JIPB Journal of Integrative Plant Biology

Transcription factors AS1 and AS2 interact with LHP1 to repress *KNOX* genes in *Arabidopsis*

Zhongfei Li[1†], Bin Li[1†], Jian Liu[2], Zhihao Guo[1], Yuhao Liu[1], Yan Li[3], Wen-Hui Shen[1,4], Ying Huang[3], Hai Huang[2], Yijing Zhang[2*] and Aiwu Dong[1*]

[1]State Key Laboratory of Genetic Engineering, International Associated Laboratory of CNRS-Fudan-HUNAU on Plant Epigenome Research, Collaborative Innovation Center for Genetics and Development, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, China, [2]National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China, [3]National Center for Protein Science Shanghai, State Key Laboratory of Molecular Biology, Shanghai State Key Laboratory of Molecular Andrology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China, [4]Institut de Biologie Moléculaire des Plantes du CNRS, Université de Strasbourg, 12 rue du Général Zimmer, 67084 Strasbourg Cédex, France. [†]These authors contributed equally to this work. *Correspondences: aiwudong@fudan.edu.cn (Dr. Aiwu Dong is fully responsible for distributions of all materials associated with this article); zhangyijing@sibs.ac.cn

Research Article

Abstract Polycomb group proteins are important repressors of numerous genes in higher eukaryotes. However, the mechanism by which Polycomb group proteins are recruited to specific genes is poorly understood. In *Arabidopsis*, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), also known as TERMINAL FLOWER 2, was originally proposed as a subunit of polycomb repressive complex 1 (PRC1) that could bind the tri-methylated lysine 27 of histone H3 (H3K27me3) established by the PRC2. In this work, we show that LHP1 mainly functions with PRC2 to establish H3K27me3, but not with PRC1 to catalyze monoubiquitination at lysine 119 of histone H2A. Our results show that complexes of the transcription factors ASYMMETRIC LEAVES 1 (AS1) and AS2 could help to establish the H3K27me3 modification at the chromatin regions of Class-I *KNOTTED1*-like homeobox (*KNOX*) genes *BREVIPEDICELLUS* and *KNAT2* via direct interactions with LHP1. Additionally, our transcriptome analysis indicated that there are probably more common target genes of AS1 and LHP1 besides Class-I *KNOX* genes during leaf development in *Arabidopsis*.

Keywords: *Arabidopsis thaliana*; ASYMMETRIC LEAVES 1; ASYMMETRIC LEAVES 2; LIKE HETEROCHROMATIN PROTEIN 1; H3K27me3 establishment; Class-I *KNOX* genes
Citation: Li Z, Li B, Liu J, Guo Z, Liu Y, Li Y, Shen WH, Huang Y, Huang H, Zhang Y, Dong A (2016) Transcription factors AS1 and AS2 interact with LHP1 to repress *KNOX* genes in *Arabidopsis*. **J Integr Plant Biol** XX: XX–XX doi: 10.1111/jipb.12485
Edited by: Catherine Kidner, University of Edinburgh, UK
Received Mar. 28, 2016; **Accepted** May 31, 2016
Available online on Jun. 7, 2016 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

Polycomb group (PcG) proteins are important regulators involved in numerous developmental processes in higher eukaryotes. They function by implementing the transcriptional silencing of specific genes. PcG proteins were first identified in *Drosophila* (Lewis 1978), and PcG proteins from many different species including animals and plants were subsequently characterized in detail (reviewed in Margueron and Reinberg 2011; Molitor and Shen 2013; Calonje 2014). PcG proteins can form two main protein complexes: polycomb repressive complex 1 (PRC1) and PRC2. It is generally thought that PRC2s catalyze tri-methylation at lysine 27 of histone H3 (H3K27me3) at target genes, while PRC1 is responsible for monoubiquitination at lysine 119 of histone H2A (H2Aub) and for nucleosome compaction (reviewed in Margueron and Reinberg 2011; Di Croce and Helin 2013).

Drosophila PRC2 consists of Enhancer of Zeste (E(z), a methyltransferase for H3K27me3), Suppressor of Zeste 12 (Su(z)12), Extra sex combs (Esc), and P55 (Simon and Kingston 2013). Subunits of PRC2 in *Arabidopsis* are conserved with those in *Drosophila* and mammals. CURLY LEAF (CLF), MEDEA (MEA/FIS), and SWINGER (SWN) are three E(z) homologs in *Arabidopsis* and all are thought to have H3K27me3 methyltransferase activity (Goodrich et al. 1997; Grossniklaus 1998; Chanvivattana 2004). The three homologs of Su(z)12 are EMBRYONIC FLOWER 2 (EMF2), VERNALIZATION 2 (VRN2), and FERTILIZATION INDEPENDENT SEED (FIS2). In *Arabidopsis*, the Esc homolog is FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and the P55 homolog is MULTIPLE SUPPRESSOR OF IRA 1 (MSI1). These various PRC2 subunits likely form three PRC2s: EMF-PRC2 (including CLF/SWN, EMF2, FIE and MSI1), VRN-PRC2 (including CLF/SWN, VRN2, FIE and MSI1), and FIS-PRC2 (including MEA, FIS2, FIE and MSI1). EMF-PRC2, VRN-PRC2 and FIS-PRC2 control sporophytic development, flowering transition induced by vernalization, and female gametophyte and seed development, respectively (Mozgova et al. 2015).

Compared with those of PRC2, the core components of PRC1 are less conserved among *Drosophila*, mammals and plants. The classical PRC1 core components in *Drosophila* include polycomb (Pc, can associate with H3K27me3), polyhomeotic (Ph), posterior sex comb (Psc) or Su(z)2, and dRing1 (Shao et al. 1999; Francis et al. 2001). To date, two homologs of dRing1 (AtRING1a and AtRING1b) and three homologs of Psc (AtBMI1a, AtBMI1b and AtBMI1c) have been

identified in *Arabidopsis*. They all have a RING-domain, show E3 monoubiquitin ligase activity for H2A, and mediate PcG-related gene silencing (Xu and Shen 2008; Bratzel et al. 2010; Li et al. 2011). Based on the results of sequence alignment analyses, there are no homologs of Pc or Ph in *Arabidopsis*. However, an *Arabidopsis* protein, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), also called TERMINAL FLOWER 2 (TFL2), was found to have a Pc-like function. LHP1 is a homolog of HETEROCHROMATIN PROTEIN 1 (HP1) in animals. HP1 has an N-terminal CHROMO domain that binds to H3K9me3, and a C-terminal CHROMOSHADOW domain (CSD) (Kotake et al. 2003). Unlike HP1, LHP1 binds to both methylated H3K9 and H3K27 *in vitro*, and specifically co-localizes with H3K27me3-enriched chromatin regions (Turck et al. 2007; Zhang et al. 2007a). In addition, LHP1 was shown to physically interact with all five RING-domain proteins (Xu and Shen 2008; Bratzel et al. 2010; *Arabidopsis* Interactome Mapping Consortium 2011), suggesting that LHP1 is a component of PRC1 in *Arabidopsis*. However, a research has shown that LHP1 is needed to establish full H3K27me3 levels at PcG targets through direct interaction with the PRC2 subunit MSI1 (Derkacheva et al. 2013), and more recently, LHP1 was proved to be co-purified with PRC2 and impact H3K27me3 levels at thousands of loci (Wang et al. 2016).

Although PcG proteins are known to play important roles in transcriptional silencing, less is known about how PcG proteins are recruited to target genes. Both in *Drosophila* and mammals, some transcription factors recruit PRC2 to specific targets via binding to characteristic DNA sequences, the so-called polycomb response elements (PREs) (Schwartz et al. 2006; Sing et al. 2009; Woo et al. 2010). Several studies in mammals suggested that long ncRNAs participate in recruiting PRC2 to target genes through physical interactions (Zhao et al. 2008; Maenner et al. 2010). Similarly, in *Arabidopsis*, an ncRNA, COLDAIR, conscribes PRC2 to *FLOWERING LOCUS C* chromatin during vernalization (Heo and Sung 2011). The recruitment of PRC1 was thought to result from the interaction between Pc or Pc-like proteins and H3K27me3 established by PRC2. However, this hierarchical model has been challenged by the results of recent studies, in which the recruitment of the PRC1 complex was shown to be independent of H3K27me3 both in animals and plants (Schoeftner et al. 2006; Yu et al. 2012; Yang et al. 2013). In such cases, the subunits of PRC1 can be led to target loci by either transcription factors or ncRNAs and mediate gene repression independent of, or even prior to, the establishment of H3K27me3 by PRC2 (Schoeftner et al. 2006; Yu et al. 2012; Yang et al. 2013).

BREVIPEDICELLUS (*BP*/*KNAT1*) and *KNAT2* are members of the Class-I *KNOTTED 1*-like homeobox (*KNOX*) family, which are expressed in the shoot apical meristem (SAM) to maintain SAM activity but strictly repressed in the leaf (Hay and Tsiantis 2010). In leaves, these two genes are marked with high levels of H3K27me3 and are repressed by both PRC2 and PRC1 (Zhang et al. 2007b; Xu and Shen 2008; Bouyer et al. 2011). However, little is known about how PRC2 and PRC1 are recruited to *KNOX* genes. ASYMMETRIC LEAVES 1 (AS1) and AS2 are two transcription factors that can form protein complex and directly bind to the promoters of *BP* and *KNAT2* to silence these genes in leaves of *Arabidopsis* (Guo et al. 2008). A further study showed that AS1 and AS2 can recruit the PRC2 to *BP* and *KNAT2* loci to build H3K27me3 (Lodha et al. 2013).

