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Connecting PRC1 and miRNA regulation

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Deciphering the role of Polycomb Repressive Complex 1 (PRC1) variants in regulating the acquisition of flowering competence in Arabidopsis

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One-sentence Summary

Two different PRC1 variants coordinate the acquisition of flowering competence during juvenile-to adult phase transition in Arabidopsis through the regulation of miR156 and miR172 levels.

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Abstract

Polycomb Group (PcG) proteins play important roles in regulating developmental phase transitions in plants; however, little is known about the role the PcG machinery in regulating the transition from juvenile to adult phase. Here, we show that Arabidopsis BMI1 (AtBMI1) PRC1 components participate in the repression of miR156. Loss of AtBMI1 function leads to upregulation of *pri-MIR156A/C* 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 *SPL* and *MIR172* genes. Accordingly, plants impaired in EMF1 function displayed misexpression of these genes early in development, which contributes to a CONSTANS (CO)-independent upregulation 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 miRNA, assemble to achieve a developmental outcome.

Introduction

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, PcGs associate in two multi-protein complexes in Arabidopsis: Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2, respectively). The combined activity of the two complexes is required for stable repression of the target genes.

The major function of PRC2 is to trimethylate lysine 27 on histone H3 (H3K27me3) through the methyltransferase activity of CURLY LEAF (CLF) and SWINGER (SWN) during sporophyte development or of MEDEA (MEA) in the endosperm (Chanvivattana et al., 2004). Other PRC2 components are the VEFS domain containing proteins VERNALIZATION 2 (VRN2), EMBRYONIC FLOWER 2 (EMF2) and FERTILIZATION INDEPENDENT SEED 2 (FIS2), which confer specificity to the resulting PRC2s even though they have some overlapping functions (Chanvivattana et al., 2004); and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) and FERTILIZATION INDEPENDENT ENDOSPERM (FIE), 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 AtBMI1A/B/C and AtRING1A/B, or H2Aub-independent, which requires the activity of the PRC1 component EMBRYONIC FLOWER 1 (EMF1) (Bratzel et al., 2010; Bratzel et al., 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 putative PRC1 component is LIKE-HETEROCHROMATIN PROTEIN 1 (LHP1) that has the ability to bind H3K27me3 marks (Turck et al., 2007); however, it has been recently shown that LHP1 co-purifies 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 is required for H3K27me3 marking at some target genes, which challenges the classical hierarchical model for 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 has been 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 flowering 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 PcGs 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 *APETALA 2* (*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 *SPLs* expression. In the shoot apical meristem (SAM), the SPL proteins activate the floral pathway integrators *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *AGAMOUS-LIKE 24* (*AGL24*) and the floral meristem identity genes *FRUITFULL (FUL)*, *LEAFY* (*LFY)*, and *APETALA1* (*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 plants 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 night, CO is rapidly degraded by the proteasome and *FT* expression is repressed (Valverde et al., 2004). Upon its production in 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 in 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 (SVP), which downregulates the levels of miR172 (Cho et al., 2012), GIGANTEA (GI), 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 evidences indicate that the seed maturation gene *FUSCA3* (*FUS3*) contributes to the direct expression of primary transcript of *MIR156A* and *C (pri-MIR156A* and *C)* 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. Accumulation of metabolically active sugars, such as sucrose and glucose, acts as a signal to selectively repress the expression of the *miR156A* and *miR156C* genes (Wahl et al., 2013; Yu et al., 2013; Yang et al., 2013b), 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 upregulation 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* upregulation, 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 *atbmi1b* allele (Bratzel et al., 2010) that causes a gradient of phenotypes in *atbmi1a/b* mutants. 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. 1B, C) to seedlings with WT-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 *emf1-1* mutant produced a small inflorescence after developing few cauline leaves, which is the same phenotype displayed by *emf2-2* (Fig. 1G).

To understand the differences in the flowering phenotype 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 Columbia (WT Col) plants. For this purpose, seven and fourteen day-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-day-old FRIGIDA (FRI)-Col plants in which a functional *FRI* allele was introgressed into Col. FRI upregulates the flowering repressor

