

The ASYMMETRIC LEAVES complex maintains repression of KNOX homeobox genes via direct recruitment of Polycomb-repressive complex2

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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 complex2 (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

(Köhler and Hennig 2010; Bemer and Grossniklaus 2012). In *Drosophila*, PRC2 is recruited to specific targets by DNA-binding proteins that bind conserved sequence motifs known as Polycomb response elements (PREs) (Schwartz et al. 2006; Schuettengruber and Cavalli 2009). In addition to PREs, long noncoding RNAs have been identified as important participants in Polycomb recruitment in mammals (Margueron and Reinberg 2011). Given the recent evidence that the noncoding RNA *COLDAIR* recruits PRC2 to *FLOWERING LOCUS C* (*FLC*) during vernalization (Heo and Sung 2011), PRC recruitment via long noncoding RNAs appears conserved between plants and animals. Whether the PRE-based recruitment mechanism is likewise conserved is unclear, as the mechanisms underlying the correct spatiotemporal recruitment of PRC2 to plant targets other than *FLC* are unknown.

Members of the class I KNOTTED1-like homeobox (KNOX) gene family are among the many predicted Polycomb targets identified in genome-wide H3K27me3 profiling studies (Zhang et al. 2007a; Bouyer et al. 2011; Roudier et al. 2011). KNOX genes promote stem cell activity, and their precise spatiotemporal regulation is essential to maintain the delicate balance between stem cell self-renewal and differentiation (for review, see Barton 2010; Hay and Tsiantis 2010). KNOX activity is required in indeterminate cells within shoot meristems, the stem cell niches positioned at the growing tips of the plant, but needs to be stably repressed in determinate lateral organs, such as leaves, to allow cellular differentiation. The *Arabidopsis* KNOX family member *SHOOTMERISTEMLESS* (*STM*) is a verified Polycomb target (Schubert et al. 2006; Zhang et al. 2007b; Xu and Shen 2008; Bratzel et al. 2010), although the mechanism via which PRC2 is recruited to *STM* is not currently known. A role for PRC2 and PRC1 in the stable repression of the KNOX genes *BREVIPEDICELLUS* (*BP*) and *KNAT2* in leaves is less clear. H3K27me3 levels are enriched at these loci in whole seedlings and roots specifically. However, no extensive enrichment of LIKE-HETEROCHROMATIN PROTEIN1 (LHP1), the chromo-domain component of *Arabidopsis* PRC1, was observed at either locus in leaves (Zhang et al. 2007a,b; Roudier et al. 2011).

The persistent silencing of KNOX activity in determinate lateral organs may indeed involve distinct cellular memory systems. In contrast to the stable repression of *STM*, silencing of *BP* and *KNAT2* in developing leaves is mediated by ASYMMETRIC LEAVES1 (AS1) and AS2 (Lodha et al. 2008). These DNA-binding proteins bind as a complex to the promoters of *BP* and *KNAT2* and act after organ initiation to maintain both KNOX genes repressed throughout subsequent leaf development (Phelps-Durr et al. 2005; Guo et al. 2008). Here, we show that AS1 and AS2 physically interact with multiple core components of PRC2. The AS1–AS2 complex recruits PRC2 to *BP* and *KNAT2* to establish the somatically heritable repressed chromatin state required for leaf differentiation. The recruitment mechanism is reminiscent of the PRE-based recruitment of PRC2 originally defined in flies (Schwartz et al. 2006; Schuettengruber and Cavalli 2009) and provides the first such example in plants. Combined with studies describing PRE-based Polycomb recruitment mechanisms in the regulation of homeobox genes in flies

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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 (Viré 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

