# Deciphering the Role of POLYCOMB REPRESSIVE COMPLEX1 Variants in Regulating the Acquisition of Flowering Competence in Arabidopsis[1]

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Polycomb group (PcG) proteins play important roles in regulating developmental phase transitions in plants; however, little is known about the role of the PcG machinery in regulating the transition from juvenile to adult phase. Here, we show that Arabidopsis (*Arabidopsis thaliana*) B lymphoma Moloney murine leukemia virus insertion region1 homolog (BMI1) POLYCOMB REPRESSIVE COMPLEX1 (PRC1) components participate in the repression of microRNA156 (miR156). Loss of AtBMI1 function leads to the up-regulation of the primary transcript of *MIR156A* and *MIR156C* at the time the levels of miR156 should decline, resulting in an extended juvenile phase and delayed flowering. Conversely, the PRC1 component EMBRYONIC FLOWER (EMF1) participates in the regulation of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* and *MIR172* genes. Accordingly, plants impaired in EMF1 function displayed misexpression of these genes early in development, which contributes to a CONSTANS-independent up-regulation of *FLOWERING LOCUS T* (*FT*) leading to the earliest flowering phenotype described in Arabidopsis. Our findings show how the different regulatory roles of two functional PRC1 variants coordinate the acquisition of flowering competence and help to reach the threshold of *FT* necessary to flower. Furthermore, we show how two central regulatory mechanisms, such as PcG and microRNA, assemble to achieve a developmental outcome.

Polycomb group (PcG) proteins are conserved epigenetic regulators that mediate gene repression through the incorporation of histone-modifying marks (Calonje, 2014). As far as it is known, PcG proteins associate in two multiprotein complexes in Arabidopsis (*Arabidopsis thaliana*): POLYCOMB REPRESSIVE COMPLEX1 (PRC1) and PRC2. The combined activity of the two complexes is required for stable repression of the target genes.

The major function of PRC2 is to perform histone H3 lysine-27 trimethylation (H3K27me3) through the methyltransferase activity of CURLY LEAF (CLF) and SWINGER (SWN) during sporophyte development or of MEDEA in the endosperm (Chanvivattana et al., 2004). Other PRC2 components are the *Drosophila melanogaster* suppressor of zeste12 homologs VERNALIZATION2 (VRN2), EMBRYONIC FLOWER2 (EMF2), and FERTILIZATION-INDEPENDENT SEED2, which confer specificity to the resulting PRC2s even though they have some overlapping functions (Chanvivattana et al., 2004), and finally MULTICOPY SUPPRESSOR OF INHIBITORY REGULATOR OF THE RAS-CYCLIC AMP PATHWAY and FERTILIZATION-INDEPENDENT ENDOSPERM, which are common subunits for the different PRC2s (Derkacheva and Hennig, 2014). On the other hand, the identity of Arabidopsis PRC1 is not defined yet. PRC1-mediated function can be histone 2A monoubiquitination (H2Aub) dependent, through the E3 ubiquitin ligase activity of the PRC1 RING finger proteins Arabidopsis B lymphoma Moloney murine leukemia virus insertion region1 homolog 1A (AtBMI1A)/B/C and AtRING1A/B, or H2Aub independent, which requires the activity of the PRC1 component EMF1 (Bratzel et al., 2010, 2012; Yang et al., 2013a; Calonje, 2014). These different PRC1 activities suggest the existence of PRC1 functional variants that may target different subsets of genes (Merini and Calonje, 2015). Another protein used to be considered as a putative PRC1 component is LIKE-HETEROCHROMATIN PROTEIN1 (LHP1), which has the ability to bind H3K27me3 marks (Turck et al., 2007); however, it was recently shown that LHP1 copurifies with PRC2, changing the notion of LHP1 as a PRC1 component (Derkacheva et al., 2013).

From a mechanistic point of view, recent data indicated that the binding and activity of PRC1 are required for H3K27me3 marking at some target genes,

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which challenges the classical hierarchical model for the recruitment of PcG complexes (Yang et al., 2013a; Calonje, 2014; Merini and Calonje, 2015). Whether this happens at all PcG targets is not yet known. In any case, both PRC1 and PRC2 play important roles in regulating developmental phase transitions in Arabidopsis. For instance, the combined activity of AtBMI1 and PRC2 is crucial for the transition from embryonic to vegetative development (Bratzel et al., 2010; Bouyer et al., 2011; Yang et al., 2013a); EMF1 and PRC2 regulate the transition from vegetative to reproductive development (Sung et al., 1992; Kinoshita et al., 2001; Schubert et al., 2006); and AtRING1A was recently shown to be involved in the regulation of several flowering repressors, suggesting its participation in the transition to flowering (Shen et al., 2014). However, thus far, little is known about the implication of PcG proteins in another important developmental change, the transition from juvenile to adult phase that marks the acquisition of reproductive competence.

Following germination, plants pass through a phase of vegetative growth that can be further divided into a juvenile and an adult vegetative phase. During the juvenile-to-adult phase transition, plants acquire competence to flower as well as undergo changes in multiple traits, such as leaf size and shape, internode length, and trichome distribution (Huijser and Schmid, 2011; Poethig, 2013). Although PcG proteins may have a role in regulating this developmental transition, the severity of the phenotype in some PcG mutants or the lack of phenotype in others has concealed their possible implication. Conversely, two microRNAs (miRNAs), miR156 and miR172, and their targets have been identified as key components of the mechanisms that underlie juvenile-to-adult phase changes. The miR156 targets transcripts of a subset of *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) transcription factors that have been shown to promote the transition from juvenile to adult and to flowering (Wu and Poethig, 2006; Schwarz et al., 2008). By contrast, miR172 targets *APETALA2* (*AP2*)-like factors that have been shown to repress both the transition to flowering and flower development (Aukerman and Sakai, 2003; Schmid et al., 2003; Jung et al., 2007; Mathieu et al., 2009). The expression of these miRNAs is temporally regulated by age; thus, as the plant ages, miR156 levels decrease, resulting in an increase in *SPL* expression. In the shoot apical meristem (SAM), the SPL proteins activate the floral pathway integrators *SUPPRESSOR OF CONSTANS1* (*SOC1*) and *AGAMOUS-LIKE24* (*AGL24*) and the floral meristem identity genes *FRUITFULL*, *LEAFY* (*LFY*), and *AP1*; and in leaves, the SPLs activate miR172 expression that in turn down-regulates the *AP2*-like floral repressors, which inhibit the floral integrator *FLOWERING LOCUS T* (*FT*; Wang, 2014). The so-called age pathway is proposed to prevent flowering during the juvenile phase and ensure plant flowering even in the absence of exogenous inductive cues.

*FT*, in addition to being regulated by the age pathway, is strongly controlled by photoperiod; in fact, the level of *FT* expression at the end of long days plays a primary role in determining when Arabidopsis flowers (Turck et al., 2008; Wigge, 2011). The circadian clock sets a high *CONSTANS* (*CO*) mRNA expression in the late afternoon in long days, which coincides with light exposure, resulting in CO protein accumulation as light stabilizes the CO protein. The vasculature-expressed CO protein promotes *FT* expression activation in the phloem companion cells, specifically at the end of long days (Imaizumi and Kay, 2006; Turck et al., 2008). During the night, CO is rapidly degraded by the proteasome and *FT* expression is repressed (Valverde et al., 2004). Upon its production at dusk, the FT protein moves from phloem to the SAM, where it interacts with the locally transcribed FLOWERING LOCUS D (FD) transcription factor to activate floral integrators like *SOC1* and *AGL24* to induce flowering (Amasino, 2010; Matsoukas et al., 2012). Accordingly, genetic studies have placed the age pathway parallel with the photoperiodic pathway (Wang, 2014), both being required to determine the threshold of *FT* necessary for flowering competence.