Here, we showed that the transcription factors AS1/AS2 directly interact with LHP1 *in vitro* and *in vivo*. Chromatin immunoprecipitation (ChIP) analyses revealed that LHP1 functions in the establishment of H3K27me3 but not H2Aub on many PcG target genes. Loss-of-function of AS1 or AS2 impaired the enrichment of both LHP1 and H3K27me3 at *BP* and *KNAT2* loci. Since recent studies showed that LHP1 also directly interacts with MSI1 and can be co-purified with PRC2 (Derkacheva et al. 2013; Wang et al. 2016), we propose that AS1/AS2 can recruit PRC2 to *BP* and *KNAT2* loci to establish H3K27me3 via a direct interaction with LHP1. Furthermore, analyses of large-scale transcriptome data indicated that this kind of gene regulation is likely to be wide-ranging.

RESULTS

AS1 and AS2 interact with LHP1 *in vitro* and *in vivo*

The AS1-AS2 pair is always used as the positive control for yeast two-hybrid assays in our laboratory (Xu et al. 2003). Once when we tested the interaction between LHP1 and its binding proteins, the LHP1-AS1 pair was designed as the negative control, but to our surprise, we found that LHP1 interacted with AS1 in yeast cells (Figure 1A). This unexpected finding drove us to search for the function of the LHP1-AS1 interaction. We also tested the interaction between LHP1 and AS2, and found that they directly interacted, although the binding activity of the AS2-LHP1 pair was not as strong as that of the AS1-LHP1 pair (Figure 1A). To confirm the interaction between the bait and prey, we performed a quantitative measurement by using the β-galactosidase activity assay. Consistent with the yeast two-hybrid assay, the β-galactosidase activity assay showed that there was a relatively strong AS1-LHP1 interaction but a weaker AS2-LHP1 interaction (Figure 1A).

To provide additional evidence for the direct interaction between AS1/AS2 and LHP1, we conducted glutathione S-transferase (GST) pulldown assays using beads coated with purified GST or GST-LHP1 and purified recombinant proteins of his-tagged AS1, AS2, and NRP1. Here, GST and NRP1 served as negative controls. GST-LHP1 directly bound to AS1 and AS2, but not to NRP1, while GST alone failed to bind to AS1, AS2, or NRP (Figure 1B). To further confirm the protein–protein interactions between AS1/AS2 and LHP1, we performed a GST pulldown assay using beads coated with purified GST, GST-AS1, or GST-AS2, and total protein extracts from transgenic plants expressing YFP or LHP1-YFP under the control of the estrogen-inducible promoter (Zuo et al. 2000). As shown in Figure 1C, a LHP1-YFP signal was detected in the fraction pulled down by GST-AS1 or GST-AS2 beads, but not in that pulled down by GST beads, and YFP alone was not pulled down by any of the GST, GST-AS1, or GST-AS2 beads.

To test the interactions between AS1/AS2 and LHP1 *in planta*, we performed bimolecular fluorescence complementation (BiFC) assays. The coding regions of *AS1*/*AS2* and *LHP1* were fused with the N- and C-terminal fragment of *YFP*, respectively, and *AS1*/*AS2*-n*YFP* in combination with c*YFP-LHP1* was co-transfected into tobacco leaves. The constructs of n*YFP* or c*YFP* together with c*YFP-LHP1* or *AS1*/*AS2*-n*YFP* were used as negative controls. We observed YFP fluorescence in

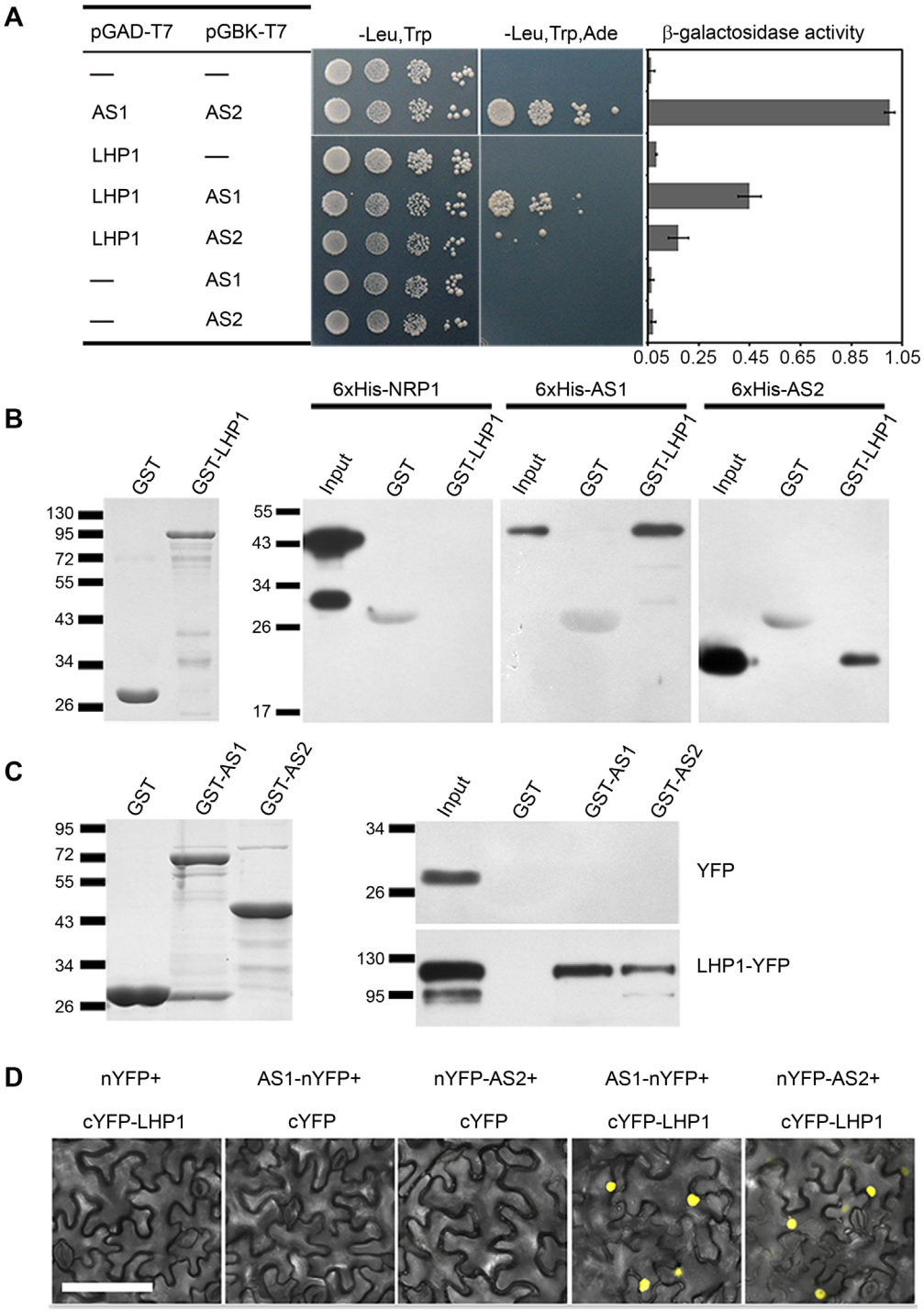

Figure 1. AS1 and AS2 physically interact with LHP1 *in vitro* and *in vivo*

(**A**) Yeast two-hybrid assays showing interactions between AS1/AS2 and LHP1. Yeast cells carrying different fusion protein combinations are listed in left panels. Middle panels show growth of diluted (×10) yeast cells expressing indicated proteins on media lacking leucine and tryptophan (–Leu, Trp) or lacking leucine, tryptophan, and adenine (–Leu, Trp, Ade). Right-hand panel shows relative β-galactosidase activities in yeast cells expressing different fusion proteins. AS1/AS2 pair served as positive control. (**B**) *In vitro* GST pull-down assay. Purified His-tagged AS1/AS2 or NRP1 (negative control) was incubated with equal quantity of beads coated with GST or GST-LHP1, respectively. One fifth of input was loaded in the input lanes. (**C**) *In vitro* GST pull-down assay. Total protein extracts from transgenic *Arabidopsis* plants expressing YFP or LHP1-YFP (right panel) were incubated with an equal quantity of beads coated with GST, GST-AS1, or GST-AS2 proteins (left panel), respectively. Pull-down fractions were detected by polyclonal anti-GFP antibody, which cross-reacts with YFP. (**D**) BiFC analysis of interactions between AS1/AS2 and LHP1 in tobacco leaf cells. Bar = 50 μm.

the nuclei of tobacco leaves co-transformed with *AS1*/*AS2*-n*YFP* and c*YFP-LHP1*, but not in those of tobacco leaves transformed with the negative controls (Figure 1D). These BiFC results indicated that AS1/AS2 can interact with LHP1 *in planta*.

We further analyzed the domains through which LHP1 interacted with AS1 and AS2 by yeast two-hybrid and pulldown assays. The yeast two-hybrid results showed that the C-terminal of LHP1 (LHP1-C, 162−445 AA), not the N terminal (LHP1-N, 1−194 AA), interacted with AS1/AS2, and the CSD of LHP1 (LHP1-CSD, 378−445 AA) was sufficient for the interaction (Figure S1A). The pulldown experiments confirmed that SUMO-LHP1-CSD but not SUMO pulled down purified AS1 (1−158 AA) and AS2 (Figure S1B). Here we used AS1 (1−158 AA) which also interacted with LHP1 similar to the full-length AS1.

Taken together, our results prove that AS1/AS2 physically interact with LHP1 *in vitro* and *in vivo*.

