The putative PRC1 RING-finger protein AtRING1A regulates flowering through repressing MADS AFFECTING FLOWERING genes in Arabidopsis

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ABSTRACT
Polycomb group proteins play essential roles in the epigenetic control of gene expression in plants and animals. Although some components of Polycomb repressive complex 1 (PRC1)-like complexes have recently been reported in the model plant Arabidopsis, how they contribute to gene repression remains largely unknown. Here we show that a putative PRC1 RING-finger protein, AtRING1A, plays a hitherto unknown role in mediating the transition from vegetative to reproductive development in Arabidopsis. Loss of function of AtRING1A results in the late-flowering phenotype, which is attributed to derepression of two floral repressors, MADS AFFECTING FLOWERING 4/5 (MAF4/5), in which turn downregulate two floral pathway integrators, FLOWERING LOCUS T and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1. Levels of the H3K27me3 repressive mark at MAF4 and MAF5 loci, which is deposited by CURLY LEAF (CLF)-containing PRC2-like complexes and bound by LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), are affected by AtRING1A, which interacts with both CLF and LHP1. Levels of the H3K4me3 activation mark correlate inversely with H3K27me3 levels at MAF4 and MAF5 loci. Our results suggest that AtRING1A suppresses the expression of MAF4 and MAF5 through affecting H3K27me3 levels at these loci to regulate the floral transition in Arabidopsis.

KEY WORDS: Polycomb repressive complex, Histone modification, Flowering time

INTRODUCTION
The transition from vegetative to reproductive growth, known as the floral transition, represents one of the most dramatic phase changes in flowering plants. In Arabidopsis, this process is regulated by a complex network of genetic pathways, including the photoperiod, vernalization, thermosensory, autonomous and gibberellin pathways (Amasino, 2004; Andrés and Coupland, 2012; Boss et al., 2004; Mouradov et al., 2002; Simpson and Dean, 2002; Srikanth and Schmid, 2011). FLOWERING LOCUS C (FLC) is a potent repressor of the flowering regulatory network that directly suppresses the expression of two floral pathway integrators, FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) (Helliwell et al., 2006; Hepworth et al., 2002; Li et al., 2008; Michaels and Amasino, 1999; Searle et al., 2006; Sheldon et al., 2000). FRIGIDA (FRI) functions as a scaffold protein to interact with FRI-LIKE 1, FRI ESSENTIAL 1, SUPPRESSOR OF FRIGIDA 4 and FLC EXPRESSOR, and the resulting transactivation complex (FRI-C) elevates FLC expression to inhibit flowering (Choi et al., 2011; Johanson et al., 2000; Michaels et al., 2004). By contrast, the vernalization and autonomous pathways repress FLC expression to promote flowering in response to prolonged cold exposure and developmental age, respectively (Michaels and Amasino, 2001; Sheldon et al., 2000). FLC and five close homologs, named MADS AFFECTING FLOWERING 1-5 (MAF1-5), belong to a small family of closely related MADS-box transcription factors (Parenicová et al., 2003). These MAF genes also repress the floral transition and their expression is influenced by vernalization (Gu et al., 2009; Kim and Sung, 2010; Ratcliffe et al., 2003; Ratcliffe et al., 2001; Sheldon et al., 2009; Sung et al., 2006).

Regulation of FLC and MAF genes involves extensive chromatin modifications at their loci (Amasino, 2004; He, 2009; He, 2012). For example, histone H3 lysine 4 (H3K4) trimethylation, H2B monoubiquitylation and H3K6 di- and trimethylation are associated with actively transcribed FLC chromatin, whereas repressive histone modifications, including histone deacetylation, H3K4 demethylation, H3K9 and H3K27 di- and trimethylation, and H4 arginine 3 symmetric dimethylation, are coupled with repression of FLC. In particular, the chromatin of FLC, MAF4 and MAF5 is associated with H3K27me3, which is a mark of transcriptionally silent chromatin (Alexandre and Henning, 2008).

In Drosophila, in which Polycomb group (PcG) proteins were first identified, deposition of the repressive H3K27me3 mark is mediated by Polycomb repressive complex 2 (PRC2) (Schuettengruber et al., 2007). The PRC2 complex contains four core components: Enhancer of zeste [E(z)], Extra sex combs (Esc), Suppressor of zeste 12 [Su(z)12] and p55 (also known as Caf1 – FlyBase). PRC2 components are evolutionarily conserved in animals and plants. The homologs of PRC2 subunits have been identified in Arabidopsis, and have been shown to play crucial roles in regulating various developmental processes including the floral transition. For example, CURLY LEAF (CLF) is a homolog of E(z), the loss-of-function mutants of which flower precociously (Goodrich et al., 1997), and directly mediates the deposition of H3K27me3 at FT, FLC, MAF4 and MAF5, thus repressing their mRNA expression (Jiang et al., 2008).

The PRC2 complex trimethylates H3K27. H3K27me3 is recognized and bound by the PRC1 complex that catalyzes the ubiquitylation of histone H2AK119, a mark for stabilizing the silenced state of H3K27me3-marked loci (Wang et al., 2004). The founding core PRC1 complex in Drosophila is composed of Polycomb (Pc), dRING1 [also known as Sex combs extra (Sce) – FlyBase], Posterior sex combs (Psc) and Polyhomeotic (Ph) (Francis et al., 2001; Shao et al., 1999), which have the corresponding
mammalian homologs HPC, RING1A/B, BMI1 and HPH, respectively (Schwartz and Pirrotta, 2007). Unlike PRC2-like complexes, which have been extensively studied, the components of the PRC1-like complex were only recently identified in Arabidopsis. Although there is no homolog of Pc in Arabidopsis, a plant chromodomain protein, LIKE HETEROOCHROMATIN PROTEIN 1 [LHP1; also known as TERMINAL FLOWER 2 (TFL2)], has been proposed to play a Pc-analogous function in binding H3K27me3 (Turck et al., 2007; Zhang et al., 2007). LHP1 regulates flowering time and is necessary for FT repression and maintaining vernalization-mediated FLC silencing after the plant resumes growth in warm conditions (Kotake et al., 2003; Mylne et al., 2006; Sung et al., 2006; Takada and Goto, 2003). Five PRC1 RING-finger proteins have been identified in the Arabidopsis genome (Sanchez-Pulido et al., 2008). AtRING1A and AtRING1B are homologous to RING1A/B, whereas AtBMI1A, AtBMI1B and AtBMI1C are homologous to BMI1. Investigations of mutants impaired in AtRING1A, AtRING1B, AtBMI1A, AtBMI1B and AtBMI1C have suggested that these PRC1 components are mainly involved in repressing embryonic and stem cell regulators (Bratzel et al., 2010; Chen et al., 2010; Xu and Shen, 2008; Yang et al., 2013). Although overexpression of AtBMI1C influences flowering time, downregulation of its expression does not show any defect (Li et al., 2011). In addition, AtBMI1C is an imprinted gene that is only expressed during endosperm and stamen development and in roots (Bratzel et al., 2012; Yang et al., 2013). Thus, AtBMI1C is unlikely to play an endogenous role in regulating flowering time.