Several direct regulators of miR172-encoding genes have been identified, including the MADS box factor SHORT VEGETATIVE PHASE, which downregulates the levels of miR172 (Cho et al., 2012), GIGANTEA, which mediates the photoperiod activation of miR172 (Jung et al., 2007), and SPL9, which leads to an accumulation of miR172 (Wu et al., 2009). On the other hand, recent evidence indicates that the seed maturation gene *FUSCA3* (*FUS3*) contributes to the direct expression of the primary transcripts of *MIR156A* and *MIR156C* (*pri-MIR156A* and *pri-MIR156C*) in the developing seed and that this expression is important after germination to delay the juvenile-to-adult vegetative phase transition (Wang and Perry, 2013). However, upstream effectors mediating the age-dependent decline in miR156 levels are largely unknown. Interestingly, several recent studies showed a correlation between plant nutritional status and miR156 levels. The accumulation of metabolically active sugars, such as Suc and Glc, acts as a signal to selectively repress the expression of the *miR156A* and *miR156C* genes (Wahl et al., 2013; Yang et al., 2013b; Yu et al., 2013), but the molecular mechanism by which this repression take place and is maintained is not yet understood.

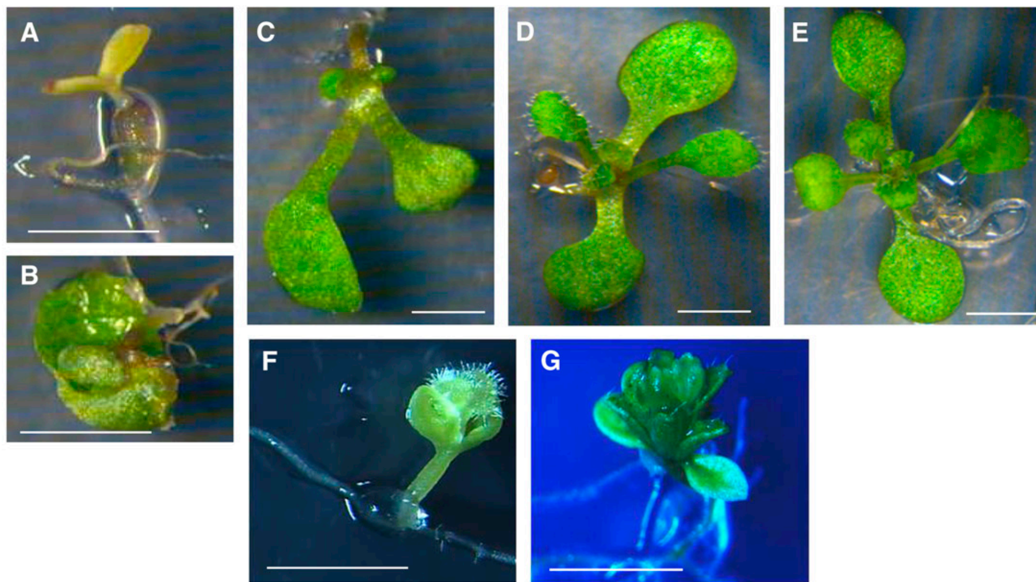
In this work, we show that loss of function of the PRC1 component AtBMI1 leads to the up-regulation of *pri-MIR156A/C* at the time the levels of miR156 should decline, resulting in an extended juvenile phase and delayed flowering. We found that *atbmi1a/b* mutants display reduced levels of H3K27me3 marks at the transcriptional start site (TSS) of these genes, suggesting the participation of the PcG machinery in regulating miR156 expression. According to our results, AtBMI1-mediated repression of *pri-MIR156A/C* allows the age-dependent expression of *FT* and the development of adult traits. Interestingly, the PRC1 component EMF1 does not regulate *pri-MIR156A/C* expression; instead, EMF1 participates in the regulation

of miR172. Our findings show how the combined regulatory roles of two functional PRC1 variants are crucial to coordinate the acquisition of flowering competence.

## RESULTS

### Loss of EMF1 Function Leads to CO-Independent *FT* Up-Regulation, But Not the Loss of *AtBMI1* Function

Mutant plants severely compromised in AtBMI1 activity do not undergo the transition from embryonic to vegetative development, remaining in an embryonic stage similar to that of mutants impaired in PRC2 function, like *clf/swn* (Chanvivattana et al., 2004). Unfortunately, the severity of *atbmi1* strong mutant phenotypes or the lack of phenotype in *atbmi1* single mutants has masked the possible implication of the AtBMI1 proteins in regulating other developmental transitions. To explore other possible roles of AtBMI1 proteins, we took advantage of the different penetrance of the *atbmi1b* allele (Bratzel et al., 2010) that causes a gradient of phenotypes in *atbmi1a/b* mutants.



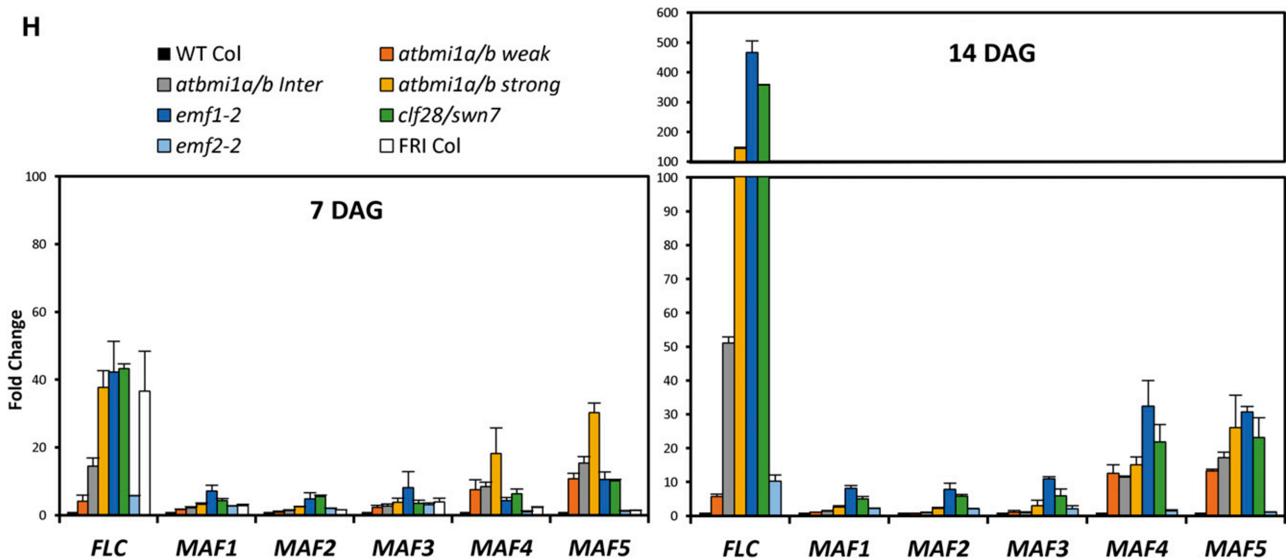




**Figure 1.** *FLC*, *MAF4*, and *MAF5* expression is significantly altered in *atbmi1* mutants. A to G, Phenotypes of strong (A), intermediate (B and C), and weak (D) *atbmi1a/b*, wild-type (WT) Columbia (Col; E), *emf1-2* (F), and *emf2-2* (G) at 10 d after germination (DAG). Bars = 2 mm. H, Expression levels of *FLC*, *MAF1*, *MAF2*, *MAF3*, *MAF4*, and *MAF5* in 7- and 14-d-old plants at ZT1 under LD conditions. The expression levels of these genes were also analyzed in 7-d-old FRI-Col seedlings. Quantifications were normalized to *ACTIN2* (*ACT2*). The *y* axis indicates fold change compared with wild-type Col.