Genetic interactions between *as1-1*/*as2-1* and *tfl2-1*

To investigate the biological significance of the physical interactions between AS1/AS2 and LHP1, we constructed *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants by genetic crossing. Compared with the wild-type Col-0, *as1-1* and *as2-1* single mutants produced smaller rosette leaves with asymmetrical leaf lobes and leaflet-like structures on some petioles (Figure 2A–C, J, K), which were caused by the derepression of Class-I *KNOX* genes. The single *tfl2-1* mutant also produced smaller rosette leaves (Figure 2D, L). These phenotypes were enhanced in *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants, which produced much smaller rosette leaves and more lobes on rosette leaves, compared with those of single mutants (Figures 2E–H, M–P, S2). The relationship between the derepression of Class-I *KNOX* genes and the phenotypes of the single and double mutants prompted us to analyze the expression levels of *BP*, *KNAT2*, and *KNAT6* in 12-day-old seedlings of these mutants by quantitative RT-PCR. As shown in Figure 3A, compared with those in wild-type, the expression levels of Class-I *KNOX* genes were elevated in *as1-1*, *as2-1*, *tfl2-1* single mutants, and further elevated in *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants. These data were consistent with the enhanced leaf phenotypes of the double mutants.

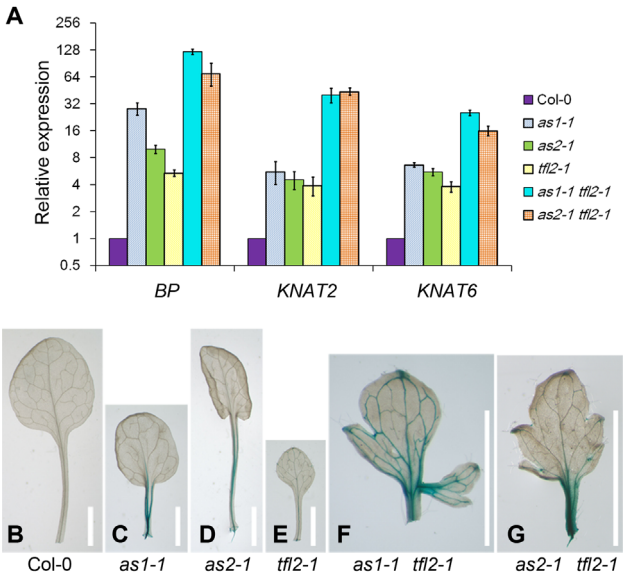

Figure 3. Enhanced ectopic expression of Class-I *KNOX* genes in *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants

(**A**) Results of qRT-PCR analyses of transcription levels of mRNA of *BP*, *KNAT2*, and *KNAT6* in rosette leaves. Transcript level of each gene was normalized to that of *UBQ10*, then to value of the wild-type Col-0. Error bars show standard deviation from three biological replicates. (**B–G**) Expression patterns of plants harboring BP:GUS fusion: GUS staining in fifth rosette leaf of Col-0 (**B**), *as1-1* (**C**), *as2-1* (**D**), *tfl2-1* (**E**), *as1-1 tfl2-1* (**F**), and *as2-1 tfl2-1* (**G**). Scale bars: 1 cm.

To further examine the spatiotemporal expression pattern of *BP* in detail, we introduced the β-glucuronidase (GUS) expression reporter *pBP:GUS* (Li et al. 2012) into the *as1-1*, *as2-1*, *tfl2-1*, *as1-1 tfl2-1* and *as2-1 tfl2-1* backgrounds. The reporter gene was fully silenced in wild-type leaves (Figure 3B), whereas GUS activity was detected in the petioles of *as1-1* and *as2-1* (Figure 3C, D), consistent with the results of a previous study (Ori et al. 2000). GUS activity was also detected in *tfl2-1*

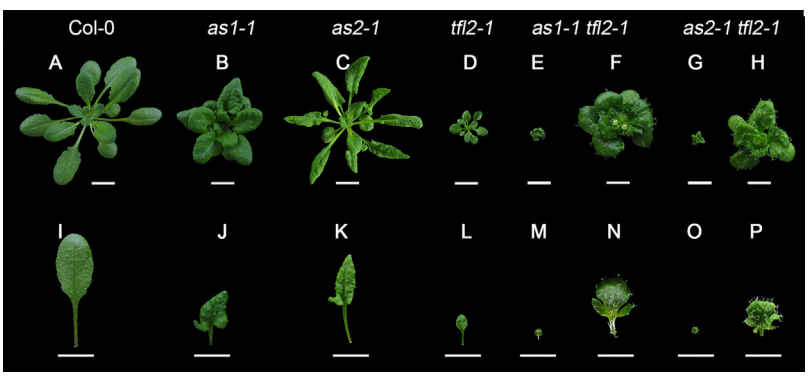

Figure 2. Enhanced *as1-1*, *as2-1* and *tfl2-1* leaf phenotypes in *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants

Twenty-two-day-old plants of wild-type Col-0 (**A**), *as1-1* (**B**), *as2-1* (**C**), *tfl2-1* (**D**), *as1-1 tfl2-1* (**E, F**) and *as2-1 tfl2-1* (**G, H**). Fourth rosette leaf of 22-day-old Col-0 (**I**), *as1-1* (**J**), *as2-1* (**K**), *tfl2-1* (**L**), *as1-1 tfl2-1* (**M, N**), or *as2-1 tfl2-1* (**O, P**). Scale bars: 1 cm in (**A, B, C, D, E, G, I, J, K, L, M, O**), 0.2 cm in (**F, H, N, P**).

(Figure 3E). GUS staining was markedly stronger in the *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants than in the single mutants, and the GUS staining extended into almost all leaf veins (Figure 3F, G).

These results together indicated that LHP1 and AS1/AS2 synergistically regulated the transcription of class I *KNOX* genes.

AS1/AS2-dependent establishment of H3K27me3 at *BP* and *KNAT2* loci via the interaction between AS1/AS2 and LHP1

LHP1 is thought to be a component of PRC1 in *Arabidopsis* as it recognizes H3K27me3 and binds to other PRC1 subunits such as AtRINGs and AtBMI1s. Recently, however, a study showed that LHP1 co-purified with PRC2 and participated in the establishment of H3K27me3 on PcG targets in dividing cells (Derkacheva et al. 2013), raising the question as to whether LHP1 functions with PRC1 or with PRC2. To determine whether LHP1 functions with PRC1, we first checked the levels of H2Aub, which is established by the PRC1 complex, on several known PcG target genes, including *LEAFY COTYLEDON 1* (*LEC1*), *ABA INSENSITIVE 3* (*ABI3*), *BABY BOOM* (*BBM*), *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), and *AGAMOUS* (*AG*), as well as *BP* and *KNAT2*, using whole seedlings. Compared with the H2Aub levels in wild-type, those in *tfl2-1* or *clf-29* (here *clf-29* was used as a control) were significantly increased at the transcriptional start site (TSS) regions of the above genes (Figure 4A), similar to those in the *clf/swn* double mutant (Yang et al. 2013), indicating that LHP1 likely does not function with PRC1. In contrast, the H3K27me3 levels on PcG targets in *tfl2-1* or *clf-29* were obviously decreased (Figure 4B), consistent with previous studies (Derkacheva et al. 2013; Wang et al. 2016), supporting the idea that LHP1 is involved in establishing H3K27me3 in plant. These results supported that LHP1 does not function as a classic PRC1 member to mediate H2Aub, but functions as a component of PRC2 to participate in the establishment of H3K27me3.

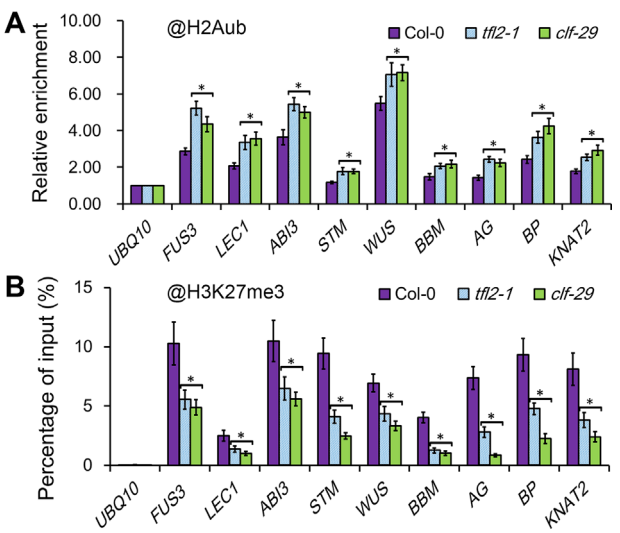

Figure 4. LHP1 does not function in PRC1 to catalyze H2Aub but with PRC2 to establish H3K27me3

(**A**) ChIP analyses using rabbit monoclonal anti-hH2Aub antibody. Results were normalized to that of *UBQ10*. (**B**) ChIP analyses using polyclonal anti-trimethyl-H3K27 antibody. In ChIP experiments, 12-day-old plants grown on agar-solidified MS medium were used. Error bars show standard deviation from three biological replicates. Asterisks indicate statistically significant differences between indicated genotypes and wild-type (Student's t-test, $P < 0.05$).