Despite the progress in understanding PRC1 complexes in Arabidopsis, whether their different components regulate specific targets and how they interact with PRC2 to mediate gene repression are still largely unknown. It has recently been shown that LHP1 interacts with MULTICOPY SUPPRESSOR OF IRA1, a subunit of all PRC2 complexes in Arabidopsis, to facilitate the recruitment of PRC2 to target genes (Derkacheva et al., 2013). In this study, we report that AtRING1A plays a previously unidentified role in regulating the floral transition in Arabidopsis. We show that AtRING1A acts in conjunction with LHP1 and the key PRC2 component CLF to affect the levels of the H3K27me3 repressive mark at the MAF4 and MAF5 loci, thus repressing MAF4 and MAF5, which in turn regulate two floral pathway integrators, FT and SOC1, to control flowering time in Arabidopsis.

RESULTS

Loss of function of AtRING1A shows late flowering

To study the role of PRC1 RING-finger proteins in plant development, we examined the phenotypes of the previously described mutant alleles of AtRING1A, AtBMI1A and AtBMI1B (Bratzel et al., 2010; Xu and Shen, 2008) and a novel T-DNA insertional mutant of AtRING1B obtained from the Arabidopsis Biological Resource Center, and found that only Atring1a (AL_945948) showed the obvious late-flowering phenotype under both long days (LDs) and short days (SDs) (Fig. 1A-D; supplementary material Fig. S1). As reported previously (Xu and Shen, 2008), Atring1a contained a T-DNA insertion at the end of the second intron (Fig. 1A) and did not produce Atring1a transcripts spanning the T-DNA insertion site (Fig. 1B). To test whether the late-flowering phenotype of Atring1a is caused by loss of AtRING1A function, we transformed Atring1a mutants with a genomic construct (gAtRING1A-4HA) that contains a 5.4 kb AtRING1A genomic region including the 2.0 kb 5’ upstream sequence, the entire 3.0 kb coding sequence plus introns fused in frame with a 4HA tag, and the 0.4 kb 3’ untranslated region (UTR). For the majority of Atring1a gAtRING1A-4HA T1 transformants, flowering time was comparable to that of wild-type plants (Fig. 1E). This suggests that disruption of Atring1a is responsible for the late-flowering phenotype of Atring1a.

In order to confirm the function of Atring1a in flowering time regulation, we used artificial microRNA (AmiR) interference to knockdown AtRING1A transcripts in Atring1a AtRING1A

Fig. 1. Atring1a regulates flowering time in Arabidopsis. (A) The T-DNA insertion in Atring1a (AL_945948) and the target site of the AmiR in AmiR-Atring1a. Exons and untranslated regions are represented by black and gray boxes, respectively, and introns are represented by black lines. Arrowheads indicate the positions of primers used for amplifying Atring1a transcripts as shown in B. (B) RT-PCR showing that Atring1a transcripts are not detectable in Atring1a plants. Exons and untranslated regions are represented by black and gray boxes, respectively, and introns are represented by black lines. Arrowheads indicate the positions of primers used for amplifying Atring1a transcripts as shown in B. (C) Atring1a mutant show late flowering under LDs. Scale bar: 1 cm. (D) Flowering time of Atring1a and AmiR-Atring1a grown under LDs and SDs. Values were scored from at least 15 plants of each genotype. Error bars indicate s.d. (E) Distribution of flowering time in T1 transgenic lines carrying the gAtRING1A-4HA construct in an Atring1a background grown under LDs.
(Schwab et al., 2006) to knockdown AtRING1A, creating 18 independent AmiR-Atring1a lines that expressed an AmiR that specifically targeted AtRING1A exon 5 (Fig. 1A). Different levels of late flowering were displayed by 15 of these lines under LDs, and the line showing the latest flowering was chosen as a representative for further investigations. As expected, there were no detectable AtRING1A transcripts in this AmiR-Atring1a line that showed a comparable late-flowering phenotype to AtRING1a mutants under both LDs and SDs (Fig. 1B,D), substantiating that AtRING1A functions in the promotion of flowering. We also created 25 transgenic plants overexpressing AtRING1A, all of which showed normal flowering time (data not shown), implying that overexpression of AtRING1A might not influence flowering.

Expression of AtRING1A during the floral transition
To examine the detailed tissue-specific expression pattern of AtRING1A during the floral transition, we generated a reporter construct with the genomic region of AtRING1A that was used for the complementation experiment (Fig. 1E), but without the 3’ UTR, fused to GUS (gAtRING1A:GUS). The staining patterns shown by most of the gAtRING1A:GUS transgenic plants were similar, and a representative line was chosen to further analyze AtRING1A expression during the floral transition. A 3-day-old gAtRING1A:GUS seedling showed specific GUS staining in the shoot apex and vascular tissues of cotyledons (Fig. 2A). In developing seedlings before, during and immediately after the floral transition occurring 9 to 13 days after germination under our growth conditions, GUS signals were consistently strong in shoot apices and vascular and mesophyll tissues of young leaves, but weak and finally absent in vascular tissues of older cotyledons or leaves (Fig. 2A; supplementary material Fig. S2). These patterns demonstrate that AtRING1A is highly expressed in actively proliferating cells during the floral transition.

