Overexpression of AtBMI1C, a Polycomb Group Protein Gene, Accelerates Flowering in Arabidopsis

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Abstract
Polycomb group protein (PcG)-mediated gene silencing is emerging as an essential developmental regulatory mechanism in eukaryotic organisms. PcGs inactivate or maintain the silenced state of their target chromatin by forming complexes, including Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2). Three PRC2 complexes have been identified and characterized in Arabidopsis; of these, the EMF and VRN complexes suppress flowering by catalyzing the trimethylation of lysine 27 on histone H3 of FLOWER LOCUS T (FT) and FLOWER LOCUS C (FLC). However, little is known about the role of PRC1 in regulating the floral transition, although AtRING1A, AtRING1B, AtBMI1A, and AtBMI1B are believed to regulate shoot apical meristem and embryonic development as components of PRC1. Moreover, among the five RING finger PcGs in the Arabidopsis genome, four have been characterized. Here, we report that the fifth, AtBMI1C, is a novel, ubiquitously expressed nuclear PcG protein and part of PRC1, which is evolutionarily conserved with Psc and BMI1. Overexpression of AtBMI1C caused increased H2A monoubiquitination and flowering defects in Arabidopsis. Both the suppression of FLC and activation of FT were observed in AtBMI1C-overexpressing lines, resulting in early flowering. No change in the H3K27me3 level in FLC chromatin was detected in an AtBMI1C-overexpressing line. Our results suggest that AtBMI1C participates in flowering time control by regulating the expression of FLC; moreover, the repression of FLC by AtBMI1C is not due to the activity of PRC2. Instead, it is likely the result of PRC1 activity, into which AtBMI1C is integrated.

Introduction
Polycomb group proteins (PcGs) were first identified in Drosophila melanogaster, which maintains the repressed state of homeotic (Hox) genes during embryogenesis via histone methylation [1]. The PcGs identified to date form at least two different complexes, Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2), which have repressive functions in flies, humans, and plants [2,3]. In Drosophila, PRC2 is composed of four core proteins, including Enhancer of zeste E(z), an H3K27 methyltransferase, Extra sex comb (Esc), Suppressor of zeste 12 (Su[z]12), and p55. This complex increases trimethyl H3K27 levels, thereby silencing Hox gene expression [4] and providing a recruitment site for PRC1 [5]. The PRC1 core in Drosophila is composed of Polycomb (Pc), Posteru sex combs (Psc), Ring or Sex comb extra (Sce), and Polyhomoetic (Ph) [6,7]. The mammalian PRC1 complex contains HPC, HPH, RING1A/B, and BMI1, which are homologs of fly Pc, Psc, and Psc [2]. Among the RING finger proteins, dRING/ Sce and human RING1B have been shown to act as an E3 ubiquitination ligase that monoubiquitylates lysine 119 in histone H2A [8]. The other two RING domain-containing proteins, RING1A and BMI1, are involved in the positive regulation of RING1B E3 ligase activity; however, they do not possess E3 ligase activity. All three RING domain-containing proteins are required for PcG-mediated silencing [9,10].

In Arabidopsis, there are twelve homologs of Drosophila PRC2 subunits, which form three distinct PRC2-like complexes: EMBRYONIC FLOWER (EMF), VERNALIZATION (VRN), and FERTILIZATION INDEPENDENT SEED (FIS). These complexes play important roles in multifaceted developmental processes, including the vegetative phase transition, gametogenesis, embryogenesis and early seed development, and flowering time control [11].

The first PRC2-like complex to be identified and biochemically characterized in Arabidopsis was the FIS complex, which prevents endosperm and seed development in the absence of fertilization [12]. The FIS complex, which is composed of MEDEA (MEA), FERTILIZATION INDEPENDENT ENDOSPERM (FIE), FIS2, and MULTICOPY SUPPRESSOR of IRA1 (MSI1) [13,14], represses the expression of PHERES1 (PHE1) to prevent the central cell from initiating endosperm development before pollination by trimethylating H3K27 in PHE1 chromatin [15]. Inactivation of the FIS complex by fertilization or the delivery of other unknown factors results in the release of PHE1 and triggers endosperm development [16].

The EMF complex, which is composed of CURLY LEAF (CLF), FIE, EMF2, and MSI1, functions before and after...
flowering by targeting different branches of genes for silencing [17,10]. During vegetative development, the EMF complex suppresses precocious flowering and enables vegetative development by repressing the transcription of FT and of the flower MADS box genes AGAMOUS and AGAMOUS-LIKE 19 by mediating the deposition of H3K27me3 at their chromatin [18,19,20]. Late during vegetative development and after flowering, CLF in the EMF complex binds directly to the chromatin of the floral repressor FLC and its relatives MAF4 and MAF5, leading to H3K27me3 modification and the repression of FLC under warm conditions [19]. These results imply that the EMF complex regulates flowering in Arabidopsis by repressing the expression of these flowering genes at different stages of development [19,20].

The VRN complex containing VRN2, CLF, FIE, and MSI1 is another PRC2-like complex that controls flowering and enables Arabidopsis to flower after vernalization [11]. Cold exposure or vernalization promotes the formation and targeting of the PHD-VRN complex to FLC chromatin. The PHD-VRN complex increases H3K27me3 levels at FLC chromatin, leading to sustainable silencing of FLC [21]. The repressed state of FLC, then, is maintained epigenetically during subsequent plant development until it is reset during embryogenesis [22].

Although several PRC2-like complexes that control Arabidopsis development have been reported, PRC1-like complexes were identified only recently, primarily because there is no homolog of Drosophila Psc in the Arabidopsis genome [5]. The existence of PRC1 in Arabidopsis was proposed recently based on evidence showing that the plant chromodomain protein LIKE HETEROCHROMATIN 1 (LHP1) binds H3K27me3 in vitro and colocalizes genome-wide with H3K27me3 profiles in euchromatin to turn off gene expression [23].