Early in development, *atbmi1a/b* phenotypes ranged from seedlings arrested in an embryo-like stage (strong mutants; Fig. 1A) and seedlings with twisted or embraced green cotyledons (intermediate mutants; Fig. 1, B and C) to seedlings with a wild-type-like phenotype (weak mutants; Fig. 1D). Later on, strong and intermediate *atbmi1a/b* mutants remained in an embryonic stage, in which they generated embryo-like structures, while *atbmi1a/b* weak mutants were able to flower and generate viable seeds (Bratzel et al., 2010), allowing us to analyze other developmental processes.

Interestingly, *atbmi1a/b* weak mutants did not show an early-flowering phenotype as other PcG mutants like *emf1* or *emf2* (Sung et al., 1992; Kinoshita et al., 2001). It is noteworthy that *emf1* and *emf2* display the earliest flowering phenotypes described in Arabidopsis. *emf1-2* strong mutants produce a carpel right after germination without developing any leaf (Fig. 1F), and the *emf1-1* mutant produced a small inflorescence after developing a few sesile leaves, which is the same phenotype displayed by *emf2-2* (Fig. 1G).

To understand the differences in the flowering phenotypes among these PcG mutants, we examined the expression levels of several flowering time master regulators in *atbmi1a/b*, *emf1-2*, *emf2-2*, *clf-28/swn-7*, and wild-type Col plants. For this purpose, 7- and 14-d-old seedlings growing under long-day (LD) conditions were collected at Zeitgeber time 1 (ZT1; i.e. 1 h after light on; Fig. 1H). We included in the analysis 7-d-old FRIGIDA (FRI)-Col plants in which a functional *FRI* allele was introgressed into Col. FRI up-regulates the flowering repressor *FLOWERING LOCUS C* (*FLC*), which represses the expression of the flowering promoter gene *FT*, leading to late flowering (Searle et al., 2006).

We found that *FLC* was strongly up-regulated in the *atbmi1a/b* intermediate and strong phenotypes, *emf1-2*, *clf-28/swn-7*, and FRI-Col, compared with wild-type Col. The expression of *FLC* was also increased in *atbmi1a/b* weak and *emf2-2* mutants, although to a lesser extent (Fig. 1H). When we measured the expression levels of the *FLC*-related flowering genes *MADS AFFECTING FLOWERING1* (*MAF1*) to *MAF5* (Scortecci et al., 2001; Ratcliffe et al., 2003), we found that the levels of *MAF1*, *MAF2*, and *MAF3* were not or were slightly altered in the analyzed mutants with the exception of *emf1-2* and *clf-28/swn-7*. On the other hand, *MAF4* and *MAF5* expression levels were dramatically increased in the different *atbmi1a/b* phenotypes, *emf1-2* and *clf-28/swn-7*, whereas they were not significantly affected in *emf2-2* and FRI-Col (Fig. 1H). The fact that *emf2-2* did not show misregulation of *MAF4* and *MAF5* while *clf-28/swn-7* did can indicate that these genes are regulated by a different paralog, such as VRN2 (Chen et al., 2009). Interestingly, *atring1a/b* mutants displayed similar expression levels of *FLC*, *MAF4*, and *MAF5* to those of *atbmi1a/b* and *emf1-2* mutants (Supplemental Fig. S1), suggesting that the PRC1 components AtBMI1, AtRING1, and EMF1 act together in the repression of these genes.

Consistent with the misexpression of *FLC*, *MAF4*, and *MAF5* in the mutants, it has been shown previously that the levels of H3K27me3 marks at these genes were altered in PRC2 mutants (Jiang et al., 2008), *emf1*, and *atring1a* (Kim et al., 2012b; Shen et al., 2014). Therefore, to investigate whether AtBMI1 loss of function also affected the levels of H3K27me3 marks at *FLC*, *MAF4*, and *MAF5*, we examined the levels of this histone modification in *atbmi1a/b* mutants at the first intron of the genes, which has been shown to display an enrichment of H3K27me3 marks in wild-type seedlings at 9 to 10 DAG (Shen et al., 2014; Fig. 2A). Indeed, we found that the levels of H3K27me3 were decreased in *atbmi1a/b* weak mutants (Fig. 2B); furthermore, the H3K27me3 marks were eliminated in the very strong *atbmi1a/b/c* mutants (Fig. 2B), indicating that the loss of AtBMI1 function causes the loss of H3K27me3 marks at *FLC*, *MAF4*, and *MAF5*.

Then, we assessed the levels of *FT* in the different seedlings. In agreement with their early-flowering

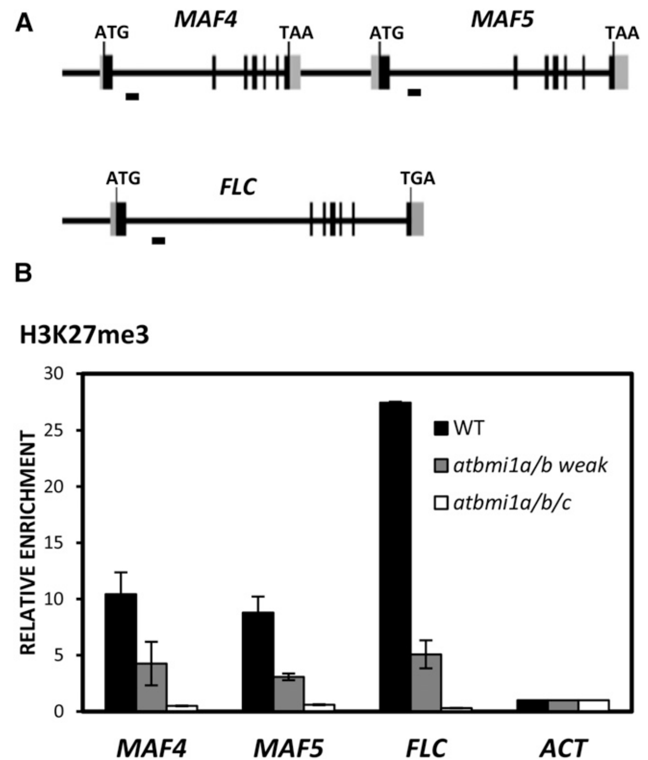



**Figure 2.** H3K27me3 levels at *MAF4*, *MAF5*, and *FLC* are altered in *atbmi1* mutants. A, Schematic diagram of *MAF4*, *MAF5*, and *FLC* genomic regions. Exons and untranslated regions are represented by black and gray boxes, respectively, while introns and other genomic regions are represented by black lines. The translation start site (ATG) and stop codon (TAA or TAG) are indicated. DNA fragments amplified in chromatin immunoprecipitation (ChIP) assays are indicated below the genomic regions. B, ChIP analysis of H3K27me3 levels at the *FLC*, *MAF4*, and *MAF5* first intron region in wild-type (WT), *atbmi1a/b* weak, and *atbmi1a/b/c* seedlings at 10 DAG. *ACT7* was used as a negative control. The immunoprecipitated DNAs were quantified and normalized to *ACT7*. Error bars indicate the SD of two biological replicates.