Given that AtRING1A is involved in flowering time control, we further examined the effect of various flowering genetic pathways on AtRING1A expression during the floral transition. AtRING1A expression remained unchanged before and during the floral transition in wild-type plants grown under LDs (supplementary material Fig. S3A). Its expression was also unaffected in loss-of-function mutants of several key regulators in the photoperiod pathway (supplementary material Fig. S3B), suggesting that AtRING1A expression is not regulated by this pathway. AtRING1A was expressed at similar levels in GA-deficient ga1-3 mutants and wild-type plants grown under SDs (supplementary material Fig. S4A). Consistently, GA treatment did not affect AtRING1A expression (supplementary material Fig. S4B), indicating that AtRING1A is not transcriptionally regulated by the GA pathway. Similarly, AtRING1A expression was unaffected in various mutants of the autonomous pathway (supplementary material Fig. S5A). Vernalization treatment did not affect AtRING1A expression, and Atring1a displayed a normal response to vernalization under both LDs and SDs (supplementary material Fig. S5B-D). These observations suggest that neither the autonomous pathway nor the vernalization pathway regulates AtRING1A. In addition, several other important flowering regulators, such as SHORT VEGETATIVE PHASE (SVP), SOC1, AGAMOUS-LIKE 24 and FLC, which mediate flowering signals from various genetic pathways, did not affect AtRING1A expression (supplementary material Fig. S6). Taken together, these results suggest that AtRING1A is expressed in developing seedlings at fairly steady levels during the floral transition regardless of environmental and endogenous flowering signals, and that its mRNA expression is unaffected by floral pathway integrators, such as SOC1 and FT.

AtRING1A promotes flowering through repressing MAF4 and MAF5
Since AtRING1A is a PRC1 RING-finger protein that could affect the transcription of target genes, we proceeded to identify downstream targets of AtRING1A that might be responsible for its effects in promoting flowering. We examined the temporal expression of two floral pathways integrators, SOC1 and FT (Blázquez and Weigel, 2000; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000), and other known important flowering regulators, including GIGANTEA (GI), CONSTANS (CO), AGL24, FLC, MAF genes, or

Fig. 2. Expression analysis of several key flowering time genes in Atring1a mutants during the floral transition. (A) GUS staining of developing gAtring1a:GUS seedlings at the vegetative phase (3 and 6 days old) and during the floral transition (9 and 12 days old). Scale bars: 1 mm. (B-F) Temporal expression of SOC1 (B), FT (C), MAF4 (D), MAF5 (E) and FLC (F) during the floral transition as determined by quantitative real-time PCR in developing wild-type and Atring1a seedlings grown under LDs. The levels of gene expression normalized to TUB2 expression are shown relative to the maximal expression level set at 100%. Error bars indicate s.d.
TEMPRANILLO 1 (TEM1), TEM2, SCHLAFMUTZE (SMZ), SCHNARCHZAPFEN (SNZ), TARGET OF EAT 1 (TOE1), TOE2 and TOE3 (Aukerman and Sakai, 2003; Castillejo and Pelaz, 2008; Hartmann et al., 2000; Li et al., 2008; Mathieu et al., 2009; Michaels and Amasino, 1999; Park et al., 1999; Putterill et al., 1995; Yant et al., 2010; Yu et al., 2002), in wild-type and Atring1a plants. Consistent with the late-flowering phenotype of Atring1a, both FT and SOC1 were downregulated in developing Atring1a seedlings during the floral transition (Fig. 2B,C). It is noteworthy that, among all the floral repressors tested, only the expression of MAF4 and MAF5 was substantially upregulated in Atring1a mutants as compared with wild-type plants (Fig. 2D,E), whereas there was only a relatively moderate increase in FLC expression in Atring1a (Fig. 2F). The expression of the other MAF genes, MAF1, MAF2 and MAF3, and of other flowering regulators was not obviously affected (supplementary material Fig. S7).

To study the regulatory hierarchy among AtRING1A, MAF4, MAF5, FT and SOC1 during the floral transition, we analyzed the genetic interactions among these genes. We identified new mutant alleles of MAF4 (maf4-2) and MAF5 (maf5-3), each carrying a T-DNA insertion in their first intron (Fig. 3A), in which MAF4 and MAF5 DNAs were not detectable (supplementary material Fig. S8). Consistent with previous studies (Gu et al., 2009; Kim and Sung, 2010), neither maf4-2 nor maf5-3 showed obvious flowering defects, but significantly rescued the late-flowering phenotype of Atring1a (Fig. 3B). These results demonstrate genetically that late flowering of Atring1a is mainly attributable to derepression of MAF4 and MAF5. Overexpression of SOC1 or FT greatly suppressed the late-flowering phenotype of Atring1a, whereas both ft-10 and soc1-2 enhanced the late-flowering phenotype (Fig. 3B). These observations, together with the expression analysis showing downregulation of SOC1 and FT in Atring1a (Fig. 2B,C), indicate that SOC1 and FT act downstream of AtRING1A.

To test whether AtRING1A promotes FT and SOC1 expression through repressing MAF4 and MAF5, we further compared the expression of FT and SOC1 in 9-day-old maf4-2 Atring1a and maf5-3 Atring1a double mutants and their respective single mutants. Whereas FT and SOC1 were downregulated in Atring1a as compared with wild-type seedlings, their expression in maf4-2 Atring1a and maf5-3 Atring1a was restored to levels close to those of wild-type seedlings (Fig. 3C). These expression data, together with the results of genetic crosses (Fig. 3B), strongly suggest that AtRING1A represses MAF4 and MAF5, which in turn derepresses FT and SOC1 to promote flowering.

AtRING1A affects H3K27me3 and H3K4me3 levels at MAF4 and MAF5

Previous studies have suggested that histone modifications, such as H3K27me3 and H3K4me3, play important roles in regulating the expression of MAF4 and MAF5 (Jiang et al., 2011; Kim and Sung, 2010). As PRC1 subunits in animals and Arabidopsis have been shown to exert different effects on H3K27me3 at their target loci (Cao et al., 2005; Endoh et al., 2008; Wang et al., 2004; Yang et al., 2013), we examined whether AtRING1A, as a component of the PRC1 complexes, is involved in affecting H3K27me3 and H3K4me3 levels at MAF4 and MAF5.