In Arabidopsis, five RING domain-containing PcGs have been predicted [24], and the functions of two RING1 homologs (AtRING1A and AtRING1B) were recently characterized [25]. Severe cotyledon, rosette leaf, shoot apical meristem (SAM), flower morphology, and floral organ identity defects were observed in AtRING1A/AtRING1B, implying that a loss of function of both AtRING1A/B perturbs cell-fate determination [25]. KNOX gene release was detected in AtRING1A/AtRING1B leaves, but the level of H3K27 trimethylation at KNOX genes was unchanged, indicating that the suppression of KNOX genes by AtRING1A/B is independent of the H3K27 trimethylation activity of PRC2 [25]. However, the biochemical function of AtRING1A/B in PRC1-mediated KNOX gene silencing has not been determined.

There are three BMI1 homologs in Arabidopsis: AtBMI1A (At2G30580), AtRING1B (At1G06770), and AtBMI1C (At3G23060) [24]. AtBMI1A/B regulate plant embryonic and stem cell development by functioning as E3 ubiquitin ligases and components of PRC1 [26,27]. Specifically, AtBMI1A/B mediate the ubiquitination of DREB2A in response to water stress, leading to degradation of the protein by the 26S proteasome [26]. In addition, AtBMI1A/B were recently identified as components of PRC1 [27]. AtBMI1A/B mediate H2A monoubiquitination, and some stem cell regulator genes were found to be expressed ectopically in Atbmi1a/Atbmi1b cotyledons, implying that AtBMI1A/B are involved in silencing stem cell regulators and sustaining the differentiated state of somatic cells [27]. In comparison, the function of the third BMI1 homolog, AtBMI1C, is unknown.

Here we report that AtBMI1C is a component of a PRC1-like complex and that it exhibits H2A monoubiquitination activity. AtBMI1C overexpression causes early flowering in Arabidopsis via silencing of the flowering repressor FLC and by promoting expression of the flowering activator FT.

**Results**

Three AtBMI1s are the homologs of human BMI1, a key component of PRC1

The mammalian PRC1 ubiquitin E3 ligase complex consists of several PcGs, including three RING domain-containing proteins (RING1/RING1A, RING2/RING1B, and BMI1) [8]. To characterize the functions of BMI1 homologs in Arabidopsis, we screened a whole-genome Arabidopsis sequence database using the protein or RING-domain sequence of Psc from Drosophila and its human homolog, BMI1. The existence of three human BMI1-like

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**Figure 1. Phylogenetic relationship and conservation of AtBMI1s.**

(A) Phylogenetic tree based on the full-length sequence of AtBMI1 from Arabidopsis and its human and fly homologs. (B) Tree showing the phylogenetic relationships among RING domain-containing AtBMI1s from Arabidopsis and their human and fly homologs. The GenBank accessions of the sequences are: NM_128610 (At2g30580) for AtBMI1A, NM_202046 (At1g06770) for AtBMI1B, AY099845 (At3g23060) for AtBMI1C from Arabidopsis thaliana, NM_002931 for hRING1, NM_007212 for hRING2 from Homo sapiens, and NM_079001 for Psc from Drosophila melanogaster (dPsc).

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proteins in *Arabidopsis*, designated AtBMI1A (At2G30580), AtBMI1B (At1G06770), and AtBMI1C (At3G23060), was revealed (Figure 1A); notably, each of these proteins was previously identified as a homolog of BMI1 [24,27].

The RING domain of the BMI1s is conserved between *Arabidopsis* and animals (Figure 1B and C). The evolutionary conservation of a protein sequence implies functional similarity among distinct organisms. Both the full-length protein and RING-domain sequences of AtBMI1A were closely related to AtBMI1B, but slightly far from AtBMI1C in terms of their evolution (Figure 1A and B), indicating that AtBMI1C may function differently in *Arabidopsis* development. To date, only roles for AtBMI1A/B have been documented; the function of AtBMI1C in plant development remains unknown [27]. Thus, we focused on AtBMI1C in our subsequent experiments.

**AtBMI1C is a ubiquitously expressed nuclear protein**

The expression pattern and subcellular localization of AtBMI1C were examined to elucidate the biological functions of the protein. To determine the subcellular localization of AtBMI1C, a reporter gene (Yellow Fluorescence Protein [YFP]) was fused to the AtBMI1C coding region under the control of the CAULIFLOWER MOSAIC VIRUS (CaMV) 35S promoter to generate stable transgenic plants carrying p35S::AtBMI1C-YFP or p35S::YFP (control). YFP signals were detected in the nucleus and cytoplasm in the roots of p35S::YFP transgenic plants (Figure 2A); in comparison, YFP signals in the roots or petals of p35S::AtBMI1C-YFP transgenic plants were detected only in the nucleus (Figure 2B and C). Thus, AtBMI1C encodes a nuclear-localized protein.

The spatial expression pattern of AtBMI1C was examined in various tissues by semiquantitative RT-PCR. Our results indicate weak expression of AtBMI1C in the SAM, juvenile leaves, adult leaves, stems, and cauline leaves, and abundant expression in cotyledons, inflorescences, siliques, seedlings, and roots (Figure 2D). Thus, AtBMI1C may function in multiple tissues during development.

**AtBMI1C is a component of PRC1 with H2A monoubiquitination activity**

AtBMI1C was localized in the nucleus (Figure 2B and C), similar to AtRING1A and AtRING1B [25]; thus, AtBMI1C was colocalized with AtRING1A and AtRING1B. To address whether AtBMI1C is a component of PRC1, we first explored the

![Figure 2. Nuclear localization and expression pattern of AtBMI1C.](image-url)
interactions of AtBMI1C with AtRING1A and AtRING1B, which are known components of PRC1 in Arabidopsis [25], using a yeast two-hybrid assay. AtBMI1C interacted physically with AtRING1A and AtRING1B in yeast (Figure 3B); the N-terminal domain of AtBMI1C, including the conserved RING domain, was required for this interaction (data not shown). The interaction of AtBMI1C with AtRING1A and AtRING1B was further confirmed by a pull-down assay. Both AtRING1A and AtRING1B could be pulled down by AtBMI1C (Figure 3C), suggesting that AtBMI1C and AtRING1 are in the same PRC1 complex.