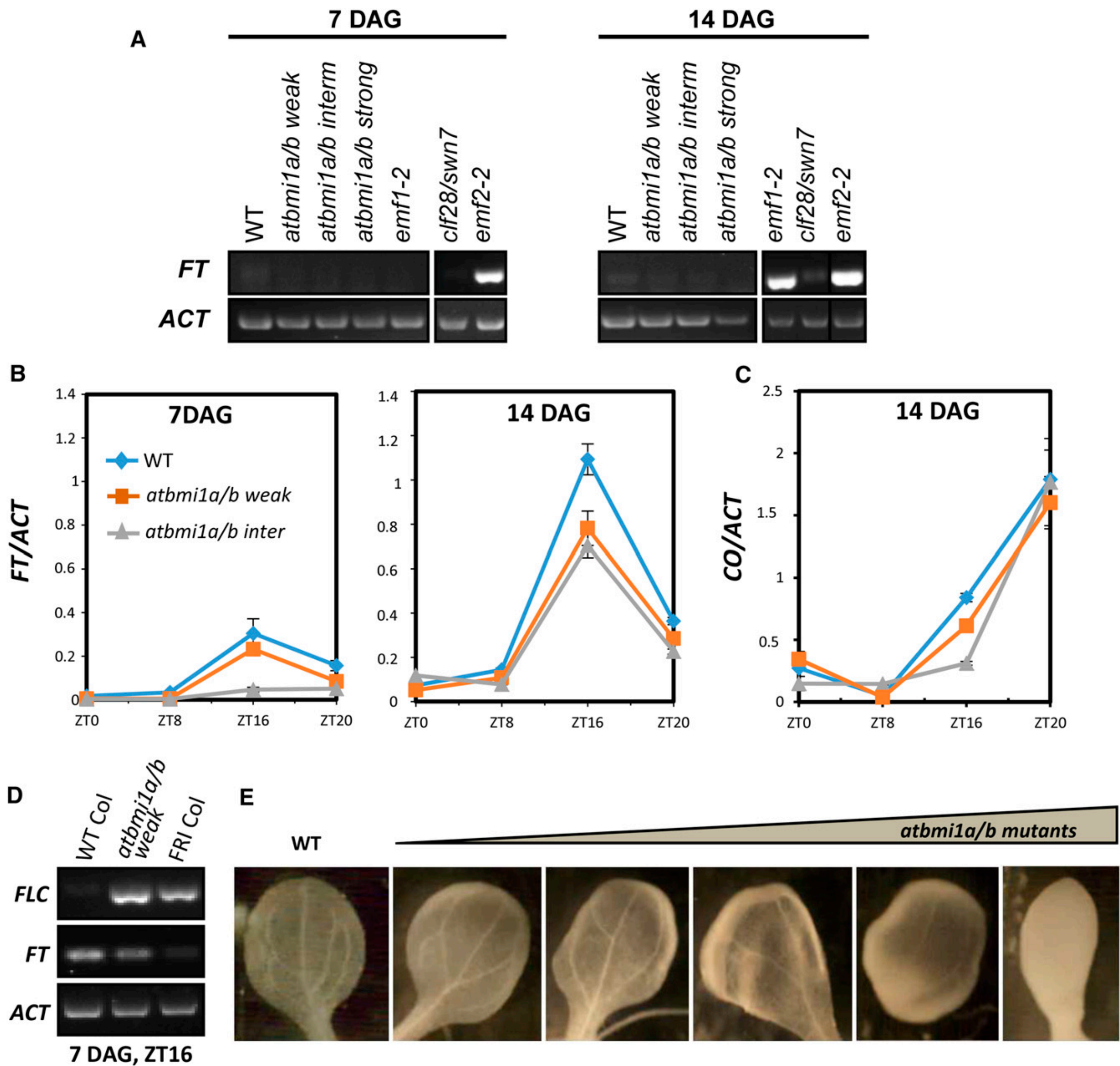



**Figure 3.** *FT* expression in *atbmi1* mutants is CO dependent. A, Expression levels of *FT* in 7- and 14-d-old plants at ZT1 under LD conditions. *ACT2* was used as an internal control (samples are as in Fig. 1H). B, *FT* mRNA levels in the indicated seedlings over an LD cycle at 7 and 14 DAG. C, *CO* mRNA levels over an LD cycle at 14 DAG. *FT* and *CO* transcript levels were normalized to *ACT2*; error bars indicate the SD of two biological repeats. D, *FLC* and *FT* transcript levels in 7-d-old wild-type (WT) Col, *atbmi1a/b* weak, and FRI-Col seedlings under LD conditions at Zeitgeber time 16 (ZT16). E, Vasculature organization of 10-d-old cotyledons from wild-type Col and different *atbmi1a/b* phenotypes.

phenotype (Sung et al., 1992), *emf1-2* and *emf2-2* displayed a strong up-regulation of *FT*, despite the high levels of *FLC* expression (Fig. 3A). A recent report proposed that FLC recruits a PRC1-containing EMF1 (EMF1-PRC1) to *FT* chromatin for PcG repression and that CO activity antagonizes this repression by reducing the levels of EMF1-PRC1 at *FT* in the evening (Wang et al., 2014). This would explain why *FLC* up-regulation did not lead to *FT* repression in *emf1*, as FLC could not mediate *FT* repression in the absence of EMF1, and also in *emf2* mutants, as EMF1 activity may be required for PRC2 recruitment. Since the Arabidopsis Col accession contains a nonfunctional *FRI* allele, and therefore the levels of *FLC* expression are very low (Kim and Sung, 2014; Fig. 1H), other FLC-related genes might recruit the EMF1-PRC1 for *FT* repression in this background, which could explain why *emf1* mutants are also unresponsive to *MAF4* and *MAF5* overexpression.

As *CO* transcription is low at ZT1 and its expression is not altered in *emf1* and *emf2* mutants (Kim et al., 2010), the *FT* misexpression in these mutants may be CO independent. In support of this, it has been shown that *emf1-1/co* and *emf2/co* double mutant phenotypes were indistinguishable from their respective *emf1* and *emf2* single mutant parents, while *emf1-1/ft* double mutants usually did not flower and *emf2/ft* double mutants bolted after producing a higher number of sessile leaves than *emf2* single mutants (Haung and Yang, 1998).

Surprisingly, we did not find a significant *FT* expression in any of the *atbmi1a/b* phenotypes at ZT1 (Fig. 3A); hence, we wondered whether *FT* levels were altered at other times of the day. When we measured the levels of *FT* transcripts over a 24-h LD cycle in *atbmi1a/b* weak, intermediate, and wild-type Col seedlings (Fig. 3B), we found that the expression of *FT* was photoperiod dependent in both the wild-type and *atbmi1a/b* mutants, but the levels of *FT* in *atbmi1a/b* were lower than in wild-type plants, despite the fact that *CO* levels were not affected in these mutants (Fig. 3C). Also, we found that *FT* expression seemed to decrease along with the severity of the *atbmi1a/b* phenotype. It might be argued that the decrease in *FT* levels was a consequence of *FLC* up-regulation; however, the expression levels of *FLC* in *atbmi1a/b* mutants were as high as in FRI-Col plants, but *FT* was not down-regulated to FRI-Col levels (Fig. 3D). Therefore, it seems that *FLC* is not able to mediate *FT* repression in *atbmi1a/b*, *emf1*, or PRC2 mutants in spite of the differences in *FT* expression among mutants.

Interestingly, like *atbmi1a/b* mutants, *clf-28/swn-7* did not show misexpression of *FT*. Low levels of *FT* in *clf/swn* compared with *clf* single mutants have been reported before (Farrona et al., 2011). Alterations in vascular development and differentiation were proposed to be the basis for *FT* down-regulation in *clf/swn* double mutants (Farrona et al., 2011). Similarly, *atbmi1a/b* mutant phenotypes displayed different degrees of altered vascular development (Fig. 3E), which might explain the gradual decrease of *FT* expression correlated with the strength of the phenotype.