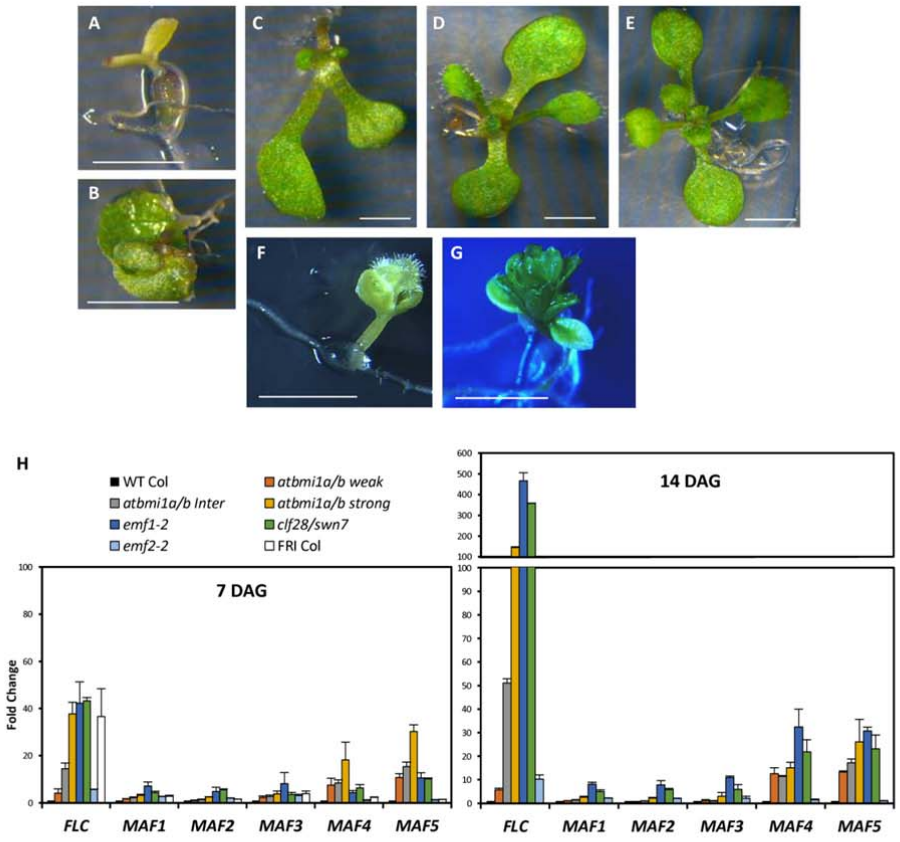

Figure 1. *FLC, MAF4 and MAF5* expression is significantly altered in *atbmi1* mutants. A to G, phenotypes of strong (A), intermediate (B, C) and weak (D) *atbmi1a/b*, WT Col (E), *emf1-2* (F) and *emf2-2* (G) at 10 DAG. H, Expression levels of *FLC*, *MAF1*, *MAF2*, *MAF3*, *MAF4 and MAF5* in 7 and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

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 upregulated in the *atbmi1a/b* intermediate and strong phenotypes, *emf1-2*, *clf-28/swn-7* and FRI-Col compared to WT 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 FLOWERING 1* to *5* (*MAF1-5*) genes (Scortecci et al., 2001; Ratcliffe et al., 2003), we found that the levels of *MAF1*, *MAF2* and *MAF3* were not or 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 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 VEFs paralog, such as VRN2 (Chen et al., 2009). Interestingly, *atring1a/b* mutants displayed similar expression levels of *FLC*, *MAF4* and *MAF5* to that 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 previously shown 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 WT seedlings at 9-10 days after germination (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, that the H3K27me3 marks were eliminated in the very strong *atbmi1a/b/c* mutants (Fig. 2B), indicating that the loss of AtBMI1 function causes 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 phenotype (Sung et al., 1992), *emf1-2* and *emf2-2* displayed a strong upregulation of *FT*, despite the high levels of *FLC* expression (Fig. 3A). A recent report proposed that FLC recruits a PRC1containing EMF1 (EMF1-PRC1) to *FT* chromatin for PcG repression, and that CO activity antagonize this repression by reducing the levels of EMF1-PRC1 at *FT* in the evening (Wang et al., 2014). This would explain why *FLC* upregulation did not lead to *FT* repression in *emf1*, as FLC could not mediate

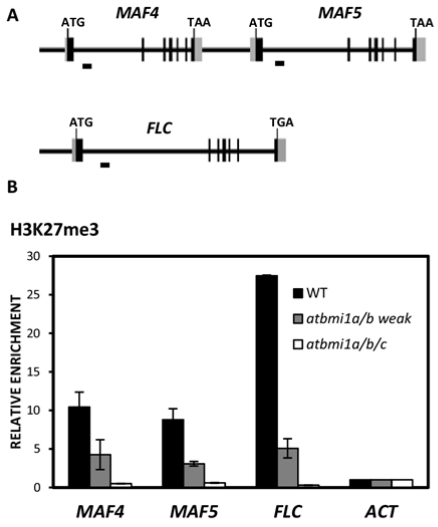

Figure 2. H3K27me3 levels at *MAF4*, *MAF5* and *FLC* are altered in *atbmi1* mutants. A, Schematic diagram of *MAF4*, *MAF5* and *FLC* genomic regions. Exons and untranslated regions are represented by black and grey boxes, respectively, while introns and other genomic regions are represented by black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

FT repression in absence of EMF1; and also in *emf2* mutants, as EMF1 activity may be required for PRC2 recruitment. Since Arabidopsis Col accession contains a non-functional *FRI* allele, and therefore the levels of *FLC* expression are very low (Kim and Sung, 2014; Fig. 1H), other FLC-related gene might be recruiting the EMF1-PRC1 for *FT* repression in this background, which could explain why *emf1* mutants are also unresponsive to *MAF4* and *MAF5* overexpression.