Figure 3. Physical interactions between AtBMI1C and AtRING1A/B, and the detection of H2A monoubiquitination activity. (A) Yeast two-hybrid assay. Positive control: pGADT7-T + pGBKT7-53 (encoding fusions between the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively). Negative control: pGADT7-T + pGBKT7-Lam (encoding a fusion of the DNA-BD with human lamin C; control for interactions between an unrelated protein and either the pGADT7-T control or AD/library plasmid). The indicated combinations of plasmids were co-transformed into the yeast reporter strain, and the interactions of AtBMI1C with AtRINGs were assessed by growth on plates lacking Leu, Trp, His, and adenine. (B) The interactions between AtBMI1C and AtRING1A/B were quantitatively evaluated based on the level of β-galactosidase activity. (C) Pull-down assay. Total protein was extracted from 2 g of eleven-day-old Myc-RING1A/ring1 or Myc-RING1B/ring1 plants, respectively. Each protein extract was divided in half and incubated with MBP- or MBP-GST-coated beads. The pulled down fractions were analyzed by Western blotting. (D) Western blot analysis of histone extracts of WT and 35S::BMI3-YFP using anti-ubiquitin and -H3 antibodies, respectively. Molecular weight (MW) markers (in kDa), monoubiquitinated H2B (uH2B), and monoubiquitinated H2A (uH2A) are indicated. Asterisks indicate cross-reacting bands.

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For a long time, scientists believed that there was no H2A monoubiquitination activity in plants [25]. However, recent data suggest the existence of an AtBMI1A- and AtBMI1B-containing PRC1 complex with H2A monoubiquitination activity [27]. To determine whether AtBMI1C-containing PRC1 possesses H2A monoubiquitination activity, we measured the monoubiquitinated H2A level in wild-type plants and an AtBMI1C-overexpressing line (Figure 3D). Anti-ubiquitin antibodies recognized two specific bands from nuclear histone extracts (Figure 3D); the upper band was recognized by anti-monoubiquitinated H2B (uH2B) antibodies as well (data not shown), suggesting that the upper band was uH2B, while the lower band was monoubiquitinated H2A (uH2A). Moreover, the level of H2A monoubiquitination activity was increased in the AtBMI1C-overexpressing line (Figure 3D). However, there was no obvious change in H2B monoubiquitination activity between the wild-type plants and AtBMI1C-overexpressing lines (Figure 3D). This result suggests that, like its homologs, the AtBMI1C-containing complex exhibits H2A monoubiquitination activity in Arabidopsis.

No loss-of-function T-DNA insertion mutant of AtBMI1C was isolated

To investigate the biological function of AtBMI1C in Arabidopsis, T-DNA insertion mutants of AtBMI1C were ordered from the Arabidopsis Biological Resource Center (ABRC). A homozygous T-DNA insertion allele of AtBMI1C was identified (Atbmi1c-1, SALK_148143), in which the T-DNA was inserted upstream of the start codon (Figure 4A). However, the T-DNA insertion in Atbmi1c-1 did not abolish the expression of AtBMI1C (Figure 4B). Not surprisingly, no visible phenotype was detected among the homozygous mutant plants (data not shown).

Lack of obvious defects in down-regulated RNA interference (RNAi) lines of AtBMI1C

Because no T-DNA insertion mutant of AtBMI1C was identified, RNAi was used to explore the function of AtBMI1C. Three primer pairs were designed using an online program (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl?page=1), the resulting three constructs were delivered to wild-type plants, and the expression of AtBMI1C was analyzed in T1 independent lines. Several transgenic lines (AtBMI1C-Rs) were identified, including AtBMI1C-R1, AtBMI1C-R4, and AtBMI1C-R9, in which the expression of AtBMI1C was down-regulated (Figure 4C). Defects, especially in flowering time, were monitored in the next generation; however, no visible phenotype was observed in AtBMI1C-R1, AtBMI1C-R4, and AtBMI1C-R9 compared to control (AtBMI1C-R12) and wild-type plants (Figure 4D and E). Because no T-DNA insertion mutant was available and because no visible phenotype was observed in our amiRNAi lines of AtBMI1C, we next investigated the function of the gene by overexpressing it in Arabidopsis.
AtBMI1C overexpression confers an early flowering phenotype in Arabidopsis

To examine whether the overexpression of AtBMI1C affects plant growth and development, we generated p35S::AtBMI1C-YFP transgenic plants and monitored their expression of AtBMI1C by semiquantitative RT-PCR (Figure 5B). We also recorded the phenotypes of the transgenic lines. The p35S::AtBMI1C-YFP transgenic lines showed an early flowering phenotype (Figure 5A).

Moreover, using p35S::AtBMI1C-YFP-27 (35S-27) and p35S::AtBMI1C-YFP-14 (35S-14) as representatives, the transgenic lines were found to possess far fewer rosette leaves than wild type under both long-day (LD; 16 h of light/8 h of dark) and short-day (SD; 8 h of light/16 h of dark) conditions (Figure 5C and D and Table 1). In addition, fewer days were required for emergence of the first bud and for opening of the first flower in lines 35S-27 and 35S-14 compared to wild-type plants (Table 1).

To exclude the effect of YFP on the function of AtBMI1C, a construct containing only p35S::AtBMI1C was transformed into wild-type plants. Flowering time was monitored in the T1 generation and in a number of T2 independent lines. Flowering time in the transgenic plants containing p35S::AtBMI1C coincided with that in transgenic plants harboring p35S::AtBMI1C-YFP, implying that the early flowering phenotype of the p35S::AtBMI1C-YFP transgenic plants was due to the overexpression of AtBMI1C, rather than the fusion of YFP to AtBMI1C (data not shown).