AS1/AS2 complex was reported to bind to the promoters of *BP* and *KNAT2* and recruit the PRC2 to these two genes (Guo et al. 2008; Lodha et al. 2013). Here, based on our protein–protein interaction results, we speculated that AS1/AS2 complex may also recruit the PRC2 to the *BP* and *KNAT2* loci to establish H3K27me3 via direct interaction with LHP1. Firstly, we analyzed H3K27me3 levels at the *BP* and *KNAT2* loci in *as1-1*, *as2-1*, and *tfl2-1* mutants. When 12-day-old whole seedlings grown on agar-solidified MS medium were used in the ChIP analysis, there were no differences in H3K27me3 levels at *BP* and *KNAT2* loci between the *as1-1* or *as2-1* mutants and wild-type, but the level of H3K27me3 at those loci was clearly decreased in *tfl2-1* (Figure S3). Considering that *BP* was mainly derepressed in the petioles of *as1-1* and *as2-1* (Figure 3), we suspected that these unchanged H3K27me3 levels in *as1-1* and *as2-1* resulted from the use of mixed materials (whole seedlings) in the ChIP assays. Therefore, we specifically harvested the petioles of the leaves from 22-day-old seedlings grown in soil for use in further ChIP experiments. The density of H3 was unchanged in the different mutants (Figure 5B); however, H3K27me3 levels at *BP* and *KNAT2* loci were markedly decreased in *as1-1* and *as2-1* (Figure 5C), as compared with those in wild-type. These results were consistent with those reported in a previous work (Lodha et al. 2013). The H3K27me3 levels at *BP* and *KNAT2* were also decreased in *tfl2-1*, to levels similar to those in *as1-1* and *as2-1* mutants (Figure 5C). These results indicated that AS1/AS2 complex and LHP1 are together involved in establishing H3K27me3 at *BP* and *KNAT2* loci in petioles.

Next, we investigated the binding activity of LHP1 to *BP* and *KNAT2* in *as1-1* and *as2-1* mutants. Transformation with *35S::LHP1-EYFP* perfectly complemented the phenotypes of the *tfl2-1* mutant, indicating that the LHP1-YFP fusion protein functioned well *in planta*. We crossed the transgenic *35S:LHP1-EYFP/tfl2-1* plants with *as1-1*, *as2-1* and wild-type Col-0 to express the YFP-tagged LHP1 protein in different backgrounds for further ChIP assays. Again, we specifically harvested the petioles as materials for the ChIP experiments. As shown in Figure 5D, the enrichment of LHP1-YFP at *BP* and *KNAT2* chromatin was significantly decreased in *as1-1* and *as2-1* mutants. These results were also consistent with Lodha's previous work, which reported that the occupancy levels of LHP1 at *BP* and *KNAT2* decreased in *as2-1* (Lodha et al. 2013). Combining the LHP1 occupancy analyses and H3K27me3 enrichment analyses described above, we propose that AS1/AS2 complex directly recruits PRC2 to the chromatin of *BP* and *KNAT2* through an interaction with LHP1, and then establishes H3K27me3.

AS1 and LHP1 co-repress a set of genes through a PcG-related pathway

Given that AS1 and LHP1 could form a complex to repress *KNOX* genes, we wondered whether AS1 and LHP1 could also co-regulate other genes. We compared the transcriptomic

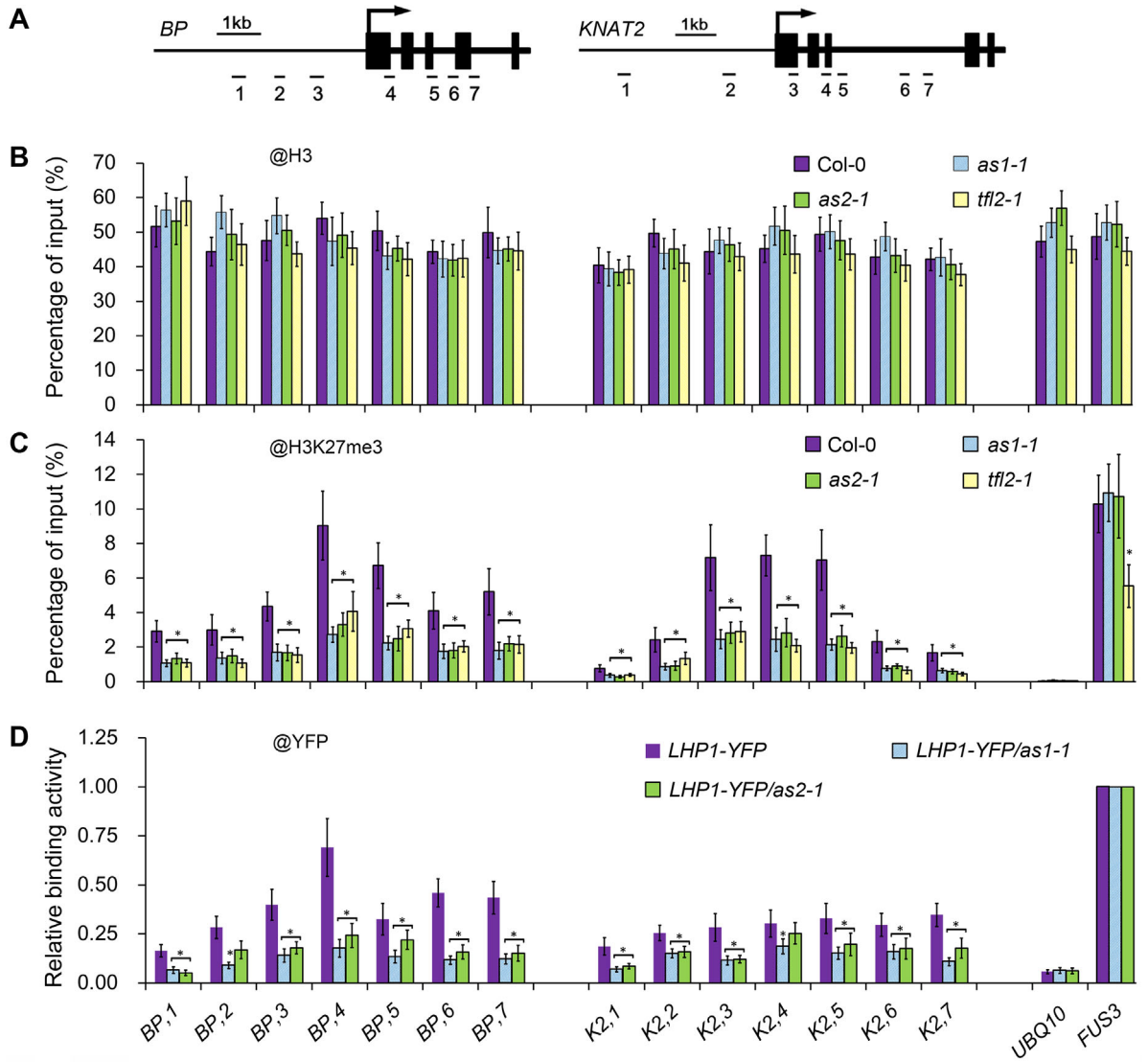

Figure 5. AS1-AS2 complex establishes H3K27me3 at *BP* and *KNAT2* loci via interacting with LHP1
(**A**) Diagrams of *BP* and *KNAT2* gene structures. Black boxes indicate exons, long and thin black lines show promoter regions, thick black lines indicate introns, and numbers with short black lines indicate PCR fragments corresponding to genomic regions. (**B**) ChIP analyses using polyclonal anti-H3 antibody. (**C**) ChIP analyses using polyclonal anti-trimethyl-H3K27 antibody. (**D**) ChIP analyses using polyclonal anti-YFP antibody. Results were normalized to that of *FUS3*. In these ChIP experiments, petioles from plants grown on soil were used. Error bars show standard deviation from three biological replicates. Asterisks indicate statistically significant differences between indicated genotypes and wild-type (in **C**) or *35S::LHP1-Y* (in **D**) (Student's *t*-test, $P < 0.05$).

changes in 12-day-old seedlings of *as1-1* and *tfl2-1* single mutants and the *as1-1 tfl2-1* double mutant. The 3,459 genes with differential expression were partitioned into five groups based on gene expression changes across the three mutants (Figure 6A, also see Materials and Methods). Because AS1 was reported to function as a transcriptional repressor (Guo et al. 2008) and the polycomb subunit LHP1 also participates in gene silencing, we focused on the 279 co-upregulated genes in *as1-1*, *tfl2-1* and *as1-1 tfl2-1* mutants (genes in group II), as potential common target genes of LHP1 and AS1. To find evidence that AS1 and LHP1 regulate these genes in the same way as they regulate *BP* and *KNAT2*, we compared a total of 279 genes in group II with published H3K27me3 ChIP-seq data. We found that 99 genes (35.5%) in group II were marked with the H3K27me3 modification (Figure 6B), a significantly higher proportion than that of H3K27me3-marked genes (16.3%) on a genome-wide scale in *Arabidopsis* (Zhang et al. 2007b). To further validate the above analyses, we randomly selected 10 genes from group II with the H3K27me3 modification (*LEA*, *CYP71B31*, *ERF12*, *LecRK-III.2*, *IPT7*, *At5g21960*, *At1g15330*, *At5g28520*, *At2g35460* and *At5g17730*) for qRT-PCR analysis. The results showed that these 10 genes were all up-

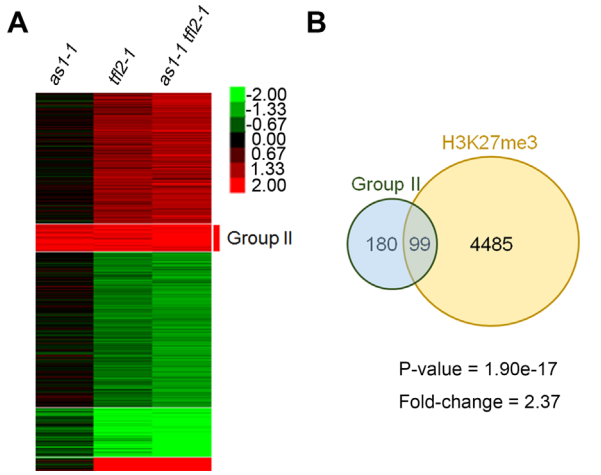

Figure 6. AS1 and LHP1 co-repress a set of target genes through a PcG-related pathway
(**A**) K-means clustering of transcriptomic changes in *as1-1*, *tfl2-1*, and *as1-1 tfl2-1* as compared with Col-0. (**B**) Genes in group II are preferentially marked by H3K27me3 in Col-0.

regulated in *as1-1*, *tfl2-1* and *as1-1 tfl2-1* (Figure 7A), consistent with our microarray data. There was obvious enrichment of H3K27me3 at the 5' ends of these genes (Figure 7B). Furthermore, LHP1 associated with these genes (Figure 7C), indicating that they were targets of PcG proteins. Taken together, these data suggest that AS1 and LHP1 likely co-repress a wide range of target genes through a PcG-related pathway.