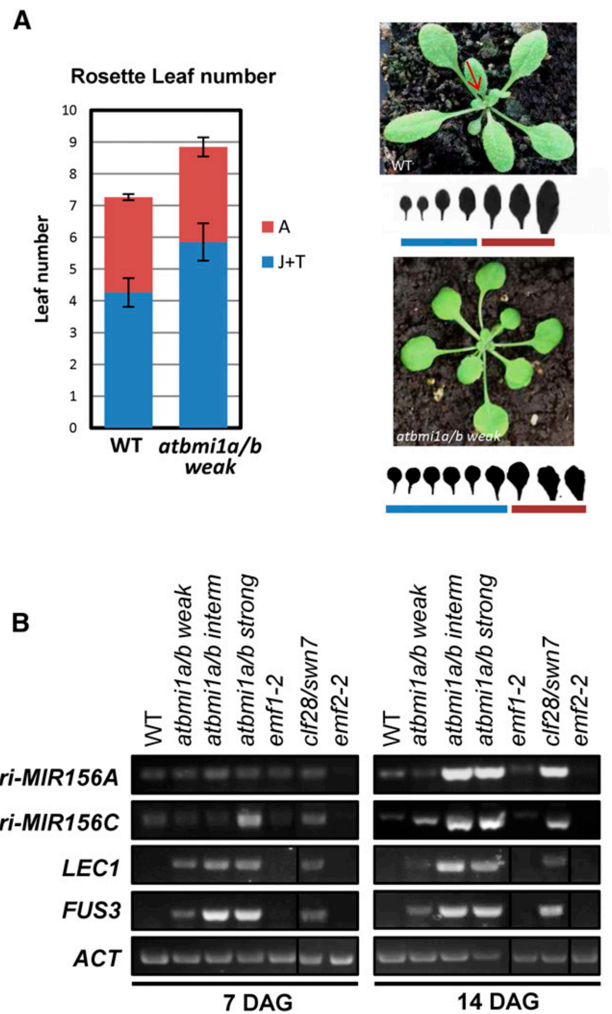



**Figure 4.** *atbmi1a/b* mutants misexpress *MIR156A* and *MIR156C*. A, Flowering time of wild-type (WT) Col and *atbmi1a/b* weak plants (left). The time was measured by the number of rosette leaves produced from the SAM prior to flowering; 16 to 20 plants for each line were scored. Error bars indicate SD. Juvenile (J) and transition (T) leaves were differentiated from adult leaves (A) by shape (right). B, Expression levels of *pri-MIR156A*, *pri-MIR156C*, and the seed maturation genes *LEAFY COTYLEDON1* (*LEC1*) and *FUS3* in the different mutants at 7 and 14 DAG growing under LD conditions at ZT1.

### *atbmi1a/b* Mutants Have an Extended Juvenile Phase

As we mentioned before, in contrast to *emf1* or PRC2 mutants like *emf2*, *atbmi1a/b* weak mutants did not show an early-flowering phenotype; moreover, the most affected mutants never flowered. To investigate if flowering time was altered in *atbmi1a/b* weak mutants, we compared the flowering time in days and number of rosette leaves before bolting between *atbmi1a/b* weak mutant and wild-type Col plants under LD conditions (Fig. 4A). We found that flowering was delayed for 3 d in *atbmi1a/b* weak mutants compared with wild-type plants (22 ± 1 and 19 ± 1 d, respectively) and that the mutants generated two extra leaves before bolting (Fig. 4A, left), which was consistent with *FT* levels in the mutants but not with *FLC*, *MAF4*, or *MAF5* levels. Surprisingly, these two extra leaves displayed round shape and a long petiole (Fig. 4A, right), which are considered juvenile traits (Wu et al., 2009), suggesting a prolonged juvenile phase in the mutants.

Overexpression of miR156 prolongs the expression of juvenile vegetative traits and delays flowering. miR156 is encoded by eight genes in Arabidopsis (*MIR156A* to *MIR156H*; Reinhart et al., 2002). Among these genes, *MIR156A* and *MIR156C* were recently shown to be direct targets of the seed maturation gene *FUS3*. FUS3 activates *MIR156A/C* expression during seed development, and this expression is important after germination to delay the juvenile-to-adult

vegetative phase transition (Wang and Perry, 2013). *MIR156A* and *MIR156C* contain RY elements at their 5′ end and into/through the gene, which are DNA elements specifically recognized by the B3 DNA-binding domain of *FUS3* (Wang and Perry, 2013).

Since *FUS3* is misexpressed in *atbmi1* mutants and *clf-28/swn-7* but not in *emf1* or *emf2* (Yang et al., 2013a; Fig. 4B), we investigated levels of the *pri-MIR156A/C* transcripts in these mutants (Fig. 4B). Strikingly, we found that the levels of *pri-MIR156A/C* displayed a drastic increase at 14 DAG in the three *atbmi1a/b* mutants, especially in intermediate and strong phenotypes, and in *clf-28/swn-7* (Fig. 3B), but they were not altered in *emf1-2* and *emf2-2* (Fig. 4B). In addition, we found that the *pri-MIR156s* displayed similar levels in *atring1a/b* mutants than in *atbmi1a/b* weak mutants (Supplemental Fig. S2), indicating that both AtBMI1 and AtRING1 proteins are required to regulate miR156 levels. According to these results, the prolonged juvenile phase in *atbmi1a/b* weak mutants may be a consequence of miR156 misexpression; however, since *FUS3* is ectopically expressed in these mutants, the high levels of *pri-MIR156A/C* might be an indirect effect of AtBMI1 loss of function.