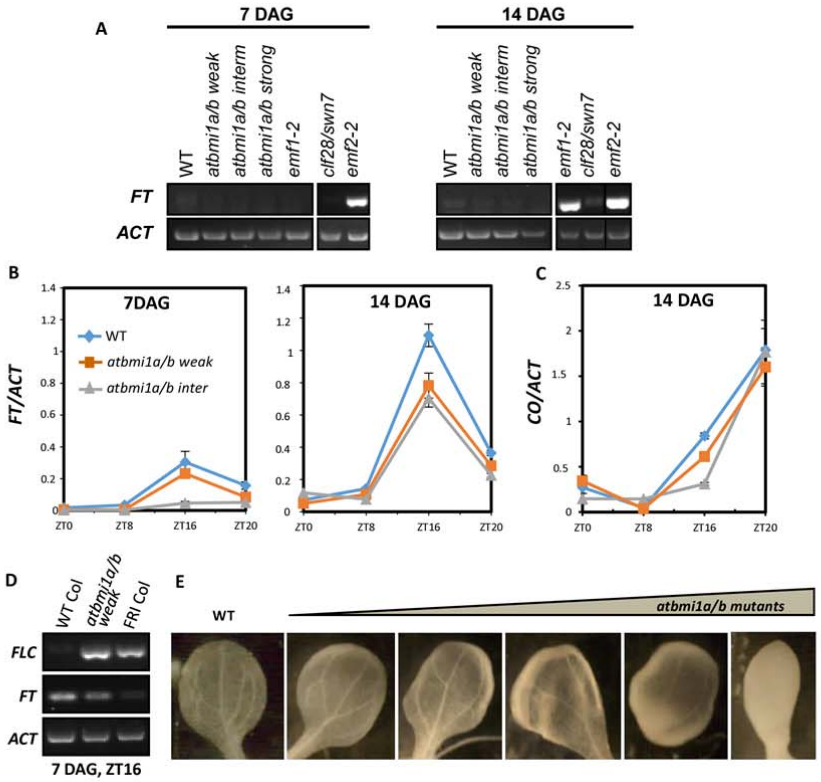

Figure 3. *FT* expression in *atbmi1* mutants is CO-dependent. A, Expression levels of *FT* in 7 and 14-day-old plants at ZT1 under LD conditions. *ACT2* was used as internal control (samples are as in Fig. 1H). B, *FT* mRNA levels in the indicated seedlings over a LD cycle at 7 and 14 DAG. C, *CO* mRNA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

As *CO* transcription is low at ZT1 and it 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 WT Col seedlings (Fig. 3B), we found that the expression of *FT* was photoperiod-dependent in both WT and *atbmi1a/b* mutants, but the levels of *FT* in *atbmi1a/b* were lower than in WT 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 *atbmi1a/b* phenotype. It might be argued that the decrease in *FT* levels was a consequence of *FLC* upregulation; however, the expression levels of *FLC* in *atbmi1a/b* mutants were as high as in FRI-Col plants but *FT* was not downregulated to FRI-Col levels (Fig. 3D). Therefore, it seems that *FLC* is not able to mediate *FT* repression either 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 to *clf* single mutants has 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.

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 WT Col plants under LD (Fig. 4A). We found that flowering was delayed for 3 days in *atbmi1a/b* weak mutants compared to WT plants (22±1 and 19±1 days, respectively), and that the mutants generated 2 extra-leaves before bolting (Fig. 4A, left panel), which was consistent with *FT* levels in the