To test whether AtRING1A regulates the floral transition through affecting histone modifications, we first compared global methylation levels of H3K27 and H3K4 in 9-day-old Atring1a versus wild-type seedlings. There were no differences in mono-, di- or trimethylation levels at H3K4 and H3K27 in Atring1a and wild-type seedlings (supplementary material Fig. S9), indicating that AtRING1A does not affect the global methylation levels of H3K4 and H3K27 during the floral transition. We then measured H3K27me3 and H3K4me3 levels at the MAF4 and MAF5 loci by ChIP assays of 9-day-old wild-type and Atring1a seedlings. In wild-type seedlings, H3K27me3 was...
AtRING1A shows that H3K27me3 and H3K4me3 levels at the genomic regions of MAF4 and MAF5 are clearly enriched in the genomic regions of MAF4 and MAF5 comprising the first exon and part of the following first intron, but not in the first exon of MAF4 that is located immediately upstream of MAF4 and MAF5 (Fig. 4A,B). This observation is consistent with a previous study showing the enrichment of H3K27me3 at MAF4 and MAF5, but not MAF3 (Jiang et al., 2008). Notably, levels of H3K27me3 enrichment at MAF4 and MAF5 were generally reduced in Atring1a (Fig. 4A,B). Furthermore, H3K4me3 enrichment was detected around the transcription start sites of both MAF4 and MAF5 in wild-type seedlings, and the enrichment levels were strongly increased at these regions of MAF4 and MAF5 in Atring1a (Fig. 4A,C). In contrast to the change in H3K27me3 and H3K4me3 levels at MAF4 and MAF5, both histone marks remained unchanged at MAF3 and at a housekeeping gene, TUB2, in Atring1a versus wild-type seedlings (Fig. 4B,C).

The reciprocal changes in the levels of repressive H3K27me3 and permissive H3K4me3 marks at MAF4 and MAF5 correlated with upregulation of MAF4 and MAF5 in Atring1a (Fig. 2D,E). By contrast, H3K27me3 and H3K4me3 levels at MAF3 and FLC, two close relatives of MAF4 and MAF5, were not obviously altered in Atring1a (Fig. 4B,C; supplementary material Fig. S10), which is consistent with their unaltered and only slightly altered expression in Atring1a, respectively (Fig. 2F; supplementary material Fig. S7). These results suggest that AtRING1A is required for mediating H3K27me3 and H3K4me3 levels at the MAF4 and MAF5 loci to repress their transcription.

As a component of the PRC1 complex that catalyzes the ubiquitination of histone H2AK119 (Wang et al., 2004), AtRING1A was shown to mediate H2A monoubiquitlation (H2Aub) in vitro (Bratzel et al., 2010). To test the endogenous effect of AtRING1A on H2Aub, we compared global H2Aub levels in 9-day-old Atring1a versus wild-type seedlings using a specific H2Aub antibody (Yang et al., 2013), and found that H2Aub levels were substantially increased in Atring1a compared with wild-type plants (supplementary material Fig. S11A). This could be due to increased H2Aub-catalyzing activity of the other four PRC1 RING-finger proteins, namely Arabidopsis AtRING1B, AtBMI1A, AtBMI1B and AtBMI1C, all of which were upregulated in mRNA expression in Atring1a (supplementary material Fig. S11B) (Chen et al., 2010). ChIP analysis further revealed that H2Aub was enriched at the transcriptional start sites of MAF4 and MAF5 in wild-type plants (supplementary material Fig. S11C). However, despite the change in global H2Aub levels in Atring1a, H2Aub levels at MAF4 and MAF5 were not significantly changed in Atring1a (supplementary material Fig. S11C), indicating that H2Aub might not directly contribute to the modulation of MAF4 and MAF5 expression by AtRING1A.