DISCUSSION

In *Arabidopsis*, AS1 and AS2 are important regulators in leaf development. They modulate proximal-distal leaf length by directly repressing Class-I *KNOX* genes (Guo et al. 2008; Ikezaki et al. 2010), and maintain proper adaxial–abaxial polarity by directly or indirectly repressing abaxial genes, such as *ETTIN(ETT)/AUXIN RESPONSE FACTOR3(ARF3)*, *ARF4*, *KANADI2*, *YABBY5* (Iwakawa et al. 2007; Iwasaki et al. 2013; Husbands et al. 2015). The simultaneous deletion of AS1/AS2 co-factors in repression of *ARF* genes would enhance the adaxial–abaxial polarity defects in *as1*/*as2* single mutants, usually displaying filamentous leaves due to the extreme expansion of abaxialized epidermis (Li et al. 2005; Xu et al. 2006; Nakata et al. 2012; Machida et al. 2015). In this study, we demonstrated that LHP1 acts as a new co-factor of AS1/AS2. The *as1-1 tfl2-1* or *as2-1 tfl2-1* double mutants showed much smaller rosette leaves instead of filamentous leaves compared with the *as1* or *as2* single mutants, indicating that LHP1 is more likely involved in regulating proximal-distal polarity but not adaxial–abaxial polarity during leaf development via repression of Class-I *KNOX* genes.

AS1 /AS2 complex directly binds to the promoters of Class-I *KNOX* genes to repress their transcription (Guo et al. 2008). Further Co-IP and BiFC experiments showed that AS1/AS2 complex associates with PRC2, hence recruiting PRC2 to the

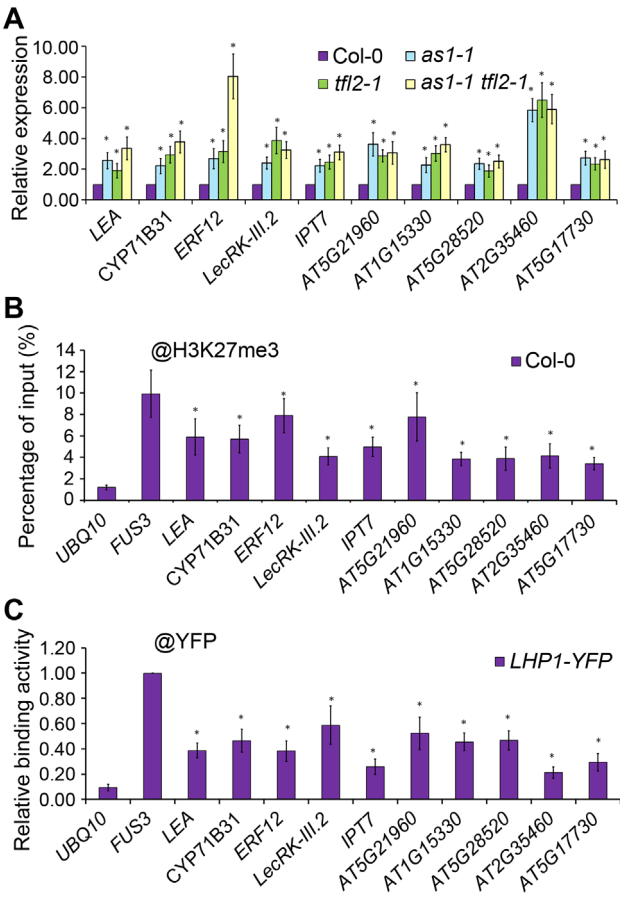

Figure 7. Validation of large-scale analysis
(**A**) qRT-PCR analyses of transcript levels of *LEA*, *CYP71B31*, *ERF12*, *LecRK-III.2*, *IPT7*, *At5g21960*, *At1g15330*, *At5g28520*, *At2g35460* and *At5g17730* in *as1-1*, *tfl2-1* and *as1-1 tfl2-1*. Transcript level of each gene was normalized to that of *UBQ10*, and then to value of the wild-type Col-0. (**B**) ChIP analyses using polyclonal anti-trimethyl-H3K27 antibody. (**C**) ChIP analyses using polyclonal anti-YFP antibody. Results were normalized to that of *FUS3*. (**A–C**) Twelve-day-old plants grown on agar-solidified MS medium were used. Error bars show standard deviation from three biological replicates. Asterisks indicate statistically significant differences between indicated genotypes and wild-type (**A**) or between indicated gene and *UBQ10* (**B** and **C**) (Student's *t*-test, $P < 0.05$).

Class-I *KNOX* genes to establish repressive H3K27me3 mark (Lodha et al. 2013). Since the direct interaction between AS1/AS2 and subunits of the PRC2 is lacking (Lodha et al. 2013), it is still unclear whether this association is direct or mediated by other protein(s). Here, we provided lines of evidence that AS1 and AS2 directly interact with LHP1 and thereby recruit PRC2 to the target genes.

Based on sequence analyses, *Arabidopsis* LHP1 was originally thought to be a member of the HP1 subfamily (Kotake et al. 2003), but its function differed from that of its orthologs in animals. Whereas HP1 functions as a reader of H3K9me3, LHP1 binds H3K27me3 via its CHROMODOMAIN and

co-localizes with H3K27me3 genome-wide (Turck et al. 2007). Furthermore, LHP1 interacts with other PRC1 proteins in *Arabidopsis*, including the RING-domain proteins AtRING1a/AtRING1b (Xu and Shen 2008) and AtBMI1a/AtBMI1b/AtBMI1c (Bratzel et al. 2010; *Arabidopsis* Interactome Mapping Consortium 2011), suggesting that LHP1 may serve as a core component of PRC1 in *Arabidopsis*, similar to the animal Pc. A recent study showed that H2Aub was almost undetectable in the PRC1 mutant *atbmia/b/c* but present at higher levels in the PRC2 mutant *clf/swn* than in WT (Yang et al. 2013). This work demonstrates that PRC1-mediated H2Aub also exists in *Arabidopsis*, and that PRC1 and PRC2 can regulate each other's activity, as the loss of one activity promotes that of the other. Strikingly, we found that in *tfl2-1*, the H2Aub levels were clearly increased on all genes we tested (Figure 4A), like those in *clf-29* or *clf/swn*. This finding indicates that LHP1 does not function with AtRINGs or AtBMIs in the classical PRC1. On the contrary, the H3K27me3 levels were significantly decreased on many PcG targets in *tfl2-1* (Figure 4B). This is consistent with recent reports that LHP1 co-purified with PRC2 and was required to establish full levels of H3K27me3 on PcG target genes (Derkacheva et al. 2013; Wang et al. 2016). The researchers also found that the changes in the transcriptomes of the *lhp* and *clf* mutants were highly correlated (Derkacheva et al. 2013), and that LHP1 functioned as a co-factor with CLF in H3K27me3 elongation (Wang et al. 2016). Together, these data and observations strongly suggest that LHP1 does not function in PRC1 but functions with PRC2 and likely helps to recruit PRC2 to target genes to reinforce, or even initiate, the H3K27me3 modification.

To date, several LHP1-binding proteins have been identified in *Arabidopsis*. Besides the direct interaction between AS1/AS2 and LHP1 observed in this study, SHORT VEGETATIVE PHASE (SVP), a MADS box transcription factor, can recruit LHP1 to the *SEPALLATA 3* (*SEP3*) gene and repress *SEP3* transcription, consequently preventing premature differentiation of floral meristems (Liu et al. 2009). Also, SCARECROW (SCR), one of the plant-specific GRAS family of transcription factors, interacts with LHP1 and co-represses *MAGPIE* to suppress premature middle cortex formation (Cui and Benfey 2009). AtCYP71, serving as a histone remodeling factor, binds to LHP1 and is needed to deposit LHP1 on some specific target genes (Li and Luan 2011). Two DNA polymerase (pol) catalytic subunits, INCURVATA2 (ICU2, a subunit of DNA pol alpha) and EARLY IN SHORT DAYS 7 (ESD7, a subunit of DNA pol epsilon) interact with LHP1 and participate in the repression of a set of target genes (Barrero et al. 2007; del Olmo et al. 2010). AG was also shown to guide LHP1 to *WUS* and then repress its expression, although in that case no direct protein interaction was observed (Liu et al. 2011). Very recently, BASIC PENTACYSTEINE6 (BPC6), a GAGA-Binding Factor, was reported to bind LHP1 and recruit it to target genes (Hecker et al. 2015). On the other hand, fewer PRC2-subunit-binding proteins have been identified, raising the possibility that LHP1 acts as a scaffold protein to recruit PRC2 to target genes. LHP1 may act via the following three pathways. First, through its recognition of H3K27me3; there is a similar example in the recruitment of mammalian PRC2, in which a core subunit of PRC2, EED, directly binds H3K27me3 to create a positive feedback loop of H3K27me3. The second pathway is through interactions with transcription factors (e.g., AS1/AS2, SVP, SCR, BPC6). Transcription factors bind to specific DNA elements and then recruit PcG proteins via LHP1 to initiate gene silencing. The third pathway is through interactions with a replication complex (e.g. ICU2, ESD7). It is likely that LHP1 can form a high-order complex with polymerase to recruit other PRC2 components to targets after DNA replication. In *Drosophila* embryos, PcG proteins but not H3K27me3 continuously associate with newly replicated DNA (Petruk et al. 2012). If this is also the case in *Arabidopsis*, LHP1 could function to recruit PRC2 to re-establish the H3K27me3 mark during cell division. Further identification of more LHP1-binding proteins will provide more information on these speculations.