Arabidopsis undergoes at least two phase transitions during its life cycle: a vegetative and a reproductive phase transition [28]. The vegetative phase transition represents a shift from the juvenile vegetative phase to the adult vegetative phase, which is usually defined by the production of leaves with abaxial trichomes; in comparison, the dramatic vegetative to reproductive phase transition, or floral transition, is characterized by bolting, flowering, and setting seeds for the next generation [28]. To determine the effects of the overexpression of AtBMI1C on vegetative and reproductive phase transitions in Arabidopsis, we examined the juvenile and adult leaf number in transgenic lines after bolting. There was no significant difference in juvenile leaf number between line 35S-27 or 35S-14 and wild type; however,

Table 1. Flowering time in p35S::AtBMI1C-YFP plants grown under LD conditions.

| Genotype | Days to the first visible bud | Days to the first open flower | Rosette leaf number | n |
|----------|-------------------------------|-------------------------------|---------------------|---|
| Wild type| 26.00 ± 0.38                  | 32.83 ± 0.38                  | 13.11 ± 0.14        | 18 |
| 35S-27   | 18.43 ± 0.47**                | 24.57 ± 0.31**                | 6.43 ± 0.14**       | 14 |
| 35S-14   | 19.61 ± 0.31**                | 25.78 ± 0.34**                | 8.22 ± 0.10**       | 18 |
| 35S-9    | 18.62 ± 0.10**                | 25.62 ± 0.10**                | 6.58 ± 0.10**       | 26 |

Data are presented as the mean ± SD; 
**represents a significant difference from wild type (t-test, p < 0.01); 
LD: 16 h of light/8 h of dark.

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Figure 6. Tissue-specific AtBMI1C overexpression promotes flowering in *Arabidopsis*. (A) Morphology of transgenic plants carrying pAP1::AtBMI1C-GFP grown under LD conditions for 28 days. The plants showed an early flowering phenotype compared with wild type. A total of 18 out of 72 independent T1 lines showed an early flowering phenotype. A few lines were chosen for the following experiments. (B) and (C) Determination of flowering time in transgenic plants containing pAP1::AtBMI1C-GFP grown under LD and SD conditions using three AtBMI1C transgenic lines as representatives. The number of rosette leaves was determined after bolting. (D) Vegetative phase transition in transgenic plants containing pAP1::AtBMI1C-GFP grown under LD conditions. Juvenile, adult, rosette, and cauline leaves were counted after flowering. Juvenile and adult leaves were distinguished based on the presence of trichomes on their abaxial surface. (E) AtBMI1C expression in transgenic lines carrying pAP1::AtBMI1C-YFP. Total RNA was extracted from leaves of pAP1::AtBMI1C-GFP and wild-type plants. AtBMI1C expression was measured by semiquantitative RT-PCR using *ACTIN2/7* as an internal control. (F) Morphology of transgenic plants carrying pKNAT1::AtBMI1C-GFP grown under LD conditions for 28 days. The plants showed an early flowering phenotype compared with wild type. A total of 20 out of 108 independent T1 lines...
showed an early flowering phenotype. (G) Determination of flowering time in transgenic plants containing pKNAT1::AtBMI1C-GFP grown under LD conditions. The number of rosette leaves was determined after bolting. (H) Morphology of transgenic plants carrying pSUC2::AtBMI1C-GFP grown under LD conditions for 28 days. The plants showed an early flowering phenotype. (I) Determination of flowering time in transgenic plants containing pSUC2::AtBMI1C-GFP grown under LD conditions. The number of rosette leaves was determined after bolting.

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the adult leaf number in 35S-27 and 35S-14 was dramatically reduced (1.07 and 1.43 adult leaves, respectively) compared with wild type (4.50 adult leaves) (Figure 5E), suggesting that the overexpression of AtBMI1C affected only the floral transition and not the vegetative phase transition.

**Tissue-specific AtBMI1C expression also produces an early flowering phenotype in Arabidopsis**

CaMT15S is a constitutive promoter with activity in various cell types, tissues, and organs. To investigate the relationship between AtBMI1C expression and the phenotype of the transgenic plants, several tissue-specific promoters were selected to drive the expression of AtBMI1C, including the APETALA1 (AP1) promoter, a floral primordium-, sepal-, and petal-specific promoter [29].

pAP1::AtBMI1C-GFP was generated and introduced to wild-type plants. A total of 18 out of 72 independent transgenic lines harboring pAP1::AtBMI1C-GFP exhibited an early flowering phenotype under LD and SD conditions (Figure 6A-C and Table 2). In the mean time, the level of AtBMI1C expression in the pAP1::AtBMI1C-GFP transgenic plants was measured by semiquantitative RT-PCR. Elevated AtBMI1C expression was detected in the early flowering transgenic plants (Figure 6E), indicating that the alteration in flowering time was caused by the overexpression of AtBMI1C.

We further investigated the impact of the overexpression of AtBMI1C using the AP1 promoter on vegetative and reproductive phase transitions in a pAP1::AtBMI1C-GFP-9 transgenic line (A-9). Our results were similar to those obtained for lines 35S-27 and 35S-14. The juvenile leaf number in A-9 was almost the same as in wild type; however, the adult leaf number in A-9 (1.83 adult leaves) was lower than that in wild type (6.33 adult leaves) (Figure 6D). Thus, the AP1-driven overexpression of AtBMI1C affected only the floral transition, and not the vegetative phase transition.

**FLC suppression and FT activation in the AtBMI1C-overexpressing lines**

FLC is a central floral repressor that blocks the expression of floral activators such as FT and SUPPRESSOR OF OVEREXPRESSSION OF CO1 (SOC1) to prevent the initiation of flowering during vegetative development. The down-regulation of FLC activates FT and SOC1 and promotes flowering [30].