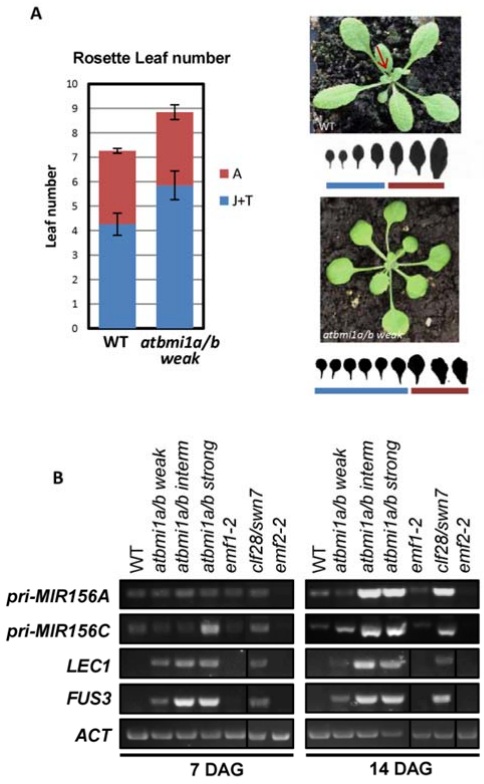

Figure 4. *atbmi1a/b* mutants misexpress *MIR156A* and *C*. A, Flowering time of WT Col and *atbmi1a/b* weak plants (left panel). The time was measured by the number of rosette leaves produced from SAM prior to flowering; 16–20 plants for each line were scored. Bars indicate SD. Juvenile (J) and transition leaves (T) were differentiated from adult leaves (A) by shape (right panel). B, Expression levels of *pri-MIR156A*, *pri-MIR156C*, and the seed maturation genes *LEAFY COTYLEDON 1* (*LEC1*) and *FUS3* in the different mutants at 7 and 14 DAG growing under LD at ZT1.

323 mutants, but not with *FLC*, *MAF4* or *MAF5* levels. Surprisingly, these 2 extra-leaves
324 displayed round shape and a long petiole (Fig. 4A, right panel), which are considered
325 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 H* (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 and (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 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.

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 WT 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);

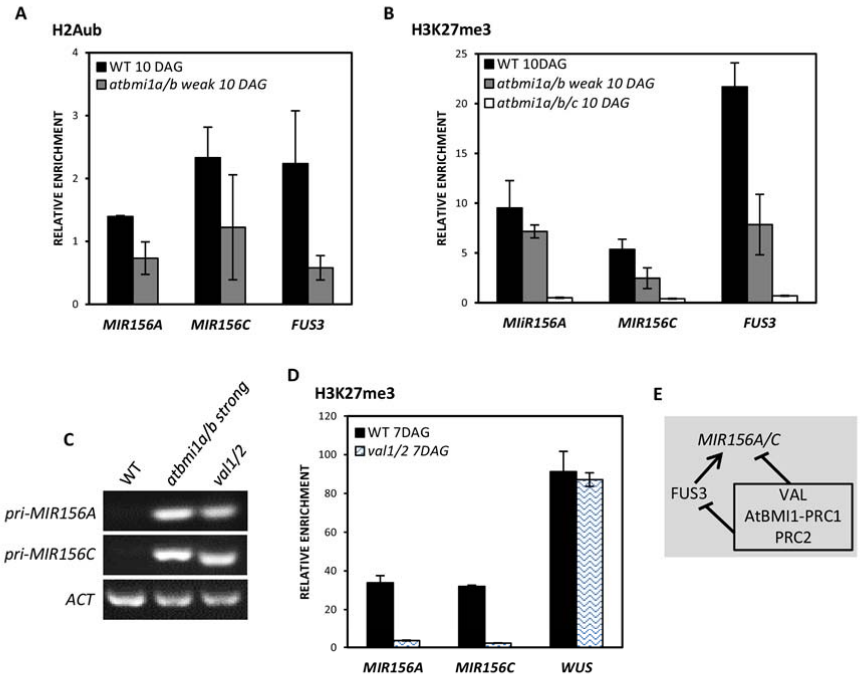

Figure 5. *MIR156A/C* are direct targets of AtBMI1. A, ChIP analysis of H2Aub levels at *MIR156A* and *MIR156C* TSS in WT and *atbmi1a/b* weak seedlings at 10 DAG. *FUS3* was used as positive control. B, ChIP analysis of H3K27me3 levels at *MIR156A* and *MIR156C* TSS in WT, *atbmi1a/b* weak and *atbmi1a/b/c*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

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 VAL (*VP1/ABI3-LIKE)*1/2/3 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

364 domain that is proposed to recognize RY-elements (Suzuki et al., 2007). Since
365 *MIR156A* and *MIR156C* contain RY motifs (Wang and Perry, 2013), we reasoned that
366 they might be targets of the VAL proteins. To investigate this, we first analyzed the
367 expression levels of the *pri-MIR156s* in *val1/2* mutants and compared to the levels in
368 WT and strong *atbmi1a/b* seedlings at 10 DAG (Fig. 5C). Indeed, we found that both
369 *pri-MIR156s* were upregulated in *val1/2* to the same levels as in *atbmi1a/b* strong
370 mutants. We further compared the levels of H3K27me3 at the TSS of *MIR156A* and *C*
371 between WT and *val1/2* mutants (Fig. 5D), and we found that the levels were
372 dramatically reduced in the mutants. All together these data suggest that the expression
373 of *pri-MIR156A/C* is regulated by VAL and the AtBMI1 proteins. Therefore, the strong
374 upregulation of *pri-MIR156* genes in *atbmi1a/b* mutants may be caused by both, the loss
375 of AtBMI1 function and the ectopic expression of *FUS3* (Fig. 5E). It might be possible
376 that the activation of *MIR156A/C* by *FUS3* only takes place in absence of VAL-PcG
377 mediated repression, as must be the case during seed development.