### AtRING1A acts in conjunction with CLF and LHP1 to repress MAF4 and MAF5

The observation that CLF, a component of PRC2-like complexes in Arabidopsis, is responsible for the deposition of H3K27me3 and the relevant change in H3K4me3 levels at MAF4 and MAF5 chromatin (Jiang et al., 2008) prompted us to investigate how AtRING1A and CLF, which have been shown to interact with each other in vitro and in yeast (Xu and Shen, 2008), act together to mediate H3K27me3 enrichment at MAF4 and MAF5 during the floral transition. We first measured H3K27me3 levels at MAF4 and MAF5 by ChIP assays of 9-day-old Atring1a, clf and clf Atring1a seedlings. As expected, H3K27me3 levels at MAF4 and MAF5 were strongly reduced in clf (Fig. 5A) (Jiang et al., 2008). In clf Atring1a, the enrichment of H3K27me3 at MAF4 and MAF5 was further reduced to levels lower than those in the respective single mutants (Fig. 5A). In agreement with the change in H3K27me3 levels at MAF4 and MAF5, the expression of MAF4 and MAF5 was higher in clf Atring1a than in single mutants (Fig. 5B).
Fig. 5. AtRING1A acts in conjunction with CLF and LHP1 to repress MAF4 and MAF5 through affecting H3K27me3 levels at these two loci. (A) ChiP analysis of H3K27me3 levels at MAF4 and MAF5 in 9-day-old wild-type, Atring1a, clf and clf Atring1a seedlings. Genomic fragments of MAF3 (3.1) and TUB2 were amplified as negative controls. *P<0.05 or **P<0.05 (two-tailed paired Student’s t-test) for ChiP fold enrichment between clf Atring1a and Atring1a or between clf Atring1a and clf, respectively. (B) Quantitative real-time PCR analysis of expression of FT, SOC1, MAF4 and MAF5 in 9-day-old Atring1a, clf and clf Atring1a mutants grown under LDs. Results were normalized against the expression levels of TUB2. Gene expression levels in wild-type seedlings are all set as 1. (C) Flowering time of Atring1a, clf and clf Atring1a mutants grown under LDs. Values were scored from at least 15 plants of each genotype. (D) Reciprocal co-immunoprecipitation assays show that AtRING1A interacts with LHP1 in vivo during the floral transition. Nuclear extracts from 9-day-old LHP1-GFP, gRING1A-4HA and LHP1-GFP gRING1A-4HA plants were incubated with either anti-HA agarose or anti-GFP antibody bound to Protein G Plus-Agarose. The input and co-immunoprecipitated proteins were detected by anti-GFP (left upper and right lower panels) or anti-HA (left lower and right upper panels) antibody. (E) ChiP analysis of LHP1-3HA binding to the genomic regions of MAF4 and MAF5. Nine-day-old lhp1-3 35S:LHP1-3HA plants were harvested for ChiP analysis. Genomic fragments of MAF3 (3.1) and TUB2 were amplified as negative controls. Fold enrichment of each fragment was calculated first by normalizing the amount of a target DNA fragment against a genomic fragment of ACTIN7 (At5g09810) as an internal control, and then by normalizing the value for lhp1-3 35S:LHP1-3HA against that for lhp1-3 plants. (F) Loss of LHP1 results in reduced H3K27me3 levels at MAF4 and MAF5 loci. ChiP analysis of H3K27me3 levels at MAF4 and MAF5 was performed on 9-day-old wild-type and lhp1-3 seedlings. *P<0.05 (two-tailed paired Student’s t-test) for ChiP fold enrichment between lhp1-3 and wild-type plants. (G) Quantitative real-time PCR analysis of expression of FT, SOC1, MAF4 and MAF5 in 9-day-old lhp1-3, Atring1a and lhp1-3 Atring1a mutants grown under LDs. Results were normalized against the expression levels of TUB2. Gene expression levels in wild-type seedlings are all set as 1. (H) Flowering time of lhp1-3, Atring1a and lhp1-3 Atring1a mutants grown under LDs. Values were scored from at least 15 plants of each genotype. Error bars indicate s.d.

As CLF-dependent H3K27me3 suppresses the expression of many flowering promoters and repressors (Jiang et al., 2008; Schönrock et al., 2006), loss of CLF function in clf simultaneously derepresses these genes, including the potent flowering promoter FT, resulting in an early-flowering phenotype (Fig. 5B,C) (Jiang et al., 2008; Schönrock et al., 2006). clf Atring1a double mutants exhibited later flowering than clf single mutants, demonstrating that Atring1a partially suppresses early flowering of clf (Fig. 5C). This observation is in line with the finding that two floral repressors, MAF4 and MAF5, are more substantially derepressed in clf Atring1a than in clf (Fig. 5B). Taken together, these results strongly suggest that AtRING1A acts in conjunction with CLF to repress MAF4 and MAF5 through affecting H3K27me3 levels at these two loci to regulate flowering time.

Although ChiP analysis of Atring1a gAtRING1A-4HA transgenic lines (Fig. 1E) did not reveal direct binding of AtRING1A to MAF4 and MAF5 genomic regions (supplementary material Fig. S12), AtRING1A has been suggested to interact with another putative PRC1 component, LHP1 (Bratzel et al., 2010; Chen et al., 2010; Xu and Shen, 2008), which may have a Pc-analogous function in binding H3K27me3-marked genomic regions (Turck et al., 2007; Zhang et al., 2007). Indeed, we detected in vivo interaction between AtRING1A-4HA and LHP1-GFP (Kotake et al., 2003) during the floral transition (Fig. 5D). Consistent with previous data from genome-wide analysis of LHP1 binding (Turck et al., 2007; Zhang et al., 2007), ChiP analysis using 9-day-old lhp1-3 35S:LHP1-3HA seedlings (Liu et al., 2009) showed that LHP1-3HA was bound to the genomic regions marked with H3K27me3 at MAF4 and MAF5.
and cells (Fig. 2A) (Kotake et al., 2003), indicate that AtRING1A and LHP1 on the expression of PcG target genes (Derkacheva et al., 2013). These results showing that LHP1 is required for establishing full H3K27me3 levels in lhp1-3 Atring1a and MAF5. However, MAF4 and MAF5 were more derepressed in lhp1-3 Atring1a than in Atring1a (Fig. 5G), showing a synergistic effect of AtRING1A and LHP1 on the expression of MAF4 and MAF5. As a result, Atring1a partially suppressed early flowering of lhp1-3 (Fig. 5H). These observations, together with the similar expression patterns of AtRING1A and LHP1 in actively proliferating cells (Fig. 2A) (Kotake et al., 2003), indicate that AtRING1A and LHP1 could act in the same PRC1-like complexes to repress MAF4 and MAF5 during the floral transition.

DISCUSSION

The PRC1 RING-finger proteins AtRING1A, AtRING1B, AtBMI1A, AtBMI1B and AtBMI1C were recently identified in the model plant Arabidopsis, and studies on these proteins have so far been focused on their function in regulating a few common targets involved in the repression of embryonic traits and meristem maintenance at the vegetative phase (Bratzel et al., 2010; Chen et al., 2010; Xu and Shen, 2008; Yang et al., 2013). Although AtBMI1A and AtBMI1B have been shown to mediate H2A monoubiquitylation and ubiquitin-dependent proteasomal degradation of a targeted transcription factor in Arabidopsis (Bratzel et al., 2010; Qin et al., 2008), how these PRC1 subunits contribute to gene silencing during plant development is still largely unknown. In this study, we report that AtRING1A acts as an important regulator of flowering in Arabidopsis, revealing a hitherto unknown role of a PRC1 RING-finger protein in mediating the transition from vegetative to reproductive development in plants. Loss of function of AtRING1A significantly delays flowering, which is due to derepression of MAF4 and MAF5 and the resulting downregulation of two floral pathway integrators, FT and SOC1.