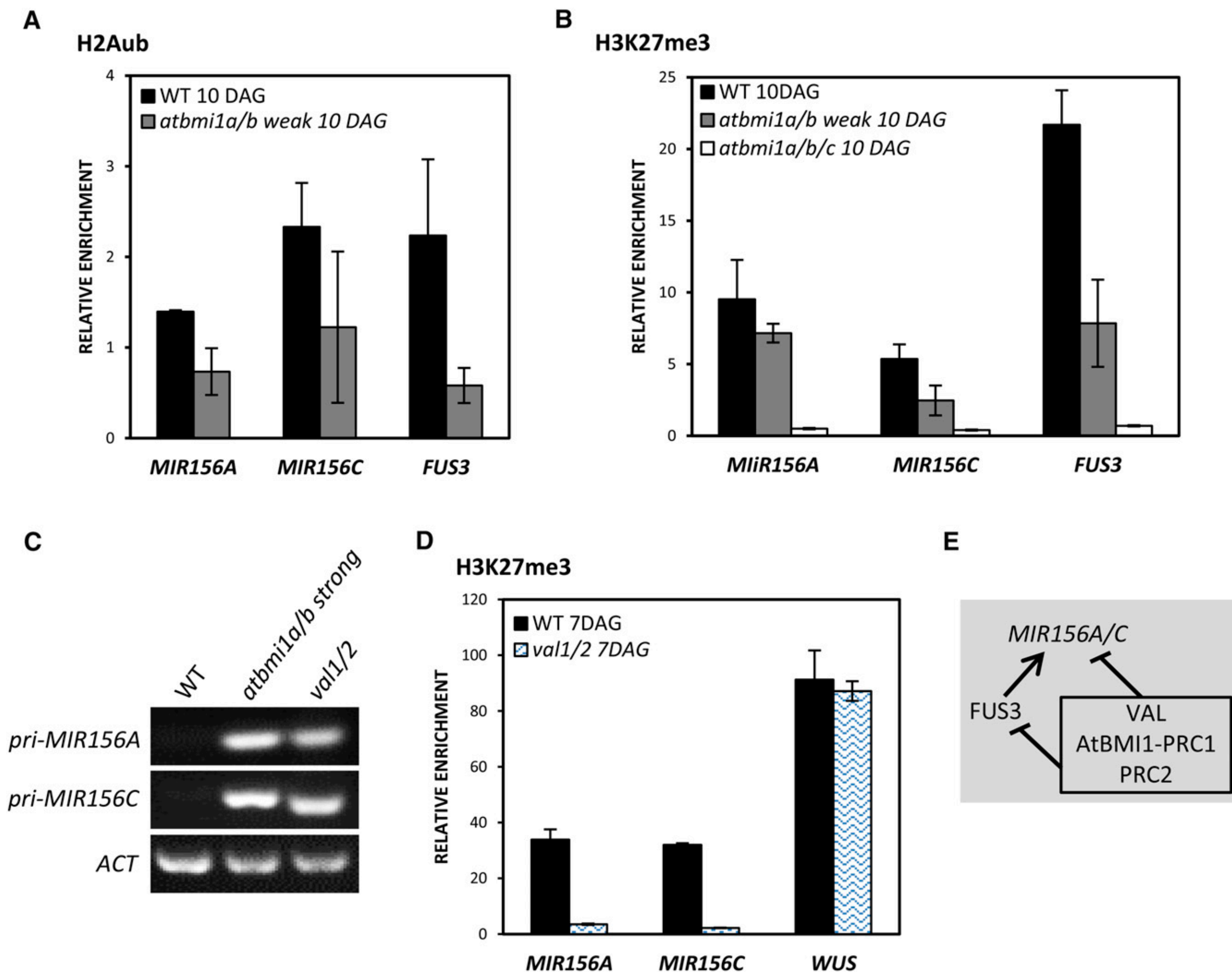



**Figure 5.** *MIR156A* and *MIR156C* are direct targets of AtBMI1. A, ChIP analysis of H2Aub levels at *MIR156A* and *MIR156C* TSS in wild-type (WT) and *atbmi1a/b* weak seedlings at 10 DAG. *FUS3* was used as a positive control. B, ChIP analysis of H3K27me3 levels at *MIR156A* and *MIR156C* TSS in wild-type, *atbmi1a/b* weak, and *atbmi1a/b/c* seedlings at 10 DAG. *FUS3* was used as a positive control. The immunoprecipitated DNAs were quantified and normalized to *ACT7*. Error bars indicate the SD of at least two biological replicates. C, Expression levels of *pri-MIR156A* and *pri-MIR156C* in the wild type, *atbmi1a/b* strong, and *val1/2* mutants at 10 DAG. *ACT2* was used as an internal control. D, ChIP analysis of H3K27me3 levels at the TSS of *MIR156A* and *MIR156C* in wild-type and *val1/2* seedlings at 7 DAG. *WUSCHEL* (*WUS*) was included as a negative target of VAL and a positive control of H3K27me3 (Yang et al., 2013a). The immunoprecipitated DNAs were quantified and normalized to *ACT7*. Error bars indicate the SD of two biological replicates. E, Schematic representation of *MIR156A/C* regulation by VAL-AtBMI1-PRC1/PRC2 and *FUS3*. Lines with bars indicate the repression of gene expression, and the line with the arrow indicates activation.

**The Levels of H2Aub and H3K27me3 Marks in *atbmi1* Mutants Are Decreased at *MIR156A*/*C***

To determine whether the AtBMI1 proteins play a role in regulating *pri-miR156A/C* expression, we investigated the levels of H2Aub marks at the TSS region of *MIR156A* and *MIR156C* in wild-type and *atbmi1a/b* weak seedlings at 10 DAG. We found that the levels of these marks at *MIR156A* were decreased in *atbmi1a/b* mutants and that the levels at *MIR156C* seemed to be reduced, although the experimental variation was large (Fig. 5A). Since AtBMI1 activity is required for PRC2-mediated H3K27me3 marking at several target genes (Yang et al., 2013a), we examined the levels of H3K27me3 marks at the TSS of these genes (Fig. 5B). We found that the levels of H3K27me3 were decreased at the TSS of all these genes in *atbmi1a/b* weak mutants (Fig. 5B); furthermore, the H3K27me3 marks were eliminated in the very strong *atbmi1a/b/c* mutants (Fig. 5B), indicating that *MIR156A* and *MIR156C* are regulated by the PcG machinery.

Then, we wondered whether the VIVIPAROUS1/ABSCISIC ACID INSENSITIVE3-LIKE1/2/3 (VAL1/2/3) proteins were involved in the recruitment of AtBMI1 and subsequently PRC2 to *MIR156A*/*C*, as is the case for the regulation of *FUS3* (Yang et al., 2013a). The VAL proteins have a B3 DNA-binding domain that is proposed to recognize RY elements (Suzuki et al., 2007). Since *MIR156A* and *MIR156C* contain RY motifs (Wang and Perry, 2013), we reasoned that they might be targets of the VAL proteins. To investigate this, we first analyzed the expression levels of the *pri-MIR156s* in *val1/2* mutants and compared them with the levels in wild-type and strong *atbmi1a/b* seedlings at 10 DAG (Fig. 5C). Indeed, we found that both *pri-MIR156s* were up-regulated in *val1/2* to the same levels as in *atbmi1a/b* strong mutants. We further compared the levels of H3K27me3 at the TSS of *MIR156A* and *MIR156C* between the wild type and *val1/2* mutants (Fig. 5D), and we found that the levels were dramatically reduced in the mutants. Together, these data suggest that the expression of *pri-MIR156A*/*C* is regulated by VAL and the AtBMI1 proteins. Therefore, the strong up-regulation of *pri-MIR156* genes in *atbmi1a/b* mutants may be caused by both the loss of AtBMI1 function and the ectopic expression of *FUS3*

