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

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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 and AS2 could help to establish the H3K27me3 modification at the chromatin regions of Class-I KNOTTED-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

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 PRC2 catalyzes 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 MSII (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 (Schoefnert 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 (Schoefnert et al. 2006; Yu et al. 2012; Yang et al. 2013).

BREVIPEDICELLUS (BP)/KNAT1 and KNAT2 are members of the Class-I KNOTTED1-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 MSII 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-YFP in combination with cYFP-LHP1 was co-transfected into tobacco leaves. The constructs of nYFP or cYFP together with cYFP-LHP1 or AS1/AS2-nYFP 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.
Figure 2. Enhanced as1-1, as2-1 and tf2-1 leaf phenotypes in as1-1 tf2-1 and as2-1 tf2-1 double mutants
Twenty-two-day-old plants of wild-type Col-0 (A), as1-1 (B), as2-1 (C), tf2-1 (D), as1-1 tf2-1 (E, F) and as2-1 tf2-1 (G, H). Fourth rosette leaf of 22-day-old Col-0 (I), as1-1 (J), as2-1 (K), tf2-1 (L), as1-1 tf2-1 (M, N), or as2-1 tf2-1 (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 3. Enhanced ectopic expression of Class-I KNOX genes in as1-1 tf2-1 and as2-1 tf2-1 double mutants
(A) Results of qRT-PCR analyses of transcription levels of mRNA of BP, KNAT2, and KNAT6 in rosette leaves. Transcription 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), tf2-1 (E), as1-1 tf2-1 (F), and as2-1 tf2-1 (G). Scale bars: 1 cm.
PRC1 complex, on several known PcG target genes, including AS1/AS2 complex directly recruits PRC2 to the chromatin of the above genes (Figure 4A), similar to those reported in a previous work (Lodha et al. 2013). The H3K27me3 levels at BP and KNAT2 loci were also decreased in tf2-1, 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-YFP perfectly complemented the phenotypes of the tf2-1 mutant, indicating that the LHP1-YFP fusion protein functioned well in plants. We crossed the transgenic 35S::LHP1-YFP/tf2-1 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 as1-1 and as2-1 mutants (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

(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).
changes in 12-day-old seedlings of as1-1 and tf2-1 single mutants and the as1-1 tf2-1 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, tf2-1 and as1-1 tf2-1 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 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).
regulated in as1-1, tf2-1 and as1-1 tf2-1 (Figure 7A), consistent with our microarray data. There was obvious enrichment of H3K27me3 at the 5' ends of these genes (Figure 7B), 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 FACTOR 3 (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 or 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 tf2-1 or as2-1 tf2-1 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 target genes.

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 atbmi/a/b/c but present at higher levels in the PRC2 mutant cfl/swm 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 tf2-1, the H2Aub levels were clearly increased on all genes we tested (Figure 4A), like those in clf-29 or clf/swm. This finding indicates that LHP1 does not function with AtRINGs or AtBMs in the classical PRC1. On the contrary, the H3K27me3 levels were significantly decreased on many Pc target genes in tf2-1 (Figure 4B). This is consistent with recent reports that LHP1 co-purified with PRC2 and was required to establish full levels of H3K27me3 on Pc target genes (Derkacheva et al. 2013; Wang et al. 2016). The researchers also found that the changes in the transcriptomes of the lhp and cfl 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 SEPALATA 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 (Barrera 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 tf2-1 and as2-1 tf2-1 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-depressed in as1-1, tf2-1, and as1-1 tf2-1 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-depressed genes in as1-1, tf2-1, and as1-1 tf2-1 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 tf2-1 (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 tf2-1 and as2-1 tf2-1, 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 Mo255 (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 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 GFP fragment to produce 35S::LHP1-YFP. This vector was then transformed into tf2-1, resulting in 35S::LHP1-YFP/tf2-1 plants. Transgenic plants of 35S::LHP1-YFP/tf2-1 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 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).

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.

Histochecmical 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 (D274; Cell Signaling Technology 8240, www.cellsignal.com), anti-H3 (ab791; 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, tf2-1, as1-1 tf2-1 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 was analyzed by using the Agilent Arabidopsis 4x44K (www. agilent.com) oligonucleotide array containing 43603 probes (Shanghai Biotechnology Corporation, www.shbiotech.org). The raw data 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) |log2(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).

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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.

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SUPPORTING INFORMATION

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**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, tf2-1, as1-1, as2-1, as1-1tf2-1, or as2-1tf2-1 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