The floral transition regulated by AtRING1A is a key developmental process that is simultaneously regulated by PRC2-like complexes in Arabidopsis. This provides a unique paradigm for studying the relationship between PRC1-like and PRC2-like complexes in plants. Enrichment of the H3K27me3 repressive mark at MAF4 and MAF5 loci, which is deposited by the CLF-containing PRC2-like complexes, is also affected by AtRING1A, which interacts with CLF. The expression levels of MAF4 and MAF5 consistently correlate with the changes in H3K27me3 levels at these two loci in Atring1a, clf and clf Atring1a (Fig. 5A,B). These findings suggest that the AtRING1A-containing PRC1-like complexes could act in conjunction with the CLF-containing PRC2 complexes to suppress the expression of MAF4 and MAF5 through affecting their H3K27me3 levels to regulate the floral transition in Arabidopsis (Fig. 6).

Interestingly, AtRING1A and CLF show additive rather than redundant effects on H3K27me3 enrichment at MAF4 and MAF5 (Fig. 5A), indicating that both proteins are required for this methylation. In the canonical model of PRC1 action, PRC2 deposits the H3K27me3 mark that sequentially recruits PRC1, which then catalyzes the ubiquitylation of H2AK119 to maintain the silenced state of H3K27me3-marked loci (Cao et al., 2005; Wang et al., 2004). This hierarchical model explains why the knockout of some PRC1 subunits in animals and plants does not affect the upstream event of H3K27me3 enrichment deposited by PRC2 at their target genes (Bratzel et al., 2010; Cao et al., 2005; Chen et al., 2010; Wang et al., 2004; Xu and Shen, 2008). However, this model of chromatin modification is challenged by the decrease in H3K27me3 levels observed at target genes in RING1A/B-deficient embryonic stem cells (Endoh et al., 2008). A recent study has shown that AtBMI1A, AtBMI1B and AtBMI1C are required for the H3K27me3 modification of seed maturation genes in Arabidopsis (Yang et al., 2013). Similarly, the impact of AtRING1A on H3K27me3 enrichment at MAF4 and MAF5 revealed in our study also implies that the concerted action of PRC1 and PRC2 could at least include their cooperative effect in mediating H3K27me3 levels at some of their common target genes.

The function of AtRING1A in mediating H3K27me3 levels could be restricted to a limited number of target genes in a specific developmental context. As shown in this study, although AtRING1A affects H3K27me3 levels at MAF4 and MAF5, global H3K27 methylation levels are not altered in Atring1a as compared with wild-type plants during the floral transition, indicating that AtRING1A might only affect H3K27me3 levels at a few specific flowering regulators. This is in contrast to the functional mode of CLF, a histone methyltransferase in PRC2-like complexes, the knockout mutants of which show a reduction in global H3K27me3 levels during the floral transition (Jiang et al., 2008). In line with the contribution of CLF and AtRING1A to global H3K27me3 levels,
CLF represses the expression of a group of flowering time genes including important flowering promoters and repressors, such as FLC, MAF4, MAF5 and FT (Jiang et al., 2008; Schürnrock et al., 2006), whereas AtRING1A only specifically represses the two flowering repressors MAF4 and MAF5 but not the other repressors tested, including their close relatives MAF1-3 and FLC. The difference in the target specificity of AtRING1A and CLF might in part correlate with the status of their physical interaction with target genes. CLF has been shown to directly bind to the chromatin of its target genes, such as FLC, MAF4, MAF5 and FT (Jiang et al., 2008). By contrast, the interaction between AtRING1A and the chromatin of MAF4 and MAF5 seems to be mediated by other regulators, potentially including LHP1, which is largely associated with H3K27me3-marked loci (Turck et al., 2007; Zhang et al., 2007). As none of the plant PRC1 RING-finger proteins has so far been reported to bind to their target genes, how their interacting partners contribute to targeting specificity of PRC1 RING-finger proteins is an intriguing question that remains to be investigated further.

The mammalian PRC1 RING-finger proteins form an E3 ubiquitin ligase complex that catalyzes histone H2A K119 ubiquitylation (H2AK119ub) (Cao et al., 2005; de Napoles et al., 2004; Wang et al., 2004). Although it has been suggested that H2AK119ub could play a role in blocking transcription elongation by restraining RNA polymerase movement through the compacted nucleosomes (Simon and Kingston, 2009), the precise underlying mechanism for PRC1-mediated gene repression is still largely unknown. All five Arabidopsis PRC1 RING-finger proteins, including AtRING1A, have also been shown to mediate H2Aub in vivo or in vitro (Bratzel et al., 2010; Bratzel et al., 2012; Li et al., 2011). However, AtRING1A is the only RING-finger protein with knockout mutants that exhibit the flowering phenotype, which is due to the elevated expression of MAF4 and MAF5. In addition, AtRING1A does not affect H2AK119ub levels at MAF4 and MAF5. These results imply that the effect of AtRING1A on the expression of MAF4 and MAF5 and on H3K27me3 levels at these two loci is at least independent of PRC1-mediated H2AK119ub during the floral transition. This example, together with other studies showing the independence of H2AK119ub and PRC1-mediated gene repression (Eskeland et al., 2010; Richly et al., 2010; Yang et al., 2013), indicate that the regulation and outcome of PRC1 activity are far more complex than previously thought.

In summary, the PRC1 RING-finger protein AtRING1A acts together with the key PRC2 component CLF to suppress the expression of two flowering repressors, MAF4 and MAF5, through affecting H3K27me3 levels at these two loci. This previously unidentified similarity in the pattern of PRC1 and PRC2 action on specific target genes in plants demonstrates the functional complexity of PcG proteins in generating chromatin-modifying complexes that target specific genes in a given developmental context.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Arabidopsis thaliana plants were grown on soil or Murashige and Skoog (MS) medium under LDs (16 hours light/8 hours dark) or SDs (8 hours light/16 hours dark). The mutants co-1, ga-1, ft-10, fve-3, soc1-2, agl24-1, syp-41, maf4-2, maf5-3, clf, Arting1a, Arting1b-2, Athmina1-1 and Athmina1b are in the Columbia (Col) background, whereas co-2, fca-1, fpa-1, fve-1 and gai-3 are in the Landsberg erecta (Ler) background (Bratzel et al., 2010; Li et al., 2008; Shen et al., 2011; Xu and Shen, 2008). Arting1a (AL_945948), Arting1b-2 (SALK_143481C), Athmina1-1 (SALK_145041), Athmina1b (CS855837), maf4-2 (CS878527), maf5-3 (SALK_015513) and clf (SALK_006658) seeds were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). All transgenic plants were generated through Agrobacterium tumefaciens-mediated transformation. Transformants harboring gAtRING1A-4HA were selected on MS medium supplemented with kanamycin, whereas those harboring gAtRING1A:GUS were selected by Basta on soil.