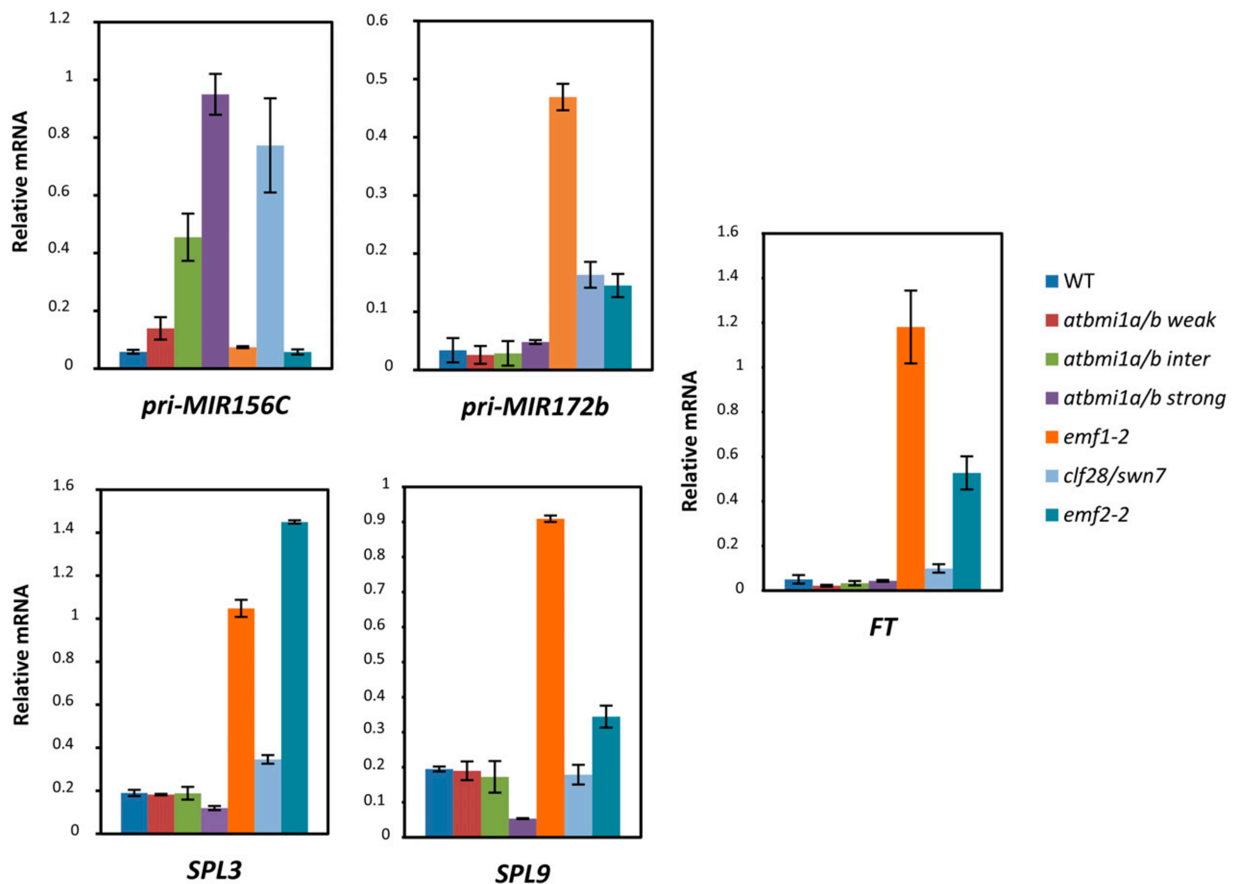



**Figure 6.** AtBMI1-PRC1- and EMF1-PRC1-mediated regulation of miR156 and miR172. Expression levels of *pri-MIR156C*, *pri-MIR172b*, *SPL3*, *SPL9*, and *FT* are shown for wild-type (WT) and mutant seedlings at 10 DAG. Quantifications were normalized to *ACT2*. Error bars represent the SD of two biological replicates.

(Fig. 5E). It is possible that the activation of *MIR156A/C* by *FUS3* only takes place in the absence of VAL-PcG-mediated repression, as must be the case during seed development.

### *emf1-2* Displays Up-Regulation of *pri-MIR172b*, *SPL3*, and *SPL9*

During the juvenile-to-adult phase transition, plants acquire competence to flower. In wild-type conditions, miR156 levels decrease as plants age, resulting in an increase in *SPL* expression. *SPL9* has been shown to activate *pri-MIR172b* expression that, in turn, down-regulates the *AP2*-like floral repressors, which inhibit *FT* (Wang, 2014). Also, SPL3 directly regulates *FT* expression (Kim et al., 2012a). Consistent with this, it has been shown that the vasculature-specific expression of *FT* was notably increased in the cotyledons and distal regions of true leaves of plants overexpressing an miR156-resistant *SPL3* and that *FT*::*GUS* expression was greatly reduced in the cotyledons and leaves of *35S*::*MIR156* plants (Kim et al., 2012a). In addition, it has been proposed that high miR156 levels reduce the ability of FT/FD to induce flowering by repressing SPL activity in the SAM (Wang et al., 2009). Therefore, SPLs and miR172 action contribute to set the threshold of *FT* necessary for flowering and to prepare the SAM to respond to the flowering signal.

To determine whether the levels of *pri-MIR156A/C* expression in the different mutants correlate with the levels of *SPL3*, *SPL9*, *pri-MIR172b*, and *FT*, and if the expression pattern of the gene in each mutant explains the different flowering times, we analyzed the expression of all these genes in 10-d-old mutants and wild-type seedlings (Fig. 6). Consistent with the *pri-MIR156A/C* levels in *atbmi1a/b* mutants, we found low expression levels of *SPL3*, *SPL9*, and *pri-MIR172b*, confirming their juvenile stage. Accordingly, we found low levels of *FT* in these mutants, which are maintained later in development, leading to a delay in flowering time in *atbmi1a/b* weak mutants. In *atbmi1a/b* intermediate and strong mutants, misexpression of these genes along with the lack of a correctly differentiated phloem may be the cause of their never-flowering phenotype.

On the other hand, *SPL3*, *SPL9*, and *pri-MIR172b* expression was high in *emf1-2* mutants. Interestingly, a recent report showed that *SPL9* is a target of EMF1 (Kim et al., 2012b); thus, derepression of *SPL9* may cause the activation of *pri-MIR172b* in *emf1-2* mutants. Also, *SPL3* is up-regulated in transgenic plants expressing an *EMF1* antisense complementary DNA under the control of the floral meristem identity gene *LFY* promoter (*LFY*:*asEMF1*; Pu et al., 2013). Moreover, it has been shown that several *MIR172* genes are direct targets of EMF1 (Kim et al., 2012b). *emf2-2* also displayed increased expression levels of *SPL3*, *SPL9*, and

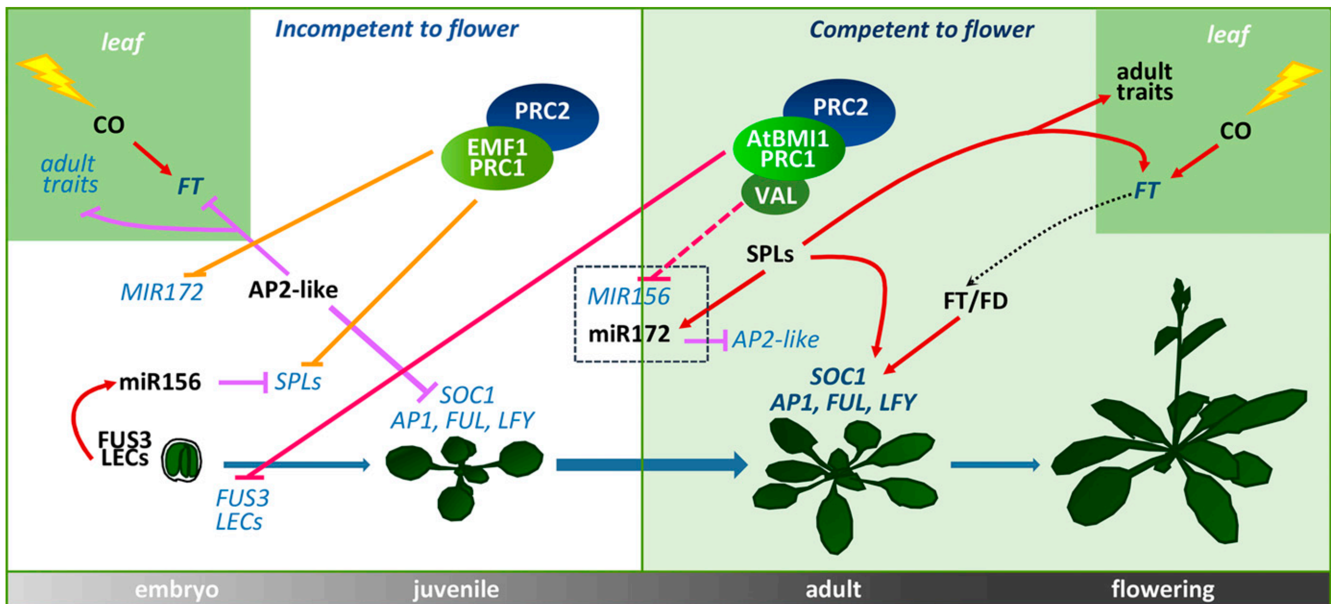



**Figure 7.** Model of the roles of AtBMI1-PRC1 and EMF1-PRC1 variants in regulating juvenile-to-adult phase transition through miR156 and miR172 repression. EMF1-PRC1 represses *MIR172* and *SPLs* to maintain the juvenile phase. As the plant ages, the levels of miR156 decrease by AtBMI1-PRC1-mediated repression, which allows the development of adult traits and the acquisition of flowering competence. Solid purple lines with bars indicate negative regulation; solid red lines with arrows indicate positive regulation; orange lines with bars indicate EMF1-PRC1/PRC2 repression; pink lines with bars indicate AtBMI1-PRC1/PRC2 repression (the dashed pink line indicates a possible negative regulation); and the dotted black line with arrow indicates the movement of FT from leaves to the SAM. Repressed genes are indicated in light blue italic type and activated genes in dark blue italic type; proteins and miRNAs are indicated in black type. *FUL*, *FRUITFULL*.