378 **_emf1-2_ displays upregulation of _pri-MIR172b_, _SPL3_ and _SPL9_**

379 During the juvenile-to-adult phase transition plants acquire competence to flowering. In
380 WT conditions, miR156 levels decrease as plants age, resulting in an increase in *SPLs*
381 expression. *SLP9* has been shown to activate *pri-MIR172b* expression that in turn down-
382 regulates the *AP2-like* floral repressors, which inhibit *FT* (Wang, 2014). Also, SPL3
383 directly regulates *FT* expression (Kim et al., 2012a). Consistent with this, it has been
384 shown that the vasculature-specific expression of *FT* was notably increased in the
385 cotyledons and distal regions of true leaves of plants overexpressing a miR156-resistant
386 *SPL3*, and that *FT::GUS* expression was greatly reduced in the cotyledons and leaves of
387 *35S::MIR156* plants (Kim et al., 2012a). In addition, it has been proposed that high
388 miR156 levels reduce the ability of FT/FD to induce flowering by repressing SPL
389 activity in the SAM (Wang et al., 2009). Therefore, SLPs and miR172 action contribute
390 to set the threshold of *FT* necessary for flowering and to prepare the SAM to respond to
391 flowering signal.

392 To determine whether the levels of *pri-MIR156A/C* expression in the different mutants
393 correlate with the levels of *SPL3*, *SPL9*, *pri-MIR172b* and *FT,* and if the expression
394 pattern of the genes in each mutant explains the different flowering times, we analyzed
395 the expression of all these genes in 10-day-old mutants and WT seedlings (Fig. 6).

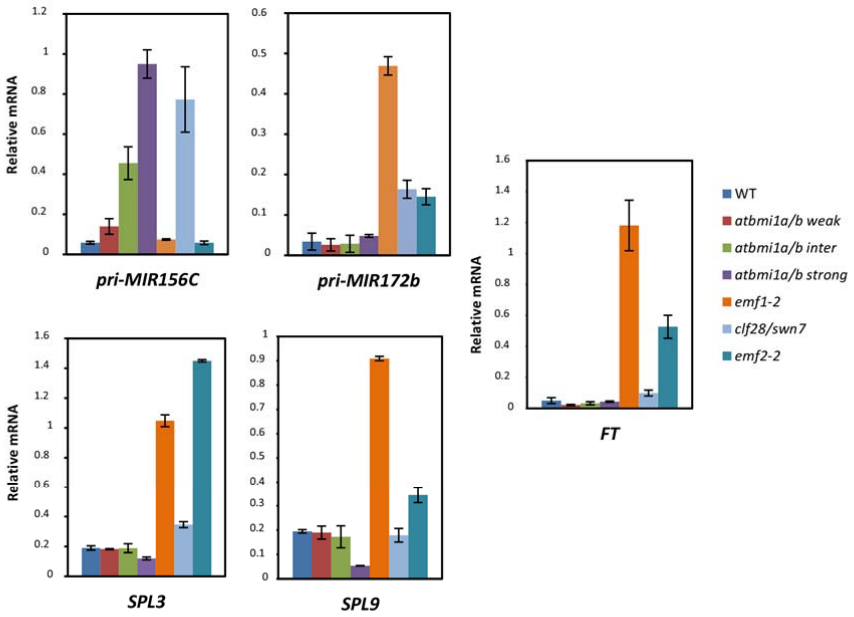

Figure 6. AtBMI1-PRC1 and EMF1-PRC1 mediated regulation of miR156 and miR172. Expression levels of *pri-MIR156C*, *pri-MIR172b*, *SPL*, *SPL9* and *FT* in WT and mutant seedlings at 10 DAG. Quantifications were normalized to *ACT2*. Bars represent SD of two biological replicates.