**Plasmid construction**

To construct gAtRING1A-4HA, a 5.4 kb gAtRING1A genomic fragment (gAtRING1A) was amplified using primers gAtRING1A-F and gAtRING1A-R and cloned into pENTR/D-TOPO (Invitrogen). Based on this construct, gAtRING1A-4HA was generated using a modified QuickChange site-directed mutagenesis approach (Geiser et al., 2001). The sequence encoding 4HA was amplified with primers gAtRING1A-4HA-F and gAtRING1A-4HA-R, and the resulting PCR products were then annealed to the methylated gAtRING1A plasmid and elongated with Phusion Hot Start II High-Fidelity DNA polymerase (Finnzymes). After DpnI digestion, the mutated plasmids containing the 4HA fragment were recovered from E. coli transformants. To construct gAtRING1A:GUS (β-glucuronidase), a 5.0 kb gAtRING1A genomic fragment was amplified with primers gAtRING1A-F (EcoRI) and gAtRING1A-R (BamHI) and cloned into pHY107 (Liu et al., 2007). The primers used for plasmid construction are listed in supplementary material Table S1.

**Expression analysis**

Total RNA was extracted using the FavorPrep Plant Total RNA Mini Kit (Favorgen) and reverse transcribed using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR was performed in triplicate on each of three independently collected samples using the 7900HT Fast Real-Time PCR System (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). The expression of TUBULIN2 (TUB2) was used as an internal control. Relative expression levels of genes were calculated as previously described (Liu et al., 2007). The primers used for real-time PCR are listed in supplementary material Table S1.

**GUS staining**

GUS staining of gAtRING1A:GUS transgenic plants was carried out as previously described (Tao et al., 2012; Yu et al., 2000). Seedlings were first fixed in ice-cold 90% acetone for 20 minutes. After three washes in rinse solution [50 mM Na2HPO4, 50 mM NaH2PO4, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6]3], the seedlings were infiltrated with staining solution (rinse solution with 2 mM X-Gluc) under vacuum and subsequently incubated at 37°C for 6 hours. The stained tissues were cleared of chlorophyll in an ethanol series and observed under a light microscope.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was performed as previously described (Shen et al., 2011). Seedlings were fixed on ice in MC buffer (10 mM potassium phosphate pH 7.0, 50 mM NaCl, 0.1 M sucrose) with 1% formaldehyde under vacuum for 45 minutes, after which the fixed seedlings were homogenized in liquid nitrogen. The chromatin was extracted and sonicated to produce DNA fragments of ~500 bp. H3K27me3 and H3K4me3 were detected by anti-H3K27me3 and anti-H3K4me3 antibodies (Upstate Biotechnology) bound to Protein A/G Plus-Agarose (Santa Cruz). Fold-enrichment of each fragment was determined by quantitative real-time PCR as previously described (Li et al., 2008). Genomic fragments of ACTIN7 (At5g09810) and MU (At4g03870) were amplified as internal controls for measurement of H3K27me3 and H3K4me3 enrichment, respectively. ChIP assays were repeated with at least three biological replicates. Unless stated otherwise, fold enrichment of each fragment was calculated first by normalizing the amount of a target DNA fragment against a genomic fragment of an internal control, and then by normalizing the value for immunoprecipitated samples against that for input. Primer pairs used for ChIP assays are listed in supplementary material Table S1.

**Co-immunoprecipitation**

Nine-day-old seedlings were ground with a mortar and pestle in liquid nitrogen and nuclear proteins were extracted. The protein extracts were then incubated overnight with anti-HA agarose conjugate (Sigma) or anti-GFP...
antibody (Invitrogen) bound to Protein G Plus-Agarose (Santa Cruz) at 4°C. The immunoprecipitated proteins and the protein extracts as inputs were resolved by SDS-PAGE and detected by anti-HA (Santa Cruz) or anti-GFP antibody (Santa Cruz).

Western blot analysis
Nuclear proteins were extracted from plant materials according to the ChIP protocol, but without the tissue fixation step. Proteins were resolved by SDS-PAGE and detected using various histone antibodies, including those detecting H3K4me1, H3K4me2, H3K4me3, H3K27me1, H3K27me2, H3K27me3, H3 (Upstate Biotechnology) and H2A K119ub (Cell Signaling).

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Competing interests
The authors declare no competing financial interests.

Author contributions
L.S. and H.Y. conceived and designed the experiments; L.S., Z.T., X.G. and Q.S. performed the experiments; L.S., Y.G. and H.Y. analyzed the data; L.S. and H.Y. wrote the manuscript.

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Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242/dev.104513/-/DC1