It is noted that the ectopic expression of Class-I *KNOX* genes were markedly elevated in *as1-1 tfl2-1* and *as2-1 tfl2-1* double mutants compared with those single mutants (Figure 3), indicating that more factors are involved in the repression of Class-I *KNOX* genes in addition to LHP1-AS1/AS2 complex. Previous studies showed that AS1 and histone deacetylase HDA6 form complex to repress Class-I *KNOX* genes' expression through deacetylation (Luo et al. 2012). Our previous work also demonstrated that AS2 interacts with several TEOSINTE BRANCHED 1-CYCLOIDEA-PCF transcription factors (TCPs) to negatively regulate Class-I *KNOX* genes (Li et al. 2012). On the other hand, LHP1 has many target genes, among which there are many important regulators for different developmental processes. Therefore, it is highly possible that LHP1 could affect the expression of Class-I *KNOX* genes through modulating other regulators. Now it is known that the repression of Class-I *KNOX* genes is a complicated process and is related to multiple components including transcription factors, PcG proteins, and histone deacetylases. More research needs to be performed in the future for understanding the molecular mechanisms how Class-I *KNOX* genes are precisely regulated during plant development.

AS1/AS2 complex is crucial regulator during leaf transition from primordium, turning off three Class-I *KNOX* genes (*BP*, *KNAT2*, and *KNAT6*) that are specifically expressed in the meristem. Indeed, AS1/AS2 complex plays additional roles in leaf transition besides their direct regulation of Class-I *KNOX* genes. Our transcriptome data revealed that 279 genes were co-derepressed in *as1-1*, *tfl2-1*, and *as1-1 tfl2-1* mutants. Detailed analyses showed that among these 279 genes, many were marked by H3K27me3 and LHP1 (Figures 6B, 7B, C), indicating that AS1/AS2 complex might regulate more target genes by recruiting PcG proteins to initiate gene silencing. When comparing the 279 co-derepressed genes in *as1-1*, *tfl2-1*, and *as1-1 tfl2-1* mutants with those differentially expressed in the meristem (Lafos et al. 2011), we found that 60 out of 279 (21.5%) genes showed higher expression in the meristem, and only eight out of 279 (0.04%) genes showed lower expression in the meristem. In wild-type, the numbers of genes with higher or lower expression in meristem were similar, 2,780 and 2,930 genes, respectively, out of a total of 24,317 genes (Figure S4). These data suggest that AS1/AS2 complex can repress many more genes with higher expression levels in the meristem besides Class-I *KNOX* genes through the PcG system, and indicate a wider function of AS1/AS2 complex in regulating the transition from meristem to leaf.

MATERIALS AND METHODS

Plant materials and growth conditions

All *Arabidopsis* alleles used in this work were derived from the Columbia ecotype. The *as1-1* (CS3374), *as2-1* (CS3117), and *tfl2-1* (CS3796) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC, www.arabidopsis.org). Seeds of *BP::GUS*/Col-0 transgenic plants were kindly shared by S. Hake. The double mutants, *as1-1 tfl2-1* and *as2-1 tfl2-1*, were obtained by genetic crossing in our laboratory. Plants were grown at 22°C under a 16-h light / 8-h dark photoperiod. Plants were grown *in vitro* on agar-solidified MS medium M0255 (Duchefa, www.duchefa.com) with 0.9% sucrose at 22°C under a 16-h light / 8-h dark photoperiod.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the GAL4 system (Invitrogen, www.invitrogen.com) with the yeast strain PJ69-4A. The pGADT7-AS1, pGBKT7-AS1, pGBKT7-AS2 and pGADT7-LHP1 constructs were described previously (Xu et al. 2003; Xu and Shen 2008). pGADT7-LHP1-N, pGADT7-LHP1-C and pGADT7-LHP1-CSD constructs were described in previous work (Valérie Gaudin et al. 2001). The yeast two-hybrid assay was performed according to the manufacturer's protocol (Clontech, www.clontech.com). For β-galactosidase assay, yeast from at least four independent colonies for each combination were collected in the middle log phase and assayed following the manufacturer's recommendations (Clontech, www.clontech.com).

Transgene constructs and plant transformation

pER8-LHP1-YFP was constructed by fusing the open reading frames (ORFs) of *LHP1* and *EYFP*, and then subcloning the fused ORFs into pER8 (Zuo et al. 2000). pER8-LHP1-YFP was transformed into *Arabidopsis* plants to produce the LHP1-YFP fusion protein after induction with 4 μM estrogen. The fused *LHP1-YFP* fragment was also inserted into the pCAMBIA1301 vector by replacing the *GUS* fragment to produce *35S::LHP1-YFP*. This vector was then transformed into *tfl2-1*, resulting in *35S::LHP1-YFP/tfl2-1* plants. Transgenic plants of *35S::LHP1-YFP/tfl2-1* were then crossed with *as1-1*, *as2-1*, and wild-type Col-0 *Arabidopsis* to obtain plants expressing YFP-tagged LHP1 in different backgrounds. The primers used for the constructs above are listed in Table S1.

Production of recombinant proteins and pulldown assays

The pET-14b-AS1, pET-14b-AS2, pGEX-4T1-AS1, pGEX-4T1-AS2, and pGEX-4T1-LHP1 constructs were described previously (Xu et al. 2003; Xu and Shen 2008). The His-tagged AS1/AS2 and GST-fused AS1/AS2/LHP1 recombinant proteins were expressed and purified as described previously (Li et al. 2012). Purified His-tagged NRP1, AS1 or AS2 were incubated with GST- or GST-LHP1-coated glutathione-Sepharose 4B beads in pulldown buffer (25 mM Tris pH 7.5, 100 mM NaCl, 15 mM MgCl2, 10 mM EDTA, 5 mM EGTA, 1 mM NaF, 1mM DTT, 0.1% Tween-20, 5% glycerol, 10% BSA) on a rotation wheel for 2 h at 4 °C. The beads were collected by centrifugation and washed by pulldown buffer and the pulldown fractions were analyzed by Western blotting using a monoclonal anti-His antibody (Catalog NO. SG4110-38, Shanghai Genomics, www.shanghaigenomics.com). The total nuclear extracts of *Arabidopsis* plants expressing YFP or LHP1-YFP were incubated with GST-, GST-AS1-, or GST-AS2-coated glutathione-Sepharose 4B beads in pulldown buffer. The pulldown fractions were analyzed by Western blotting using a polyclonal anti-GFP antibody (A-11122, Invitrogen).

For SUMO pulldown assays, the DNA fragment encoding SUMO-tag was added to *LHP1-CSD*, *AS1(1-158)* and *AS2*, and then the constructs were each subcloned into the pET28a vector (Novagen, http://www.merckmillipore.com). The 6×His-SUMO-tagged proteins were expressed in the *E. coli* BL21 (DE3) strain. Their expression was induced by adding 0.2 mM isopropyl β-D-thiogalactoside (IPTG) to *E. coli* cells at an OD600 of 0.6. After growth at 20 °C for 18 h, cells were harvested, lysed by a high pressure disruptor, and then purified using His-Trap column (GE Healthcare, http://www.gelifesciences.com). The 6×His-SUMO tag of AS1(1-158) or AS2 was cleaved by ulp1 protease and the recombinant proteins were further purified by ion-exchange and size-exclusive chromatography. The purified AS1(1-158) and AS2 proteins were incubated with 6×His-SUMO- or 6×His-SUMO-LHP1-CSD-coated complete His-Tag Purification Resin (Roche, http://www.roche.com), and the eluted fractions were separated by SDS-PAGE and stained by Coomassie Brilliant Blue (CBB).

Bimolecular fluorescence complementation (BiFC) assay

The ORFs encoding full-length AS1, AS2, and LHP1 proteins were subcloned into pXY103, pXY105 and pXY106, respectively, to produce AS1-nYFP, nYFP-AS2, and cYFP-LHP1 fusion proteins. Leaves of 4-week-old *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* GV3101 harboring different construct combinations. BiFC fluorescence was observed 3–5 days after transformation and images were acquired with a Zeiss 710 confocal microscope (www.zeiss.com). The primers used for the BiFC constructs are listed in Table S1.

Gene expression analysis

Total RNA was prepared from plant tissues using TRI Reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription was performed using standard procedures with Improm-II reverse transcriptase (Promega, www.promega.com). PCR amplifications from the cDNA template were performed using gene-specific primers (see Table S1). *UBQ10* was used as the reference gene to normalize the data.