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 activation of *pri-MIR172b* in *emf1-2* mutants. Also, *SPL3* is upregulated in transgenic plants expressing an *EMF1* antisense cDNA 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* displayed also increased expression levels of *SPL3*, *SPL9* and *pri-MIR172b*, although the levels of the transcripts were not as high as in *emf1-2*, most 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 WT (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 are most probably affecting *pri-MIR172b* expression by reducing SPLs 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 or not they are targeted to distinct subsets of targets in specific cell types or developmental stages has been a major research problem. Recent findings in 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 animal (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 subset 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 have 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, current data on PRC1 mediated gene regulation in Arabidopsis point to the existence of at least different PRC1 functional variants, Interestingly, despite AtBMI1 and EMF1 may participate in the regulation of the *FT* through the repression of *FLC*, *MAF4* and *MAF5,* loss of function in AtBMI1 and EMF1 do 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, a AtBMI1-PRC1 and a 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, albeit the roles of these miRNA have been extensively studied, 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 *SPLs* and *MIR172* genes during the juvenile phase, thereby delaying the acquisition of flowering competence (Fig.7). Accordingly, plants impaired in EMF1 function displayed upregulation *SPL3, SPL9* and *pri-MIR172* early in development, which may trigger a CO-independent upregulation 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 the PRC1 triggers H3K27 trimethylation at some target genes.

In summary, these results show how the coordinated role 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.

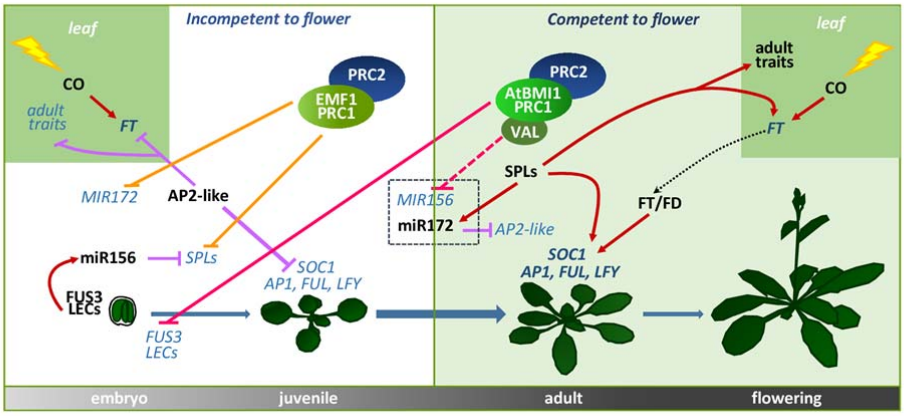

Figure 7. Model resuming the role of AtBMI1-PRC1 and EMF1-PRC1 variants in regulating juvenile-to-adult phase transition through miR156 and miR172 repression. EMF1-PRC1 represses *MIR172* and *SPLs* to maintain the juvenile phase. As plant ages, the levels of miR156 decrease by AtBMI1-PRC1 mediated repression, which allows 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 VAL/AtBMI1-PRC1/PRC2 repression (dashed pink line indicates possible negative regulation); dotted black line with arrow indicates the movement of FT from leaves to SAM; repressed genes are indicated in light blue italic and activated genes in dark blue italic; proteins and miRNAs are indicated in black.

Materials and Methods

Plant materials and growth conditions

Arabidopsis *emf1-2*, *emf2-2*, *val1/2*, *atbmi1a/b*, *clf-28/swn-7*, *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 light/8 h dark) at 21 °C on MS agar plates containing 1.5% sucrose and 0.8% 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). cDNAs were reverse-transcribed from total RNAs with QuantiTect reverse transcription kit (Qiagen). qRT-PCRs were performed using Sensi FAST SYBR & Fluorescein kit (Bioline) and an iQ5 Biorad system. Primers used are specified in Supplemental Table S1.

Chromatin immunoprecipitation

ChIP assays were carried out on fixed-chromatin extracted from seedlings at 10 DAG using anti-H2Aub monoclonal (Cell signaling #8240) and polyclonal anti-trimethyl H3-K27 (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 Sensi FAST SYBR & Fluorescein kit (Bioline) and an iQ5 Biorad 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.

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Figure Legends

Figure 1. *FLC*, *MAF4* and *MAF5* expression is significantly altered in *atbmi1* mutants. A to G, phenotypes of strong (A), intermediate (B, C) and weak (D) *atbmi1a/b*, WT Col (E), *emf1-2* (F) and *emf2-2* (G) at 10 DAG. H, Expression levels of *FLC*, *MAF1*, *MAF2*, *MAF3*, *MAF4* and *MAF5* in 7 and 14-day-old plants at ZT1 under LD conditions. The expression levels of these genes were also analyzed in 7 day-old FRI Col seedlings.

