluorescence signal. (Left panels) EYFP signal monitoring protein–protein interactions. (Middle panels) DAPI staining indicating positions of nuclei. (Right panels) Merged images.

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**Figure 4.** AS1–AS2-binding sites are necessary and sufficient for recruitment of PRC2 activity. ChIP analysis showing that H3K27me3 levels are enriched at the GFP-GUS reporter in lines where the transgene contains wild-type AS1–AS2-binding sites in the promoter. H3K27me3 levels are significantly reduced when the AS1–AS2-binding sites are mutated. (*) P < 0.05; (**) P < 0.01. Two pools comprising three independent transgenic lines were analyzed for each construct. Quantitative PCR values (mean ± SE) are normalized to H3 levels and calculated from three independent replicates. A schematic representation of the reporter lines is shown at the top. A BP promoter fragment spanning nucleotides –2707 to –1088 from the ATG was fused to the 35S minimal promoter and inserted upstream of a GFP-GUS fusion. (Black dash) Position of the ampiclon analyzed; (red ovals) AS1–AS2 complex-binding sites; (arrow) transcription start site; (violet box) the 35S minimal promoter; (green box) GFP; (blue box) GUS. Sequence of the wild-type and mutated AS1–AS2-binding sites are shown below. Controls establishing the specificity and efficiency of ChIP reactions are shown in Supplemental Figure 7.

FLC [Heo and Sung 2011], plants use at least two distinct Polycomb recruitment mechanisms: the one described here and a second involving long noncoding RNAs. As such, our findings show that, like the PRC2 core components themselves, the mechanisms via which this complex is recruited to specific targets are conceptually conserved between animals and plants.

How widespread PRE-based recruitment mechanisms are in plants remains to be seen. The genetic interactions between mutants affecting PRC2 and AS1–AS2 complex activity predict that this specific mechanism acts at a select subset of all Polycomb targets. Indeed, PRC2-activity predicts that this specific mechanism acts at between mutants affecting PRC2 and AS1–AS2 complex are in plants remains to be seen. The genetic interactions components themselves, the mechanisms via which this such, our findings show that, like the PRC2 core complex is recruited to specific targets are conceptually nonredundantly, pointing to cooperativity between AS1–AS2 complexes in the repression of these meristem regulators during organogenesis. A similar cooperativity has been reported for transcription factors acting at animal PREs [Schwartz et al. 2006; Schuettengruber and Cavalli 2009; Margueron and Reinberg 2011] and may be required to stabilize the binding of PRC2 at target loci. In this regard, it is also suggestive that AS1 and AS2 interact physically with multiple PRC2 core components. In addition, cooperativity between AS1–AS2 complexes could be important for the formation of long-range interactions between nucleosomes, which have been postulated to drive the switch from an active to a repressive chromatin state [Dodd et al. 2007; Angel et al. 2011]. Consistent with this idea, binding of the AS1–AS2 complex at the KNOX promoters leads to a peak of H3K27me3 deposition just downstream from the proximal AS1–AS2-binding sites. Once nucleated, positive feedback in PRC2 activity [Margueron et al. 2009; Suganuma and Workman 2010] is recruited to drive the maintenance of this repressive chromatin mark across BP and KNAT2. Autocatalytic activity of PRC2 along with PRC1 occupancy could further ensure that the repressive chromatin state is stably maintained through the many rounds of cell division associated with leaf development [Margueron et al. 2009; Bratzel et al. 2010; Suganuma and Workman 2010]. Thus, even though AS1–AS2 complex activity is limited to early leaf development [Guo et al. 2008], repression of the KNOX genes persists throughout organogenesis. These findings thus provide a framework for the Polycomb-based cellular memory system underlying the somatically heritable repression of stem cell-promoting homeobox genes required for cellular differentiation in leaves. Combined with studies reporting PRE-based PRC2 recruitment mechanisms in the regulation of homeobox genes in flies [Schwartz et al. 2006; Schuettengruber and Cavalli 2009] and, more recently, also in mammals [Sing et al. 2009; Woo et al. 2010], this work reveals an ancient paradigm to control the spatiotemporal expression of homeobox genes during development.

**Materials and methods**

The ChIP experiments were performed as described previously [Guo et al. 2008]. Relative enrichments were calculated as the percentage of input or as a ratio over H3 enrichment. In the MYC-CLF ChIP experiments, enrichments were calculated relative to the enrichment of CLF at AG. All experiments were performed at least three times independently, and PCR reactions were performed in duplicate. Student’s t-test was used to calculate statistical significance. Protein immunoprecipitation reactions were performed using the μMACS Epitope Tag isolation kits (Miltenyi Biotec). Tissues were processed without cross-linking, according to the manufacturer’s recommendations, with one addition. Cleared sonicated lysates were treated with 5 μl of benzamide (250 U/μl) to degrade DNA and RNA to avoid artificial protein–protein interactions. Samples were analyzed by Western as published previously [Guo et al. 2008]. For BiFc assays, 2.5 μg of each plasmid DNA was coated onto 1 μM gold particles and bombarded into onion epidermal cells using a PDS-1000/He Biolistic particle delivery system (Biorad) as per the manufacturer’s instructions. McrBC assays were performed at least three independent times. Approximately 500 ng of genomic DNA was treated with 50 U of McrBC (New England Biolabs) overnight at 37°C. Control samples were treated identically but without enzyme. Amplifiable DNA levels were subsequently quantified by quantitative PCR using standard protocols. GUS staining was performed as described previously [Guo et al. 2008], and primers used in this study are listed in Supplemental Table 2. Further details can be found in the Supplemental Material.
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