Histochemical GUS activity assay

GUS activity was assayed by incubating plant tissues in GUS staining buffer (Bu et al. 2014) for 3–6 hours at 37 °C. Plant material was cleared in 70% ethanol, and observed directly under a Leica MZ10F dissecting microscope (www.leica-microsystems.com).

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed according to a previously described method (Saleh et al. 2008), using the following antibodies: Anti-trimethyl-H3K27 (07-449; Millipore, www.millipore.com), anti-hH2Aub (D27C4; Cell Signaling Technology 8240, www.cellsignal.com), anti-H3 (ab1791; Abcam, www.abcam.com), and anti-GFP (A-11122, Invitrogen). The gene-specific primers used in PCR are listed in Table S1.

Microarray data analysis and H3K27me3 enrichment analysis Twelve-day-old seedlings of *as1-1*, *tfl2-1*, *as1-1 tfl2-1* and Col-0 grown on agar-solidified MS medium M0255 (Duchefa) under a 16-h light/8-h dark photoperiod (light intensity approx. 100 Em^{-2} s^{-1}) at 22 °C were used in transcriptome analyses. RNA was extracted using the TRIzol kit according to the manufacturer's instructions (Invitrogen). Gene expression were analyzed by using the Agilent Arabidopsis4*44K (www.agilent.com) oligonucleotide array containing 43603 probes (Shanghai Biotechnology Corporation, www.shbiotech.org). The raw data of microarray have been deposited in public database NCBI-GEO (GSE81229). Limma (Ritchie et al. 2015) was used with default settings to detect differentially expressed probes. In total, 4,756 probes (representing 3,459 unique genes) with differential expression in at least one of the three mutants were collected based on the following criteria: 1) $|\log_2(\text{fold-change})|>1$ and 2) adj. $P < 0.05$. Next, K-means clustering was used to partition these genes into five groups based on changes in gene expression among mutants. Using previously published H3K27me3 data (Deng et al. 2013), MACS (Zhang et al. 2008) was used to detect read-enriched regions (peaks). Fisher's exact test was used to test the significance of enrichment. Microarray data from GSE24507 was used for comparison with meristem-biased expressed genes (Lafos et al. 2011).

ACKNOWLEDGEMENTS

We thank Dr. Sarah Hake for providing the *BP::GUS/Col-0* seeds, and Dr. Jianxiang Liu for providing the pXY103, pXY105, and pXY106 vectors. This work was supported by the National Basic Research Program of China (2012CB910500 and 2011CB944600) and the National Natural Science Foundation of China (31370752).

AUTHOR CONTRIBUTIONS

Z.L., B.L., Z.G., Y.H.L. and Y.L. performed the experiments; J.L. and Y.Z. contributed to data analysis; Z.L., H.H., Y.H., W.H. S., Y.Z. and A.D. contributed to discussion; A.D. designed the experiments; Z.L. and A.D. wrote the paper.

REFERENCES

Arabidopsis interactome Mapping Consortium (2011) Evidence for network evolution in an *Arabidopsis* interactome map. **Science** 333: 601–606

Barrero JM, Gonzalez-Bayon R, del Pozo JC, Ponce MR, Micol JL (2007) INCURVATA2 encodes the catalytic subunit of DNA Polymerase and interacts with genes involved in chromatin-mediated cellular memory in *Arabidopsis thaliana*. **Plant Cell** 19: 2822–2838

Bemer M, Grossniklaus U (2012) Dynamic regulation of polycomb group activity during plant development. **Curr Opin Plant Biol** 15: 523–529

Bouyer D, Roudier F, Heese M, Andersen ED, Gey D, Nowack MK, Goodrich J, Renou JP, Grini PE, Colot V, Schnittger A (2011) Polycomb repressive complex 2 controls the embryo-to-seedling phase transition. **PLoS Genet** 7: e1002014

Bratzel F, Lopez-Torrejon G, Koch M, Del Pozo JC, Calonje M (2010) Keeping cell identity in *Arabidopsis* requires PRC1 RING-finger homologs that catalyze H2A monoubiquitination. **Curr Biol** 20: 1853–1859

Bu Z, Yu Y, Li Z, Liu Y, Jiang W, Huang Y, Dong AW (2014) Regulation of arabidopsis flowering by the histone mark readers MRG1/2 via interaction with CONSTANS to modulate *FT* expression. **PLoS Genet** 10: e1004617

Byrne ME, Barley R, Curtis M, Arroyo JM, Dunham M, Hudson A, Martienssen RA (2000) Asymmetric leaves1 mediates leaf patterning and stem cell function in *Arabidopsis*. **Nature** 408: 967–971

Calonje M (2014) PRC1 marks the difference in plant PcG repression. **Mol Plant** 7: 459–471

Chanvivattana Y (2004) Interaction of Polycomb-group proteins controlling flowering in *Arabidopsis*. **Development** 131: 5263–5276

Cui H, Benfey PN (2009) Interplay between SCARECROW, GA and LIKE HETEROCHROMATIN PROTEIN 1 in ground tissue patterning in the *Arabidopsis* root. **Plant J** 58: 1016–1027

del Olmo I, López-González L, Martín-Trillo MM, Martínez-Zapater JM, Piñeiro M, Jarillo JA (2010) *EARLY IN SHORT DAYS 7(ESD7)* encodes the catalytic subunit of DNA polymerase epsilon and is required for flowering repression through a mechanism involving epigenetic gene silencing. **Plant J** 61: 623–636

Deng W, Buzas DM, Hua Y, Robertson M, Taylor J, Peacock WJ, Dennis ES, Helliwell C (2013) *Arabidopsis* polycomb repressive complex 2 binding sites contain putative gaga factor binding motifs within coding regions of genes. **BMC Genomics** 14: 593

Derkacheva M, Steinbach Y, Wildhaber T, Mozgova I, Mahrez W, Nanni P, Bischof S, Gruissem W, Hennig L (2013) *Arabidopsis* MSI1 connects LHP1 to PRC2 complexes. **EMBO J** 32: 2073–2085

Di Croce L, Helin K (2013) Transcriptional regulation by polycomb group proteins. **Nat Struct Mol Biol** 20: 1147–1155

Francis NJ, Saurin AJ, Shao Z, Kingston RE (2001) Reconstitution of a functional core polycomb repressive complex. **Mol Cell** 8: 545–556

Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. **Nature** 386: 44–51

Grossniklaus U (1998) Maternal control of embryogenesis by *MEDEA*, a polycomb group gene in *Arabidopsis*. **Science** 280: 446–450

Guo M, Thomas J, Collins G, Timmermans MC (2008) Direct repression of *KNOX* loci by the ASYMMETRIC LEAVES1 complex of *Arabidopsis*. **Plant Cell** 20: 48–58

Hay A, Tsiantis M (2010) *KNOX* genes: Versatile regulators of plant development and diversity. **Development** 137: 3153–3165

Hecker A, Brand LH, Peter S, Simoncello N, Kilian J, Harter K, Gaudin V, Wanke D (2015) The *Arabidopsis* GAGA-Binding factor BASIC PENTACYSTEINE6 recruits the POLYCOMB-REPRESSIVE COMPLEX1 component LIKE HETEROCHROMATIN PROTEIN1 to GAGA DNA motifs. **Plant Physiol** 168: 1013–1024

Heo JB, Sung S (2011) Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. **Science** 331: 76–79

Husbands AY, Benkovics AH, Nogueira FTS, Lodha M, Timmermans, MCP (2015) The ASYMMETRIC LEAVES complex employs multiple modes of regulation to affect adaxial-abaxial patterning and leaf complexity. **Plant Cell** 27: 3321–3335

Ikezaki M, Kojima M, Sakakibara H, Kojima S, Ueno Y, Machida C, Machida Y (2010) Genetic networks regulated by ASYMMETRIC LEAVES1(AS1) and AS2 in leaf development in *Arabidopsis thaliana*: *KNOX* genes control five morphological events. **Plant J** 61: 70–82

Iwakawa H, Iwasaki M, Kojima S, Ueno Y, Soma T, Tanaka H, Semiarti E, Machida Y, Machida C (2007) Expression of the *ASYMMETRIC LEAVES2* gene in the adaxial domain of *Arabidopsis* leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. **Plant J** 51: 173–184

Iwasaki M, Takahashi H, Iwakawa H, Nakagawa A, Ishikawa T, Tanaka H, Matsumura Y, Pekker I, Eshed Y, Vial-Pradel S, Ito T, Watanabe Y, Ueno Y, Fukazawa H, Kojima S, Machida Y, Machida C (2013) Dual regulation of *ETTIN* (*ARF3*) gene expression by AS1-AS2, which maintains the DNA methylation level, is involved in stabilization of leaf adaxial-abaxial partitioning in *Arabidopsis*. **Development** 140: 1958–1969

Kotake T, Takada S, Nakahigashi K, Ohto M, Goto K (2003) *Arabidopsis TERMINAL FLOWER 2* gene encodes a heterochromatin protein 1 homolog and represses both *FLOWERING LOCUS T* to regulate flowering time and several floral homeotic genes. **Plant Cell Physiol** 44: 555–564

Lafos M, Kroll P, Hohenstatt ML, Thorpe FL, Clarenz O, Schubert D (2011) Dynamic regulation of H3K27 Trimethylation during *Arabidopsis* differentiation. **PLoS Genet** 7: e1002040

Lewis EB (1978) A gene complex controlling segmentation in *Drosophila*. **Nature** 276: 565–570

Li H, Xu L, Wang H, Yuan Z, Cao X, Yang Z, Zhang D, Xu Y, Huang H (2005) The putative RNA-dependent RNA polymerase RDR6 acts synergistically with ASYMMETRIC LEAVES1 and 2 to repress BREVIPEDICELLUS and MicroRNA165/166 in *Arabidopsis* leaf development. **Plant Cell** 17: 2157–2171