521 Quantifications were normalized to *ACT2*. The y-axis indicates fold change compared to
522 WT Col.

523

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

534 **Figure 3.** *FT* expression in *atbmi1* mutants is CO-dependent. A, Expression levels of
535 *FT* in 7 and 14-day-old plants at ZT1 under LD conditions. *ACT2* was used as internal
536 control (samples are as in Fig. 1H). B, *FT* mRNA levels in the indicated seedlings over
537 a LD cycle at 7 and 14 DAG. B, *CO* mRNA levels over a LD cycle at 14 DAG. *FT* and
538 *CO* transcript levels were normalized to *ACT2*; bars indicate the standard deviation
539 (SD) of two biological repeats. C, *FLC* and *FT* transcript levels in 7-day-old WT Col,
540 *atbmi1a/b* weak and FRI Col under LD at ZT16. D, Vasculature organization of 10-day-
541 old cotyledons from WT Col and different *atbmi1a/b* phenotypes.

542 **Figure 4.** *atbmi1a/b* mutants misexpress *MIR156A* and *C*. A, Flowering time of WT
543 Col and *atbmi1a/b* weak plants (left panel). The time was measured by the number of
544 rosette leaves produced from SAM prior to flowering; 16–20 plants for each line were
545 scored. Bars indicate SD. Juvenile (J) and transition leaves (T) were differentiated from
546 adult leaves (A) by shape (right panel). B, Expression levels of *pri-MIR156A*, *pri-*
547 *MIR156C*, and the seed maturation genes *LEAFY COTYLEDON 1* (*LEC1*) and *FUS3* in
548 the different mutants at 7 and 14 DAG growing under LD at ZT1.

549 **Figure 5.** *MIR156A* and *C* are direct targets of AtBMI1. A, ChIP analysis of H2Aub
550 levels at *MIR156A* and *MIR156C* TSS in WT and *atbmi1a/b* weak seedlings at 10 DAG.
551 *FUS3* was used as positive control. B, ChIP analysis of H3K27me3 levels at *MIR156A*

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

Figure 6. AtBMI1-PRC1 and EMF1-PRC1 mediated regulation of miR156 and miR172. Expression levels of *pri-MIR156C*, *pri-MIR172b*, *SPL, SPL9* and *FT* in WT and mutant seedlings at 10 DAG. Quantifications were normalized to *ACT2*. Bars represent SD of two biological replicates.

Figure 7. Model resuming the role 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 plant ages the levels of miR156 decrease by AtBMI1-PRC1 mediated repression, which allows 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 (dashed pink line indicates possible negative regulation); dotted black line with arrow indicates the movement of FT from leaves to SAM; repressed genes are indicated in light blue italic and activated genes in dark blue italic; proteins and miRNAs are indicated in black.

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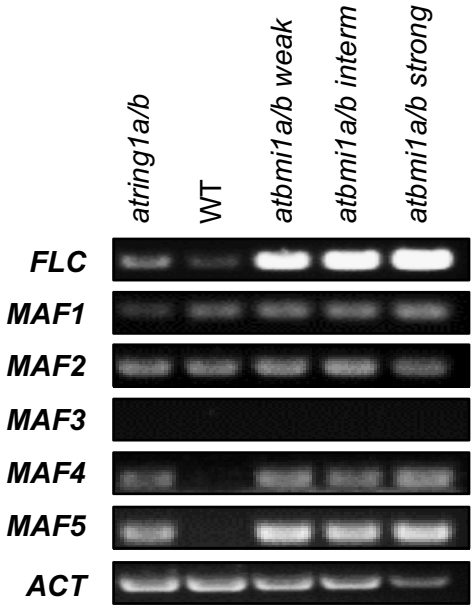
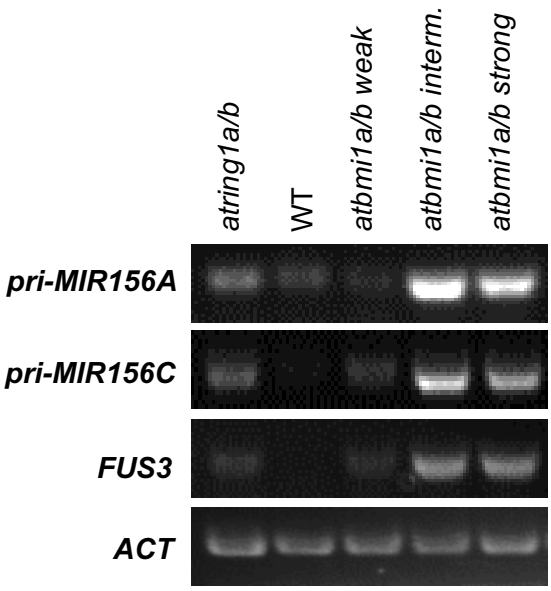


Supplemental Figure S1. Expression of *FLC*, *MAF1, MAF2, MAF3, MAF4* and *MAF5* in 14-day-old *atring1a/b,* WT and *atbmi1a/b* different phenotypes at ZT1 under LD conditions. *ACT2* was used as internal control. *MAF4* and *MAF5* are upregulated in *atring1a/b* mutants as in *atbmi1a/b* mutants. *FLC* is also upregulated but less that in *atbmi1a/b* mutants.