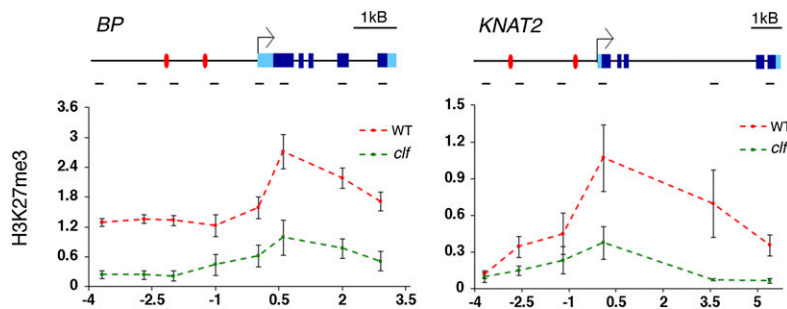


Figure 1. CLF mediates the trimethylation of H3K27 at KNOX loci. ChIP experiments show that H3K27me3 levels across *BP* (left panel) and *KNAT2* (right panel) are reduced in *clf* seedlings compared with wild type. Quantitative PCR values (mean \pm SE) are shown as percentage of input and calculated from at least three independent biological replicates. Schematic representations of the *BP* and *KNAT2* loci are shown at the top. (Black dashes) Positions of amplicons analyzed in ChIP assays; (red ovals) AS1–AS2 complex-binding sites; (arrow) transcription start site; (light blue boxes) 5' and 3' untranslated regions (UTRs); (dark blue boxes) ORF. Values on the X-axis are distances in kilobases relative to the transcription start site.

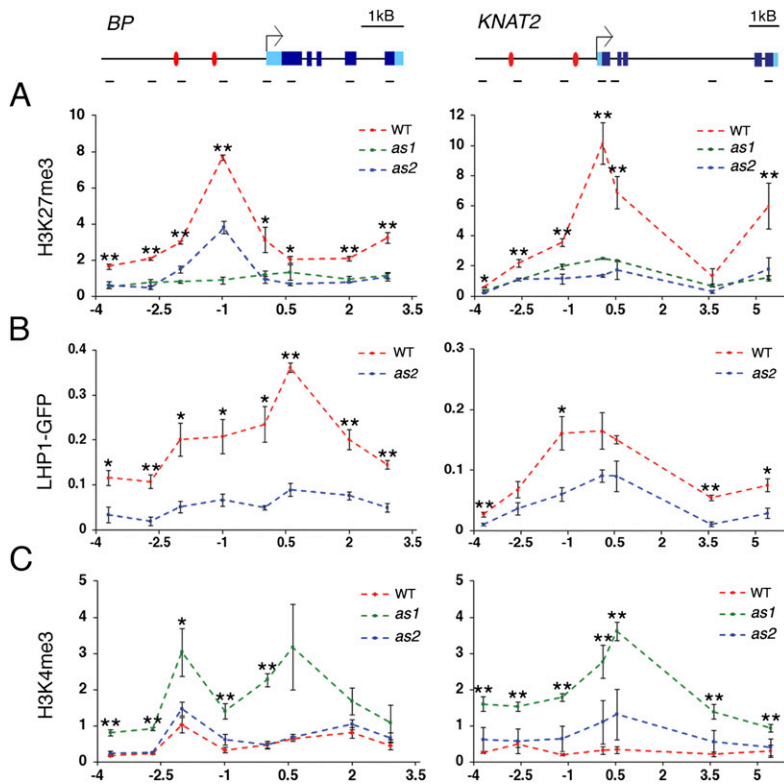


Figure 2. The AS1–AS2 complex influences chromatin structure at *BP* and *KNAT2*. (A) Levels of H3K27me3 at *BP* (left panel) and *KNAT2* (right panel) are significantly reduced in *as1* and *as2* leaves compared with wild type. (B) Occupancy levels of the PRC1 component LHP1 at *BP* (left panel) and *KNAT2* (right panel) are likewise reduced in *as2*. (C) Enrichment levels for the active chromatin mark H3K4me3 across *BP* (left panel) and *KNAT2* (right panel) in wild type, *as1*, and *as2*. Values (mean \pm SE; $n \geq 3$) are normalized to H3 levels (A,C) to correct for possible variation in nucleosome density at *BP* and *KNAT2* or calculated as percentage of input (B). Values significantly different from wild type in at least one of the mutants are indicated. (*) $P < 0.05$; (**) $P < 0.01$. Schematic representations of the *BP* and *KNAT2* loci are as in Figure 1. Control experiments establishing the specificity and efficiency of ChIP reactions are shown in Supplemental Figure 1.