Li H, Luan S (2011) The cyclophilin AtCYP71 interacts with CAF-1 and LHP1 and functions in multiple chromatin remodeling processes. **Mol Plant** 4: 748–758

Li W, Wang Z, Li J, Yang H, Cui S, Wang X, Ma L (2011) Overexpression of *AtBMI1C*, a polycomb group protein gene, accelerates flowering in *Arabidopsis*. **PLoS ONE** 6: e21364

Li Z, Li B, Shen WH, Huang H, Dong AW (2012) TCP transcription factors interact with AS2 in the repression of class-I *KNOX* genes in *Arabidopsis thaliana*. **Plant J** 71: 99–107

Liu C, Xi W, Shen L, Tan C, Yu H (2009) Regulation of floral patterning by flowering time genes. **Dev Cell** 16: 711–722

Liu X, Kim YJ, Muller R, Yumul RE, Liu C, Pan Y, Cao X, Goodrich J, Chen X (2011) AGAMOUS terminates floral stem cell maintenance in *Arabidopsis* by directly repressing WUSCHEL through recruitment of polycomb group proteins. **Plant Cell** 23: 3654–3670

Lodha M, Marco CF, Timmermans MC (2013) The ASYMMETRIC LEAVES complex maintains repression of *KNOX* homeobox genes via direct recruitment of Polycomb-repressive complex2. **Genes Dev** 27: 596–601

Luo M, Yu CW, Chen F, Zhao L, Tian G, Liu X, Cui Y, Yang J, Wu K (2012) Histone deacetylase HDA6 is functionally associated with AS1 in repression of *KNOX* genes in *Arabidopsis*. **PLoS Genet** 8: e1003114

Machida C, Nakagawa A, Kojima S, Takahashi H, Machida Y (2015) The complex of ASYMMETRIC LEAVES (AS) proteins plays a central role in antagonistic interactions of genes for leaf polarity specification in *Arabidopsis*. **WIREs Dev Biol** 4: 655–671

Maenner S, Blaud M, Fouillen L, Savoye A, Marchand V, Dubois A, Sanglier-Cianferani S, Van Dorsselaer A, Clerc P, Avner P, Visvikis A, Branlant C (2010) 2-D structure of the A region of Xist RNA and its implication for PRC2 association. **PLoS Biol** 8: e1000276

Margueron R, Reinberg D (2011) The polycomb complex PRC2 and its mark in life. **Nature** 469: 343–349

Molitor A, Shen WH (2013) The polycomb complex PRC1: Composition and function in plants. **J Genet Genomics** 40: 231–238

Mozgova I, Köhler C, Hennig L (2015) Keeping the gate closed: Functions of the polycomb repressive complex PRC2 in development. **Plant J** 83: 121–132

Nakata M, Matsumoto N, Tsugeki R, Rikirsch E, Laux T, Okada K (2012) Roles of the middle domain-specific *WUSCHEL-RELATED HOMEOBOX* genes in early development of leaves in *Arabidopsis*. **Plant Cell** 24: 519–535

Ori N, Eshed Y, Chuck G, Bowman JL, Hake S (2000) Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. **Development** 127: 5523–5532

Petruk S, Sedkov Y, Johnston, Danika M, Hodgson, Jacob W, Black, Kathryn L, Kovermann, Sina K, Beck S, Canaani E, Brock, Hugh W, Mazo A (2012) TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. **Cell** 150: 922–933

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) Limma powers differential expression analyses for RNA-sequencing and microarray studies. **Nucleic Acids Res** 43: e47

Saleh A, Alvarez-Venegas R, Avramova Z (2008) An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. **Nat Protoc** 3: 1018–1025

Schoeftner S, Sengupta AK, Kubicek S, Mechtler K, Spahn L, Koseki H, Jenuwein T, Wutz A (2006) Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. **EMBO J** 25: 3110–3122

Schwartz YB, Kahn TG, Nix DA, Li XY, Bourgon R, Biggin M, Pirrotta V (2006) Genome-wide analysis of polycomb targets in *Drosophila melanogaster*. **Nat Genet** 38: 700–705

Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT, Bender W, Kingston RE (1999) Stabilization of chromatin structure by PRC1, a polycomb complex. **Cell** 98: 37–46

Simon JA, Kingston RE (2013) Occupying chromatin: Polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. **Mol Cell** 49: 808–824

Sing A, Pannell D, Karaiskakis A, Sturgeon K, Djabali M, Ellis J, Lipshitz HD, Cordes SP (2009) A vertebrate polycomb response element governs segmentation of the posterior hindbrain. **Cell** 138: 885–897

Turck F, Roudier F, Farrona S, Martin-Magniette ML, Guillaume E, Buisine N, Gagnot S, Martienssen RA, Coupland G, Colot V (2007) *Arabidopsis* TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. **PLoS Genet** 3: e86

Valérie G, Marc L, Sylvie P, Trine J, Zhao G, Delphine L, Olivier G (2001) Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in *Arabidopsis*. **Development** 128: 4847–4858

Wang H, Liu C, Cheng J, Liu J, Zhang L, He C, Shen WH, Jin H, Xu L, Zhang Y (2016) *Arabidopsis* flower and embryo developmental genes are repressed in seedlings by different combinations of polycomb group proteins in association with distinct sets of Cis-regulatory elements. **PLoS Genet** 12: e1005771

Woo CJ, Kharchenko PV, Daheron L, Park PJ, Kingston RE (2010) A region of the human HOXD cluster that confers polycomb-group responsiveness. **Cell** 140: 99–110

Xu L, Shen WH (2008) Polycomb silencing of KNOX genes confines shoot stem cell niches in *Arabidopsis*. **Curr Biol** 18: 1966–1971

Xu L, Xu Y, Dong AW, Sun Y, Pi L, Huang H (2003) Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. **Development** 130: 4097–4107

Xu L, Yang L, Pi L, Liu Q, Ling Q, Wang H, Poethig R, Huang H (2006) Genetic interaction between the AS1-AS2 and RDR6-SGS3-AGO7 pathways for leaf morphogenesis. **Plant Cell Physiol** 47: 853–863

Yang C, Bratzel F, Hohmann N, Koch M, Turck F, Calonje M (2013) VAL- and AtBMI1-mediated H2Aub initiate the switch from embryonic to postgerminative growth in *Arabidopsis*. **Curr Biol** 23: 1324–1329

Yu M, Mazor T, Huang H, Huang HT, Kathrein KL, Woo AJ, Chouinard CR, Labadorf A, Akie TE, Moran TB, Xie H, Zacharek S, Taniuchi I, Roeder RG, Kim CF, Zon LI, Fraenkel E, Cantor AB (2012) Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. **Mol Cell** 45: 330–343

Zhang X, Germann S, Blus BJ, Khorasanizadeh S, Gaudin V, Jacobsen SE (2007a) The *Arabidopsis* LHP1 protein colocalizes with histone H3 Lys27 trimethylation. **Nat Struct Mol Biol** 14: 869–871

Zhang X, Clarenz O, Cokus S, Bernatavichute YV, Pellegrini M, Goodrich J, Jacobsen SE (2007b) Whole-genome analysis of histone H3 lysine 27 trimethylation in *Arabidopsis*. **PLoS Biol** 5: e129

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nussbaum C, Myers RM, Brown M, Li W, Liu XS (2008) Model-based analysis of ChIP-Seq MACS. **Genome Biol** 9: R137

Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT (2008) Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. **Science** 322: 750–756

Zuo J, Niu QW, Chua NH (2000) Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. **Plant J** 24: 265–273

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12485/suppinfo

Figure S1. LHP1 interacts with AS1/AS2 through its CHROMOSHADOW domain (CSD)

(**A**) Yeast two-hybrid analyses showing interactions between AS1/AS2 and truncated LHP1 proteins. (**B**) *In vitro* pulldown assay. Purified AS1(1-158AA) or AS2 was incubated with equal quantity of beads coated with SUMO or SUMO-LHP1-CSD, respectively.

Figure S2. Analysis of number of lobes on fifth rosette leaf from 22-day-old Col-0, *tfl2-1*, *as1-1*, *as2-1*, *as1-1tfl2-1*, or *as2-1tfl2-1* plants. In total one hundred leaves are used for statistics

Figure S3. In whole seedlings, H3K27me3 levels on *BP* and *KNAT2* in *as1-1* and *as2-1* were not significantly different from those in Col-0

(**A**) Diagrams of *BP* and *KNAT2* gene structures. Black boxes indicate exons, long and thin black lines show promoter regions, thick black lines indicate introns, and numbers with short black lines indicate PCR fragments corresponding to genomic regions. (**B**) ChIP analyses using polyclonal anti-trimethyl-H3 antibody. (**C**) ChIP analyses using polyclonal anti-trimethyl-H3K27 antibody. In ChIP experiments (B–C), 12-day-old plants grown on agar-solidified MS medium were used. Error bars show standard deviation from three biological replicates. Asterisks indicate statistically significant differences between indicated genotypes and wild-type (Student's t test, $P < 0.05$).

Figure S4. Genes expressed at higher levels in meristem are significantly over-represented among AS1-LHP1-co-repressed genes (Fisher's exact test *P* value < 1e-3)

(**A**) Number of genes differentially expressed between meristem and leaf. (**B**) Fraction of genes from group II (left) or all genes on array (right) that are expressed at higher levels in meristem (dark grey bars) or leaf (light grey bars).

Table S1. List of primers used in this study