To explore the molecular mechanisms responsible for the change in flowering time in our AtBMI1C-overexpressing lines, FLC and FT expression was examined by quantitative RT-PCR. The expression of FLC in lines 35S-14 and 35S-27 was 2.5 times lower than that in wild type (Figure 7A). As a result of the down-regulation of FLC, the expression of FT in lines 35S-14 and 35S-27 was about five times higher than that in wild type (Figure 7B). Similarly, the repression of FLC in lines A-9, A-13, and A-14 was also observed (Figure 7C). An increase in FT expression of 3–4.5 times in lines A-9, A-13, and A-14 was detected (Figure 7D). These results suggest that AtBMI1C overexpression promotes flowering by repressing FLC expression and activating FT expression.

**Early flowering caused by AtBMI1C overexpression in the SAM and vascular companion cells**

FLC is expressed mainly in the SAM and vascular companion cells [31]. Our initial results indicated that AtBMI1C overexpression repressed FLC and raised the expression of FT to promote flowering. Thus, we hypothesized that expressing AtBMI1C in specific tissues, including the SAM and vascular companion cells, would cause early flowering. To test this hypothesis, KNAT1, an SAM-specific promoter [32], and SUC2, a vascular companion cell-specific promoter [33], were selected to drive the tissue-specific expression of AtBMI1C.

Flowering time in T1 and T2 lines expressing pKNAT1::AtBMI1C-GFP and pSUC2::AtBMI1C-GFP was examined. As expected, AtBMI1C overexpression in either the SAM or vascular companion cells caused early flowering. Lines K-32, K-38, and K-79 containing pKNAT1::AtBMI1C-GFP flowered earlier (8.22, 8.50, and 8.63 rosette leaves) than wild type (12.65 rosette leaves) (Figure 6F and G and Table 3). Similarly, lines SU-58 and SU-142 carrying pSUC2::AtBMI1C-GFP produced fewer rosette leaves (8.33 and 8.12, respectively) compared to wild type (12.71 leaves) (Figure 6H and I and Table 4).

| Table 2. Flowering time in pAP1::AtBMI1C-GFP plants grown under LD conditions. |
|---------------------------------|-----------------|-----------------|-----------------|---|
| Genotype | Days to the first visible bud | Days to the first open flower | Rosette leaf number | n |
| Wild type | 28.48±0.23 | 34.67±0.32 | 12.00±0.19 | 27 |
| AP1-9 | 23.00±0.39** | 31.00±0.35** | 9.11±0.20** | 19 |
| AP1-13 | 22.87±0.16** | 31.00±0.18** | 9.10±0.09** | 20 |
| AP1-14 | 22.54±0.21** | 30.00±0.30** | 9.00±0.13** | 24 |

Data are presented as the mean ± SD; **represents a significant difference from wild type (t-test, p<0.01); LD: 16 h of light/8 h of dark.

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PcG Controls Flowering in Arabidopsis
AtBMI1C-overexpressing line 35S-9. There was no obvious difference in the level of H3K27me3 or H3K4me3 across FLC chromatin between wild type and line 35S-9 (Figure 8).

**Discussion**

The PcG proteins in PRC2 complexes (e.g., the EMF complex and VRN complex), which are involved in the switch from vegetative to reproductive development, have been documented, and they have been shown to be involved in the H3K27me3 modification of FT and FLC chromatin [11]. However, little is known about the impact of PRC1 components on the regulation of flowering, even though PRC1 components AtRING1A/B and AtBMI1A/B play essential roles in the repression of plant developmental regulators in Arabidopsis [25,27].

AtBMI1C is involved in flowering time control in Arabidopsis

Genetic and molecular analyses have uncovered multiple signaling pathways that integrate environmental and developmental cues to control flowering time in Arabidopsis, including the autonomous, photoperiod, vernalization, gibberellin, and PAF1 complex pathways [37]. PRC2 complexes are involved in cold-

**Table 3. Flowering time in KNAT1::AtBMI1C-GFP plants grown under LD conditions.**

| Genotype | Days to the first visible bud | Days to the first open flower | Rosette leaf number | n |
|----------|-------------------------------|-------------------------------|---------------------|---|
| Wild type| 27.00±0.35                    | 33.83±0.29                    | 12.65±0.22          | 18|
| K-32     | 22.28±0.34**                  | 28.78±0.34**                  | 8.22±0.25**         | 18|
| K-58     | 23.50±0.60**                  | 30.08±0.58**                  | 8.50±0.30**         | 12|
| K-79     | 20.47±0.29**                  | 27.11±0.25**                  | 8.63±0.14**         | 19|

Data are presented as the mean ± SD; **represents a significant difference from wild type (t-test, p<=0.01); LD: 16 h of light/8 h of dark.
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induced flowering regulation [34,37]; however, no PRC1 component has been shown to act as a floral transition regulator. Because no loss-of-function AtBMI1C mutant was available until now, and given that no visible phenotype was observed in our RNAi lines (Figure 4), we generated several transgenic lines in which AtBMI1C was overexpressed (35S:AtBMI1C-YFP, AP1-AtBMI1C-GFP, KNAT1-AtBMI1C-GFP, and SU1-AtBMI1C-GFP) in order to dissect the functions of AtBMI1C in Arabidopsis. Flowering was accelerated in all AtBMI1C overexpressors (Figures 5 and 6). FLC is a central repressor of flowering that integrates endogenous signals from the autonomous and vernalization pathways with environmental cues [37]. The repression of FLC and activation of FT were detected in our AtBMI1C overexpression lines (Figure 7). These results suggest that AtBMI1C is involved in flowering regulation. Further analysis using an AtBMI1C knockout mutant will provide additional evidence for the functions of AtBMI1C.