Supplemental Figure S2. AtRING1 together with AtBMI1 regulates the expression of *pri-MIR156A/C.* Expression levels of *pri-MIR156A*, *pri-MIR156C*, and the seed maturation gene *FUS3* in *atring1a/b*, WT and *atbmi1a/b* mutants at 14 DAG growing under LD at ZT1. *ACT2* was used as internal control. *FUS3* and *pri-MIR156A/C* are derepressed in *atring1a/b* mutants to *atbmi1a/b* weak levels.

Supplemental Table S1. Primers used in this work

qRT-PCR primers

Name	5´to 3´
FLC Fw	GCCACCTTAAATCGGCGGTTG
FLC Rev	CACAAAGTCTCTTGGCCAAAGAGAGAG
MAF1 Fw	CGGCTGAGTTTTCACCTTAAACTCAAAGCC
MAF1 Rev	GAGGAAGATAAAAGGTTTGAGATTACACAGC
MAF2 Fw	GGCTGAGCTTTCACCTTAAACTTACAGC
MAF2 Rev	CCACATTGGCGCGAGGAAGATAAAAAGG
MAF3 Fw	GGCTGAGCTTTCACCTTAAACTTACAGC
MAF3 Rev	GCTTCGTTTTGTTTTACCTTTATTTCCACATTGGG
MAF4 Fw	GCTACGGAAAAGTCATCCAAGGAGATGC
MAF4 Rev	CGAAAGTAAATACTATATCATCCTGTCTCCGAAGG
MAF5 Fw	CCACCAATCATCAACGGCTGATTTTTCATCATCC
MAF5 Rev	CCGTATGCAGGGGGAGAAGAGG
FT Fw	CGAACGGTGATGATGCCTATAGTAG
FT Rev	CACTCTCATTTTCCTCCCCCTCTC
CO Fw	CCAATGGACAGAGAAGCCAGG
CO Rev	GCATCGTGTTGAACCCTTGC
Pri-MIR156A Fw	CTTCGTTCTCTATGTCTCAATCTCTC
Pri-MIR156A Rev	TGATTAAAGGCTAAAGGTCTCCTC
Pri-MIR156C Fw	GTGATAATGAGTGATGACTGATG
Pri-MIR156C Rev	GAAAACGTGACCGGGACCGAATCG
FUS3 Fw	TCATGGTCTGCAGCTAGGTGACTT
FUS3 Rev	CGTCTACTTCTTCTTCTTCCGATGC
LEC1 Fw	TGGAGCTCCCTTCTCTCACTATCA
LEC1 Rev	CTGCTGGACCACGATACCATTGTT
pri-miR172b Fw	CGGATTAGGGCGTTAATTACAATG
pri-miR172b Rev	GGTCTCTGGACGAACTATTCTGTA
SLP3 Fw	CTTAGCTGGACACAACGAGAGAAGGC
SLP3 Rev	GAGAAACAGACAGAGACACAGAGGA
SLP9 Fw	CAAGGTTCAGTTGGTGGAGGA
SLP9 Rev	TGAAGAAGCTCGCCATGTATTG
ACT2 Fw	CACTTGCACCAAGCAGCATGAAGA
ACT2 Rev	AATGGAACCACCGATCCAGACACT

ChIP-PCR primers

Name	5´to 3´
FLC ChIP Fw	TCTGGTTATCGATTGCGATTCT
FLC ChIP Rev	CGTGCATATACAAATCCAAGAGAAC
MAF4 ChIP Fw	CCCGGTAGATTTGTTGAGAAAC
MAF4 ChIP Rev	CACTTGAAATTAACCAAGGAATGC
MAF5ChIP Fw	CAAGTCATCTTAACTTTGTCTTGCT
MAF5ChIP Rev	GGCACTCGTTTCCACTAGATT
MIR156A ChIP Fw	CTCTCAAATCTCAAGTTCATTGCC
MIR156A ChIP Rev	GGCTCTTGTCGCTTTCTTTATC
MIR156C ChIP Fw	TCTCCGGTTTTGCTTGTTTAAC
MIR156C ChIP Rev	AGAAGATTGGAAAGGAGGCAG
FUS3 ChIP Fw	ACTTTTGCTACACTTGTTCACCATG
FUS3 ChIP Rev	CGCAACAAGATCTAATGCCACT
WUS ChIP Fw	CAAACTTCTCTTTCTCTTGTTCCTCTC
WUS ChIP Rev	GGCTCCATGTGTGTTTGATTCGAC
ACT7 ChIP Fw	GCGATGTTTGAGTTTCAATAAACGCTGC
ACT7 ChIP Rev	CTCACCTTCACCATTCCAGTTCCA

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