consistent with these complexes acting in the same pathway to repress both *KNOX* genes. However, the synergistic phenotypes of double mutants between *as1*, *as2*, and components of PRC2 or PRC1 indicate that these complexes also regulate distinct target sets (Supplemental Fig. 5). Considering this, it is unlikely that AS1 and AS2 effect the Polycomb-mediated repression of *BP* and *KNAT2* via regulation of PRC components. Indeed, the levels of H3K27me3 in the overall histone H3 pool are unchanged in *as1* and *as2* (Supplemental Fig. 6). Moreover, H3K27me3 levels at *STM*, whose silencing in leaves occurs in an AS1–AS2 complex-independent manner, are unaffected in these mutants (Supplemental Fig. 6).

The ASYMMETRIC LEAVES complex recruits PRC2 to *KNOX* loci by physical interactions

Given the DNA-binding properties of AS1 and AS2 (Guo et al. 2008), their requirement for the Polycomb-mediated repression of *BP* and *KNAT2* could indicate that the AS1–AS2 complex mediates the recruitment of PRC2 to these *KNOX* loci. This possibility was assessed using ChIP assays on chromatin prepared from plants expressing a

MYC-tagged version of CLF (*p35S:MYC-CLF*) in either a wild-type or *as1* background. In nonmutant plants, CLF occupancy at *BP* and *KNAT2* is detected at regions surrounding the proximal AS1–AS2 complex-binding site (Fig. 3A), which include those *BP* and *KNAT2* fragments marked by peak levels of H3K27me3 (Fig. 2A). Enrichment of CLF at these sites is comparable with that observed at *AGAMOUS* (*AG*), which is a known direct target of CLF-containing PRC2 (Schubert et al. 2006; Heo and Sung 2011). Failure to detect CLF binding above background at other sites across *BP* and *KNAT2*, despite being marked by H3K27me3 (Fig. 2A), could suggest a relatively transient, dispersed association 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 reveal 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 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 across *BP* and *KNAT2*, which in animals is known to occur via an autocatalytic process mediated by binding of the PRC2 component *Esc* to H3K27me3 and subsequent activation of methyltransferase activity on H3K27 (Margueron et al. 2009; Suganuma and Workman 2010). Given that the propagation of PRC2 is likely to proceed discretely in individual cells, CLF occupancy beyond its sites of recruitment may be undetectable by ChIP.

To gain insight into the molecular mechanism via which the AS1–AS2 complex recruits PRC2 to *BP* and *KNAT2*, we next determined whether either DNA-binding protein interacts directly with PRC2 core components. The *Arabidopsis* genome includes one or more homologs for each of the *Drosophila* core PRC2 subunits. However, in the *Arabidopsis* seedling, the H3K27me3