References


Fig. S1. *Atr1*1b-2, *Atbmi1a-1* and *Atbmi1b* exhibit similar flowering time to wild-type plants under long days and short days. Values were scored from at least 15 plants of each genotype. Error bars indicate s.d.
Fig. S2. GUS staining of a 15-day-old *AtRING1A:GUS* seedling. Bar = 1 mm.
Fig. S3. *AtRING1A* expression is not affected by the photoperiod pathway. (A) Temporal expression of *AtRING1A* determined by quantitative real-time PCR in wild-type seedlings grown under LDs (upper panel). The expression of *SOC1*, which is regulated by the photoperiod pathway, was examined as a positive control (lower panel). Error bars indicate s.d. (B) *AtRING1A* expression determined by quantitative real-time PCR in 9-day-old mutants of the photoperiod pathway. *AtRING1A* expression was normalized to *TUB2* expression. Error bars indicate s.d.
Fig. S4. *AtRING1A* expression is not affected by the GA pathway. (A) Comparison of *AtRING1A* expression in GA-deficient mutant *gal1-3* and wild-type plants. Seedlings grown under SDs from week 2 (W2) to week 5 (W5) were collected for expression analysis. Error bars indicate s.d. (B) Effect of GA treatment on *AtRING1A* expression in wild-type plants grown under SDs. Exogenous GA (100 µM) or 0.1% ethanol (mock) was applied weekly onto wild-type Col plants grown under SDs. Seedlings treated from week 2 (W2) to week 5 (W5) were collected for expression analysis. *AtRING1A* expression was normalized to *TUB2* expression. Error bars indicate s.d.
Fig. S5. *AtRING1A* expression is not regulated by the autonomous and vernalization pathways. (A) *AtRING1A* expression in 9-day-old mutants of the autonomous pathway grown under LDs. (B) Effect of vernalization treatment on *AtRING1A* expression. For vernalization treatment, seeds were sow on MS medium and vernalized at 4°C under low light condition for 8 weeks. The 9-day-old seedlings grown under LDs were harvested for expression analysis. *AtRING1A* expression in (A) and (B) was examined by quantitative real-time PCR, and normalized to *TUB2* expression. Error bars indicate s.d. (C, D) Flowering time of *Atring1a* and wild-type plants with (V) and without (NV) vernalization treatment grown under SDs (C) and LDs (D). After vernalization treatment, the seedlings were transferred to soil and grown under SDs or LDs. Values were scored from at least 15 plants of each genotype. Error bars indicate s.d.
Fig. S6. *SVP, AGL24, SOC1 and FLC* do not affect *AtRING1A* expression. (A,B) *AtRING1A* expression in several flowering time mutants. *AtRING1A* expression was examined by quantitative real-time PCR in 7-day-old wild-type and *svp-41* seedlings (A), and 9-day-old wild-type and several other mutant seedlings (B) grown under LDs. *AtRING1A* expression was normalized to *TUB2* expression. Error bars indicate s.d.
Fig. S7. Expression of MAF1, MAF2, MAF3, and other important floral repressors is not regulated by AtRING1A. (A-C) Temporal expression of MAF1 (A), MAF2 (B) and MAF3 (C) determined by quantitative real-time PCR in developing Atring1a and wild-type seedlings grown under LDs. Gene expression was normalized to TUB2 expression. (D,E) Expression of CO, GI and AGL24 (D), and SVP, TEM1, TEM2, TOE1, TOE2, TOE3, SMZ and SNZ (E) determined by real-time PCR in 9-day-old Atring1a and wild-type seedlings grown under LDs. Gene expression was normalized to TUB2 expression, and expression levels in wild-type seedlings are all set as 1. Error bars indicate s.d.
Fig. S8. Expression of MAF4 and MAF5 is undetectable in maf4-2 and maf5-3, respectively. Gene expression was determined by quantitative real-time PCR in 9-day-old wild-type and mutant plants. Results were normalized against the expression levels of TUB2. Asterisks indicate that quantitative real-time PCR analysis of MAF4 and MAF5 in maf4-2 and maf5-3 obtains very high Ct values, respectively, because of their barely detectable levels.
Fig. S9. Analysis of global H3K27 and H3K4 methylation levels in wild-type and Atring1a plants by immunoblotting. Nuclear extracts of 9-day-old Atring1a and wild-type seedlings were subjected to Western blot analysis using various antibodies.
Fig. S10. *AtRING1A* does not obviously affect H3K27me3 and H3K4me3 enrichment at *FLC*. (A) Schematic diagram of the *FLC* genomic region. Exons and untranslated regions are represented by black and grey boxes, respectively, while introns and other genomic regions are represented by black lines. The translation start site (ATG) and stop codon (TAG) are indicated. DNA fragments amplified in ChIP assays are indicated below the *FLC* genomic region that carries both H3K27me3 and H3K4me3 marks. (B) ChIP analysis of H3K27me3 and H3K4me3 levels at *FLC* in 9-day-old wild-type and *Atring1a* seedlings. Error bars indicate s.d. of three biological replicates.
Fig. S11. *AtRING1A* does not significantly affect H2AK119ub levels at *MAF4* and *MAF5*. (A) Analysis of global H2AK119ub levels in wild-type and *Atring1a* plants by immunoblotting. Nuclear extracts of 9-day-old *Atring1a* and wild-type seedlings were subjected to Western blot analysis using anti-H2AK119ub and anti-H3 antibodies. The different bands shown in the blot indicate different H2Aub isoforms. (B) Quantitative real-time PCR analysis of expression of *AtRING1B*, *AtBMI1A*, *AtBMI1B* and *AtBMI1C* in 9-day-old *Atring1a* and wild-type seedlings grown under LDs. Results were normalized against the expression levels of *TUB2*. Gene expression levels in wild-type seedlings are all set as 1. (C) ChIP analysis of H2AK119ub levels at *MAF4* and *MAF5* in 9-day-old wild-type and *Atring1a* seedlings. A genomic fragment of *ACTIN7* (*At5g09810*) was amplified as an internal control for measurement of H2AK119ub enrichment. Error bars indicate s.d of three biological replicates. There is no statistically significant difference in ChIP enrichment fold between wild-type and *Atring1a*. 
Fig. S12. AtRING1A is not associated directly with MAF4 and MAF5 genomic regions. (A) Western blot analysis using anti-HA antibody shows the expression of AtRING1A-4HA in nuclear extracts (Input) or immunoprecipitated fractions (Eluate) of 9-day-old Atring1a gAtRING1A-4HA seedlings. (B) ChIP analysis shows no significant binding of AtRING1A-4HA to MAF4 and MAF5 genomic regions. Enrichment fold was calculated first by normalizing the amount of a target DNA fragment against a genomic fragment of ACTIN7, and then by normalizing the value for Atring1a gAtRING1A-4HA against that for Atring1a. Error bars indicate s.d. of three biological replicates.
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**Primers pairs used for gene expression analysis (quantitative real-time PCR)**

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Primers pairs used for gene expression analysis (semi-quantitative PCR)

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Primers pairs used for ChIP assays (quantitative real-time PCR)

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MU

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