AtBMI1C is a PRC1 component, and the AtBMI1C-containing complex exhibits H2A monoubiquitination activity

AtBMI1C is a homolog of the RING-domain proteins AtBMI1A/B in Arabidopsis, Psc in Drosophila, and BMI1 in humans (Figure 1B and C). In this report, we found that AtBMI1C interacts physically with AtRING1A/B (Figure 3B and C), indicating that AtBMI1C is a novel component of PRC1 in Arabidopsis.

In animals, the repression of gene expression is maintained by PRC1, which monoubiquinates H2A [8–10]. However, H2A monoubiquitination was not detected in plants until a recent report showing that AtBMI1A/B are required for H2A monoubiquitination activity [27]. AtBMI1A/B-mediated H2A monoubiquitination activity has been shown in vivo and in vitro, and defects in AtBMI1A/B have been shown to impair the production of uH2A [27]. In the present study, we found that an increase in the level of AtBMI1C raised H2A monoubiquitination activity (Figure 3D). Thus, our result supports the notion that AtBMI1C-containing PRC1 has H2A monoubiquitination activity in Arabidopsis.

AtBMI1C-containing PRC1 suppresses FLC expression independent of H3K27 methylation

PRC1 controls gene expression by altering the level of H2A monoubiquitination [8–10], while PRC2 methylates histone H3 at lysine 27 to silence gene expression [2–4]. In the present study, we found that AtBMI1C overexpression accelerated flowering and repressed the expression of FLC (Figures 5–7). In addition, the repression of FLC by AtBMI1C is independent of the level of H3K27 and H3K4 methylation (Figure 8). Therefore, AtBMI1C may function as part of the PRC1 complex to suppress the expression of FLC and promote flowering in Arabidopsis. However, we cannot rule out the possibility that FLC is not a direct target of AtBMI1C-containing PRC1 at this stage. Further study is needed to resolve this issue.

In conclusion, a novel PcG in PRC1, AtBMI1C, was characterized in this study. AtBMI1C is a universally expressed nuclear protein that participates in flowering time control by regulating the expression of FLC. The down-regulation of FLC was not due to PRC2 activity; rather, it was likely the result of PRC1 activity, which is associated with AtBMI1C.

### Materials and Methods

#### Plant materials and growth conditions

Arabidopsis thaliana plants [ecotype Columbia [Col-0]] were used in this study. Seeds were surface-sterilized with 2.25% NaHClO and plated on 1X Murashige and Skoog (MS) basal salt medium containing 0.3% agar and 1% (weight/volume) sucrose. After stratification in the dark at 4°C for two days, the plates were transferred to a growth chamber (Percival Scientific) set to 150 μmol m⁻² s⁻¹ cool white fluorescent light) or 18°C (dark). The seedlings sampled for the assay were grown under different conditions as indicated.

#### Flowering time determination

For flowering time measurement, mutant and wild-type (Col-0) plants were grown under long-day (LD; 16 h of light/8 h of dark) or short-day (SD; 8 h of light/16 h of darkness at 18°C) conditions.

#### Isolation of the AtBMI1C T-DNA insertion mutant

T-DNA insertion mutants of AtBMII1C (At3G23060) were ordered from the Arabidopsis Biological Resource Center (ABRC). Primers specific for sequences upstream and downstream of the T-DNA insertion were designed that could amplify the gene fragment without the T-DNA insertion. To amplify the T-DNA insertion, the T-DNA-specific primer LBb1 (5' - GCCTGGCAC-GCGTGGAAC-3') and a gene-specific primer were used. We used two combinations of primers, each consisting of gene-specific primers, and a combination of a gene-specific primer and T-DNA-specific primer to identify individuals that were homozygous or heterozygous for the T-DNA insertion. The position of the T-DNA insertion was determined by sequencing those products carrying T-DNA-genome junctions.

#### amiRNA interference (amiRNAi)

Three primer pairs were designed through the web (http://wmdl2.weigelworld.org/cgi-bin/mirnatools.pl?page=1) for use in
creating amiRNAi constructs according to a previously published protocol [38]. The resulting three constructs were delivered to wild-type plants via Agrobacterium-mediated transformation to generate AtBMI1C amiRNAi lines (AtBMI1C-Rs).

Vector construction and transformation

The coding sequence of AtBMI1C was amplified from the cDNA of wild-type (Col-0) plants using the primer pair AtBMI1C-SpeI/AtBMI1C-BamHI-R. The resulting product, AtBMI1C, was cloned into pEASY-Blunt using SpeI and BamHI and sequenced. Next, the fragment was cloned into the binary vector pCambia1300 harboring the CAULIFLOWER MOSAIC VIRUS (CaMV) 35S constitutive promoter and Yellow Fluorescent Protein (YFP) gene using XbaI and BamHI to produce the construct p35S::AtBMI1C-YFP. P35S::AtBMI1C without the YFP tag was also constructed to assess the effects of YFP on the function of AtBMI1C. Constructs for the expression of GFP-tagged AtBMI1C driven by the AP1, SUC2, and KNAT1 promoters were also generated (pAP1::AtBMI1C-GFP, pSUC2::AtBMI1C-GFP, and pKNAT1::AtBMI1C-GFP, respectively).

Figure 8. ChIP analysis of H3K4me3 and H3K27me3 at FLC chromatin. (A) H3K4 trimethylation levels at FLC chromatin between wild type and an ATBMI1C-overexpressing line harboring p35S::AtBMI1C-YFP. (B) H3K27 trimethylation levels at FLC chromatin between wild type and AtBMI1C-overexpressing lines harboring p35S::AtBMI1C-YFP. Anti-H3K4me3 or -H3K27me3 antibodies were used for the assay. The locations of the primer pairs used to amplify FLC fragments across the region are indicated in (C). The values are the mean ± SD of three independent experiments. doi:10.1371/journal.pone.0021364.g008
Table 5. Primers used in this study.