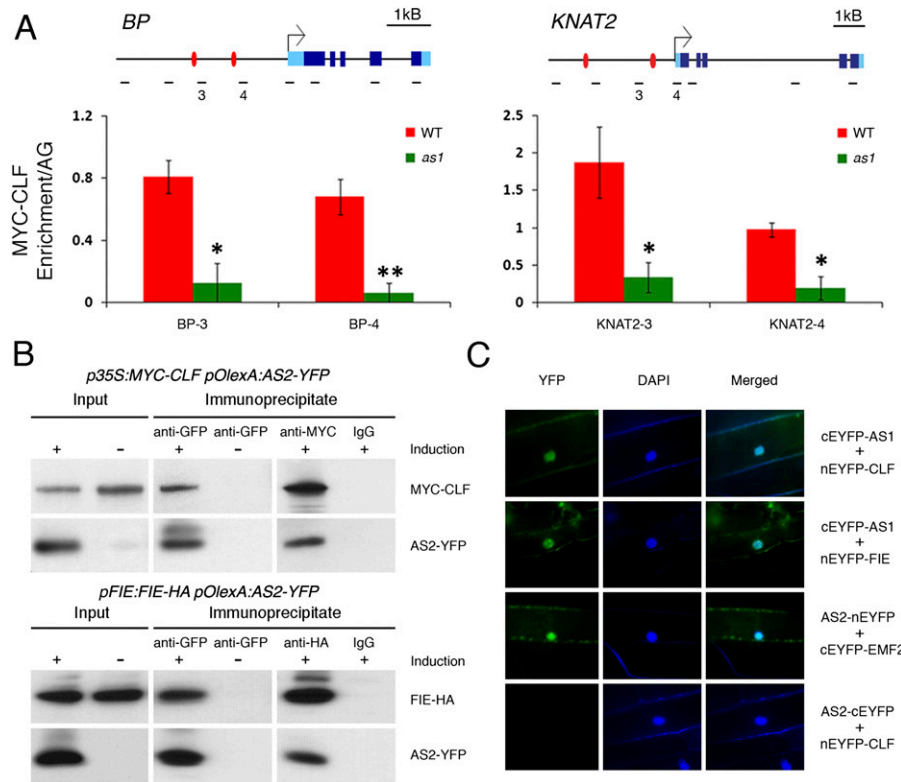


Figure 3. The AS1–AS2 complex physically interacts with PRC2 and directly recruits this complex to *BP* and *KNAT2*. (A) ChIP analyses showing CLF occupancy at specific regions of *BP* and *KNAT2*. CLF occupancy at these sites is significantly reduced in *as1* compared with wild type. (*) $P < 0.05$; (**) $P < 0.01$. Values (mean \pm 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) coimmunoprecipitate with AS2-YFP specifically upon induction. AS2-YFP also coimmunoprecipitates with MYC-CLF (*top* panel) and FIE-HA (*bottom* panel) in immunoprecipitation assays with anti-MYC and anti-HA antibody, respectively, but not in control immunoprecipitation assays with IgG. Antibodies used in immunoprecipitation 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) YFP signal monitoring protein–protein interactions. (*Middle* panels) DAPI staining indicating positions of nuclei. (*Right* panels) Merged images.

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 from 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. Given the role of *COLDAIR* in recruitment of PRC2 to

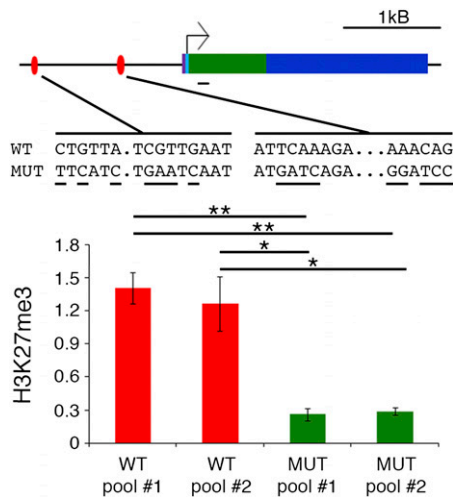


Figure 4. AS1–AS2-binding sites are necessary and sufficient for recruitment of PRC2 activity. ChIP analysis showing that H3K27me3 levels are enriched at the *GFP-GUS* reporter in lines where the transgene contains wild-type AS1–AS2-binding sites in the promoter. H3K27me3 levels are significantly reduced 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 \pm 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 amplicon 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-mediated silencing of *STM* and *WUS* occurs in an AS1–AS2 complex-independent manner (Guo et al. 2008; Liu et al. 2011). Considering the broad repertoire of processes controlled by Polycomb proteins, diversity in PRC2 recruitment mechanisms is certainly expected. Likewise, animal Polycomb proteins are known to interact with multiple distinct recruitment proteins to generate target specificity (Schwartz et al. 2006; Schuettengruber and Cavalli 2009; Margueron and Reinberg 2011).

A framework for Polycomb-mediated epigenetic repression of KNOX homeobox genes

The AS1–AS2 complex binds two sites in the *BP* and *KNAT2* promoters (Guo et al. 2008). These sites act

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 predicted to drive the propagation 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 μ MACS 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 μ L of benzonase (250 U/ μ L) 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 μ g of each plasmid DNA was coated onto 1 μ M gold particles and bombarded into onion epidermal cells using a PDS-1000/He Biolistic particle delivery system (Bio-Rad) 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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