*pri-MIR172b*, although the levels of the transcripts were not as high as in *emf1-2*, probably due to a redundant role of VRN2 in regulating these genes, as EMF2 and VRN2 regulate a common subset of targets (Lafos et al., 2011). Therefore, EMF1 and EMF2 directly and indirectly regulate miR172 levels. Remarkably, the levels of *pri-MIR156*, *SPLs*, and *pri-MIR172b* in *emf1-2* and *emf2-2* may explain the CO-independent expression of *FT* and the extremely early acquisition of flowering competence of these mutants.

Surprisingly, in the complete loss-of-PRC2-function *clf-28/swn-7* mutants, the levels of *SPL3* and *SPL9* were only slightly higher than in the wild type (Fig. 6), and *pri-MIR172b* expression was not as high as in *emf1-2*. However, the high levels of *pri-MIR156A/C* in these mutants most likely affect *pri-MIR172b* expression by reducing SPL levels, thus explaining the expression pattern in these mutants. Consistent with this, *clf-28/swn-7* did not display high levels of *FT* expression, which must be accentuated by alterations in vascular development.

## DISCUSSION

PcG proteins have been shown to play important roles in regulating developmental phase transitions in plants; however, given that PcG components are present in the nuclei of most cells, whether they are targeted to distinct subsets of targets in specific cell types or developmental stages has been a major research problem. Recent findings regarding the PcG mechanism have shown that PRC1 is required for H3K27me3 marking at some target genes in both Arabidopsis (Yang et al., 2013a; Calonje, 2014) and animals (Comet and Helin, 2014; Schwartz and Pirrotta, 2014), placing PRC1 in a decisive position for the repression of some genes. In addition, several lines of evidence have suggested the existence of different mechanisms for PRC1-mediated repression in Arabidopsis (Kim et al., 2012b; Yang et al., 2013a; Calonje, 2014); however, it is not known whether a combination of different PRC1 subunits is required to exert the different mechanisms.

According to previous results in Arabidopsis, the PRC1 RING finger proteins AtBMI1 and AtRING1 are required for the repression of the seed maturation program after germination, whereas EMF1 is required for the repression of the floral program during vegetative development (Moon et al., 2003; Calonje et al., 2008; Bratzel et al., 2010; Chen et al., 2010), indicating that different PRC1 components are crucial for the regulation of different subsets of targets. On the other hand, other results suggest that all these components are required for the regulation of a different subset of target genes. For instance, AtRING1A has been shown to participate in the repression of *FLC*, *MAF4*, and *MAF5* (Shen et al., 2014) and EMF1 in the repression of *FLC* (Kim et al., 2010). We show here that both EMF1 and AtBMI1 are required for *FLC*, *MAF4*, and *MAF5* repression, suggesting a PRC1 in which AtRING1, AtBMI1, and EMF1 are required for repression. Whether these PRC1 proteins are always associated in the same complex or not remains to be investigated. In any case, the current data on PRC1-mediated gene regulation in Arabidopsis point to the existence of at least different PRC1 functional variants. Interestingly, despite the fact that AtBMI1 and EMF1 may participate in the regulation of *FT* through the repression of *FLC*, *MAF4*, and *MAF5*, loss of function in AtBMI1 and EMF1 does not have the same effect on *FT* expression, suggesting that the coordinated activity of different PRC1 functional variants may be required to give a specific developmental outcome. Therefore, to understand the role of PcG regulation in plant development, it will be necessary to determine the particular combination of PRC1s that regulates a specific process.

By exploring other possible roles of AtBMI1 proteins during plant development besides the repression of seed maturation genes after germination, we found that these proteins play a crucial role in the regulation of the transition from juvenile to adult phase. More importantly, our results point to a model in which two different functional PRC1 variants, an AtBMI1-PRC1 and an EMF1-PRC1 variant, coordinate the acquisition of flowering competence and contribute to reach the threshold of *FT* necessary to flower through the regulation of miR156 and miR172 levels, respectively (Fig. 7).

miR156 and miR172 have been identified as key components of the mechanisms that underlie the transition from juvenile to adult phase (Huijser and Schmid, 2011); however, although the roles of these miRNAs have been studied extensively, the mechanisms involved in their regulation are still largely unknown, especially those related to the age-dependent decline of miR156. We found that plants impaired in AtBMI1 function showed increased levels of *MIR156A/C* at the time the levels of miR156 should decline, which indicates that AtBMI1 proteins are required for miR156 repression. We propose that the high miR156 levels in *atbmi1a/b* contribute to reduce the levels of *FT* in leaves and to reduce the ability of FT/FD to induce flowering in the SAM by repressing SPL activity, leading to an extended juvenile phase. Conversely, we found that EMF1-PRC1 is required to maintain the repression of several *SPL* and *MIR172* genes during the juvenile phase, thereby delaying the acquisition of flowering competence (Fig. 7). Accordingly, plants impaired in EMF1 function displayed up-regulation of *SPL3*, *SPL9*, and *pri-MIR172* early in development, which may trigger a CO-independent up-regulation of *FT* and a precocious acquisition of flowering competence. In addition, AtBMI1-PRC1 and EMF1-PRC1 seem to be required for H3K27me3 marking at miR156 and miR172, respectively, supporting the idea that PRC1 triggers H3K27me3 at some target genes.

In summary, these results show how the coordinated roles of two functional PRC1 variants are required to regulate the transition from juvenile to adult

phase; furthermore, we show how two central regulatory mechanisms, such as PcG and miRNA, assemble to control the acquisition of flowering competence, providing new insights into the paths actually used by the cell in order to achieve a developmental outcome.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) *emf1-2*, *emf2-2*, *val1/2*, *atbmi1a/b*, *clf-28/swn-7*, and *atring1a/b* mutants were described previously (Yang et al., 1995; Suzuki et al., 2007; Bratzel et al., 2010; Chen et al., 2010; Lafos et al., 2011). Plants were grown under LD conditions (16 h of light/8 h of dark) at 21°C on Murashige and Skoog agar plates containing 1.5% (w/v) Suc and 0.8% (w/v) agar. After germination, plants were transferred to soil and grown under the same conditions.

Seedlings at 10 DAG were fixed in ethanol:acetic acid (9:1, v/v) to analyze vasculature development in cotyledons.

### Gene Expression Analysis

Total RNA was extracted using the ISOLATE II RNA Plant Kit (Bioline). Complementary DNAs were reverse transcribed from total RNAs with the QuantiTect reverse transcription kit (Qiagen). Quantitative reverse transcription-PCR was performed using the SensiFAST SYBR & Fluorescein Kit (Bioline) and the Bio-Rad iQ5 system. Primers used are specified in Supplemental Table S1.

### ChIP

ChIP assays were carried out on fixed chromatin extracted from seedlings at 10 DAG using anti-H2Aub monoclonal (Cell Signaling; 8240) and anti-H3K27me3 polyclonal (Diagenode; pAb-069-050) antibodies. Buffers and procedures were as described previously (Yang et al., 2013a). Quantitative measurements of the immunoprecipitated DNA were performed using the SensiFAST SYBR & Fluorescein Kit (Bioline) and the Bio-Rad iQ5 system. Each of the immunoprecipitations was repeated independently at least once, and each sample was quantified in triplicate. Primers used are specified in Supplemental Table S1.

### Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Expression of flowering repressors in *atring1a/b* mutants.

**Supplemental Figure S2.** Expression of *pri-MIR156A/C in atring1a/b* mutants.

**Supplemental Table S1.** Primers used in this work.

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