| Primer name | Primer sequence (5’ to 3’) |
|-------------|----------------------------|
| ATBMI1C-Spel-F | ACTAGTGGGAAATCCGGAGAGGAAGATGTTA |
| ATBMI1C-BamHI-R | GGATCCCTCAAGGGGAGCACAGAGCTAGGAG |
| ATBMI1C-p1969-F | CTGCACTGAGACCATTCCAATCTG |
| ATBMI1C-p1969-R | TCAGATTCCTCCGTGATCCAAAC |
| SU2C-F | AAATACTGGTTCTATATTAATTTCA |
| SU2C-R | ATTGGAACAACAGAAGAAGTTA |
| KNAT1-F | GATCTAGGACCCCTAGTGGAGT |
| KNAT1-R | ACCACAGTGTTAAGAGTGAGT |
| AtBMI1C-RTF | CATGCCTTGCTTGCATTTAC |
| AtBMI1C-RTFR | GTCTGTCGCATTCTG |
| Actin2/7F | AGGCACCTCTTACACCTAAGGC |
| Actin2/7R | GGCCACAGGAACTCTGCAGG |
| Actin2/7RTF | GTGTCATGGTTGATCGTGC |
| Actin2/7RTFR | CTCCTTGAGTACGACGTCG |
| FLC-F | CCTCTCGGTGACTAGACGCAAG |
| FLC-R | AGGTAACATCCCTGACTCAAGT |
| FT-F | ACTATAGGCTATCATCCGGCTGTG |
| FT-R | ACAACTGGAAACACTCTTGGGAAGT |
| FLC1F | GCATTAGTGGTCTCTTCCAAAC |
| FLC1R | GCCCTACCTGATACGAGGAG |
| FLC2F | GTCCGAGGTAACTACAAAATAAAGG |
| FLC2R | GAAAAACGCTGATACACATTCCAC |
| FLC3F | TGGGGTAAACGAGATTGAT |
| FLC3R | GCCATATGCTATCCGTAGAGT |
| FLC1F | GTGCCTATCCTGGTACCTAGT |
| FLC1R | GCCCTACCTGATACGAGGAG |
| FLC2F | CGACGCGCATCAGATCG |
| FLC2R | GCCCGCGTTTGTTGTTCTCT |
| FLC3F | GACCCTGATCATATAAACAAAGAGAAC |
| FLC3R | CCTGGATACAAATGCTG |
| FLC4F | CTCTGCTGAGTACGAGC |
| FLC4R | CCTGCTGATCCGAGGGAG |
| FLC5F | CTCTCGTGAAAGACAAAGAAGTTG |
| FLC5R | AGGGTACACATCCCTGAGGTC |

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The lengths of the promoters were chosen according to the indicated reference.

To create stable transgenic materials, wild-type (Col-0) plants were transformed using Agrobacterium tumefaciens GV3101 [39]. Independent transgenic lines were obtained on selective MS medium containing hygromycin. T2 or T3 lines were used for flowering time determination and other assays.

Semi-quantitative RT-PCR and quantitative real-time RT-PCR

About 100 mg of ten-day-old seedlings grown under LD conditions were ground in liquid nitrogen. Total RNA was extracted from the seedlings using Takara RNAiso Plus (D9108A) according to the manufacturer’s protocol. The RNA was treated with RQ1 RNase-Free DNase (Promega, M6101) to remove any contaminating DNA.

Three micrograms of total RNA were used for the synthesis of full-length first strand cDNA with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, K1622) according to the manufacturer’s protocol. One microliter of cDNA was utilized for semi-quantitative RT-PCR.

Quantitative real-time RT-PCR was performed using Takara SYBR Premix Ex Taq in a 7500 fast real-time PCR instrument (Applied Biosystems). The assays were done according to the manufacturer’s instructions. ACTIN2/7 was used as an endogenous control.

Observation of YFP fluorescence by confocal microscopy

P35S::ATBMI1C-YFP transgenic lines (T3) were generated for the subcellular analysis of AtBMI1C. A Zeiss Meta confocal microscope was used to detect YFP fluorescence in the roots of ten-day-old seedlings and petals. Images in the YFP, DAPI, and brightfield channels were acquired. The final images were visualized using LSM 510 software.

Yeast two-hybrid analysis

AtBMI1C was amplified and cloned into pGBD-T7 or pGBK-T7 and co-transformed into yeast strain AH109. Transformation, yeast growth, and quantitative β-galactosidase assays were conducted according to the protocols in the Clontech Yeast Protocols Handbook.

Protein pull-down assay

AtBMI1C was amplified and cloned into the bacterial expression vector pMAL-c2X (NEB) using BamHI and SalI. The constructs were transformed into BL21 plus competent cells for protein expression. The proteins were induced overnight at 16°C. MBP and MBP-AtBMI1C were purified using amylose resin (NEB). Plant total protein was extracted using extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, and 1% NP-40) containing complete protease inhibitor cocktail (Roche), separated by SDS-PAGE, and probed with anti-GFP and -tubulin antibodies, respectively.

Chromatin immunoprecipitation (ChIP) analysis

Constructs carrying p35S::ATBMI1C-YFP were transformed into wild-type plants, and homozygous lines were identified at the T3 generation for ChIP analysis. ChIP was performed as described [40] using ten-day-old seedlings grown on MS medium under LD conditions. Anti-H3K27me3 and H3K4me3 antibodies were purchased from Upstate Biotechnology. Quantitative real-time PCR was performed to detect the FLC regions harboring H3K27me3 and H3K4me3 modifications with the primer pairs shown in Table 5. The locations of the primer pairs are given in Figure 8C. All ChIP assays were performed three times using at least three biological replicates.

Western blotting

Total protein was extracted using protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, and 1% NP-40) containing complete protease inhibitor cocktail (Roche), separated by SDS-PAGE, and probed with anti-GFP and -tubulin antibodies, respectively. Histone-enriched protein extracts were prepared as described previously using ten-day-old seedlings [41]. Nuclei were isolated.
using extraction buffers I (0.4 M sucrose, 10 mM Tris-HCl [pH 8.0], 10 mM MgCl\(_2\), 5 mM β-ME, 0.1 mM PMSF, and complete protease inhibitor cocktail), II (0.25 M sucrose, 10 mM MgCl\(_2\), 0.15% Triton X-100, 2 mM MgCl\(_2\), 5 mM β-ME, 0.1 mM PMSF, and complete protease inhibitor cocktail), and III (1.7 M sucrose, 10 mM Tris-HCl [pH 8.0], 1% Triton X-100, 10 mM MgCl\(_2\), 5 mM β-ME, 0.1 mM PMSF, and complete protease inhibitor cocktail) in turn. The chromatin was treated overnight with 0.4 M H\(_2\)SO\(_4\) at 4°C and the proteins were precipitated with 25% trichloroacetic acid. The precipitate was washed three times with acetone, air-dried, and resuspended in 4 M urea. The histone-enriched protein extracts were separated by 15% SDS-PAGE, transferred to a PVDF membrane, and probed with anti-ubiquitin and -H3 antibodies, respectively.

Alignment and phylogenetic analysis

A phylogenetic tree of RING domain-containing proteins and RING domains from different organisms was constructed using MEGA4 software [42]. The RING domains of the RING domain-containing proteins were aligned using Jalview through ClustalW [43].

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Author Contributions

Conceived and designed the experiments: LM ZW XW SC. Performed the experiments: WL ZW JL HY XW. Analyzed the data: WL ZW JL HY XW SC LM. Contributed reagents/materials/analysis tools: LM SC. Wrote the paper: XW LM.

References

1. Jurgen G (1985) A group of genes controlling the spatial expression of the bithorax complex in Drosophila. Nature 316: 153–155.
2. Schwartz YB, Pirrotta V (2007) Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet 8: 9–22.
3. Savva R, Puro R (2010) Interpretation of developmental signaling at chromatin: the Polycomb perspective. Dev Cell 19: 651–661.
4. Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, et al. (2002) Histone methyltransferase activity of a Drosophila Polycomb repressor complex. Cell 111: 197–208.
5. Fischle W, Wang Y, Jacobs SA, Kim Y, Allen CD, et al. (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev 17: 1670–1681.
6. Shao Z, Raible F, Mollaaghababa R, Goyon JR, Wu CT, et al. (1999) STB, a Polycomb group protein, is required for seed development. EMBO J 22: 4804–4814.
7. Hennig L, Derkacheva M (2009) Diversity of Polycomb group complexes in Drosophila melanogaster. Bioessays 31: 655–664.
8. Jung JH, Doyle MR, Sung S, Amasino RM (2009) Vernalization: winter and the timing of developmental transitions in plants. Cell 135: 149–156.
9. Cao R, Tsikaada Y, Zhang Y (2005) Role of Bni-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell 20: 845–854.
10. de Moraes M, Mermoud JE, Wakao R, Tang YA, Endoh M, et al. (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to histone H3 methylation. Nature 427: 873–878.
11. Hennig L, Derkacheva M (2009) Diversity of Polycomb group complexes in Drosophila melanogaster. Bioessays 31: 655–664.
12. Spillane C, MacDougall C, Stock C, Kohler C, Vielle-Calzada JP, et al. (2000) A Drosophila Polycomb repressor complex. Mol Cell 5: 545–556.
13. Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, et al. (2004) Role of histone H2A ubiquitination in Polycomb silencing. Nature 431: 873–878.
14. Cao R, Tsikaada Y, Zhang Y (2005) Role of Bni-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell 20: 845–854.
15. de Moraes M, Mermoud JE, Wakao R, Tang YA, Endoh M, et al. (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to histone H3 methylation. Nature 427: 873–878.
16. Kohler C, Lemig R, Bouvetet R, Gheysienc K, Grossniklaus U, et al. (2003) Arabidopsis MSH1 is a component of the MIF4/FIE Polycomb group complex and required for seed development. EMBO J 22: 4004–4014.
17. Makarewich G, Gierlotka V, Pquilh C, Bravdo SZ, Lippman Z, et al. (2002) Different Polycomb group complexes regulate common target genes in Arabidopsis. EMBO Rep 7: 947–952.
18. Pquilh C, Page DR, Gagliardini V, Grossniklaus U, et al. (2005) Interaction of the Arabidopsis polycomb group protein FIE and MEF mediates their common chromatin: the Polycomb perspective. Dev Cell 7: 663–676.
19. He DJ, Derkacheva M (2009) Diverse Polycomb group complex in plants: same rules, different players? Trends Genet 25: 414–423.
20. Spillane C, MacDougall C, Stock C, Kohler C, Vielle-Calzada JP, et al. (2000) A Drosophila Polycomb repressor complex. Mol Cell 5: 545–556.
21. Lu M, Bilodeau P, Dennis ES, Peacock WJ, Chaudhary A (2000) Expression and parent-of-origin effects for FIS2, MEF, and FIE in the endosperm and embryo of developing Arabidopsis seed. Proc Natl Acad Sci USA 97: 10637–10642.
22. Sheehan K, Yanai Y, Chen L, Kato Y, Hiratsuka J, et al. (2001) EMBRYONIC FLOWER2, a novel polycomb group protein homolog, modulates shoot development and flowering in Arabidopsis. Plant Cell 13: 2471–2481.
23. Tao J, Dang L, Wu Y (2008) Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the Arabidopsis Polycomb repressive complex 2 components. PLoS One 3: e3494.
24. Schonrock N, Bouvetet R, Leroy O, Borghi L, Kohler C, et al. (2006) Polycomb-group proteins repress the floral activator AGAMOUS in the FLC-independent vernalization pathway. Genes Dev 20: 1667–1678.
25. De Lucia F, Crevillen P, Jones AM, Greb T, Dean C (2008) A PHD-polycomb repressive complex 2 that triggers epigenetic silencing of FLC during vernalization. Proc Natl Acad Sci USA 105: 16351–16356.
42. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
43. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.