

## Article

# PsmiR159b-PsMYB65 module functions in the resumption of bud growth after endodormancy by affecting the cell cycle in tree peony

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## Abstract

Bud endodormancy in perennial plants is a sophisticated system that adapts to seasonal climatic changes. Growth-promoting signals such as low temperature and gibberellins (GAs) are crucial for facilitating budbreak following endodormancy release (EDR). However, the regulatory mechanisms underlying GA-mediated budbreak in tree peony (*Paeonia suffruticosa*) remain unclear. In tree peony, the expression of PsmiR159b among three differentially expressed miR159 members was inhibited with the prolonged chilling, and overexpression of PsMIR159b delayed budbreak, whereas silencing PsmiR159b promoted budbreak after dormancy. PsMYB65, a downstream transcription factor in the GA pathway, was induced by prolonged chilling and exogenous GA<sub>3</sub> treatments. PsMYB65 was identified as a target of PsmiR159b, and promoted budbreak in tree peony. RNA-seq of PsMYB65-silenced buds revealed significant enrichment in the GO terms regulation of 'cell cycle' and 'DNA replication' among differentially expressed genes. Yeast one-hybrid and electrophoretic mobility shift assays demonstrated that PsMYB65 directly bound to the promoter of the type-D cyclin gene PsCYCD3;1. Dual-luciferase reporter assay indicated that PsMYB65 positively regulate PsCYCD3;1 expression, suggesting that miR159b-PsMYB65 module contributes to budbreak by influencing the cell cycle. Our findings revealed that the PsmiR159b-PsMYB65 module functioned in budbreak after dormancy by regulating cell proliferation, providing valuable insights into the endodormancy release regulation mechanism.

## Introduction

In perennial woody plants, bud endodormancy plays a critical role in ensuring survival under the adverse environmental conditions present during winter, such as low temperature (LT) and dehydration stress. Bud dormancy consists of the following stages: endodormancy establishment, maintenance, endodormancy release (EDR), and budbreak [1, 2]. Growth cessation and endodormancy are established before winter, whereas EDR and resumption of normal growth occur only after a period of chilling in winter. Photoperiod and temperature are two main environmental factors controlling bud endodormancy in woody plant [3, 4]. In hybrid aspen (*Populus tremula* × *tremuloides*), short-day (SD) exposure leads to a reduction in PtFT2 transcription, halting growth, and initiating bud formation to protect the shoot apical meristems [5, 6]. Under long day conditions, PtFT2 interacts with PtFD-like 1 to ensure high transcriptional levels of PtAPI-like [7], which activates key D-type cyclin (PtCYCD) transcripts to promote growth [8].

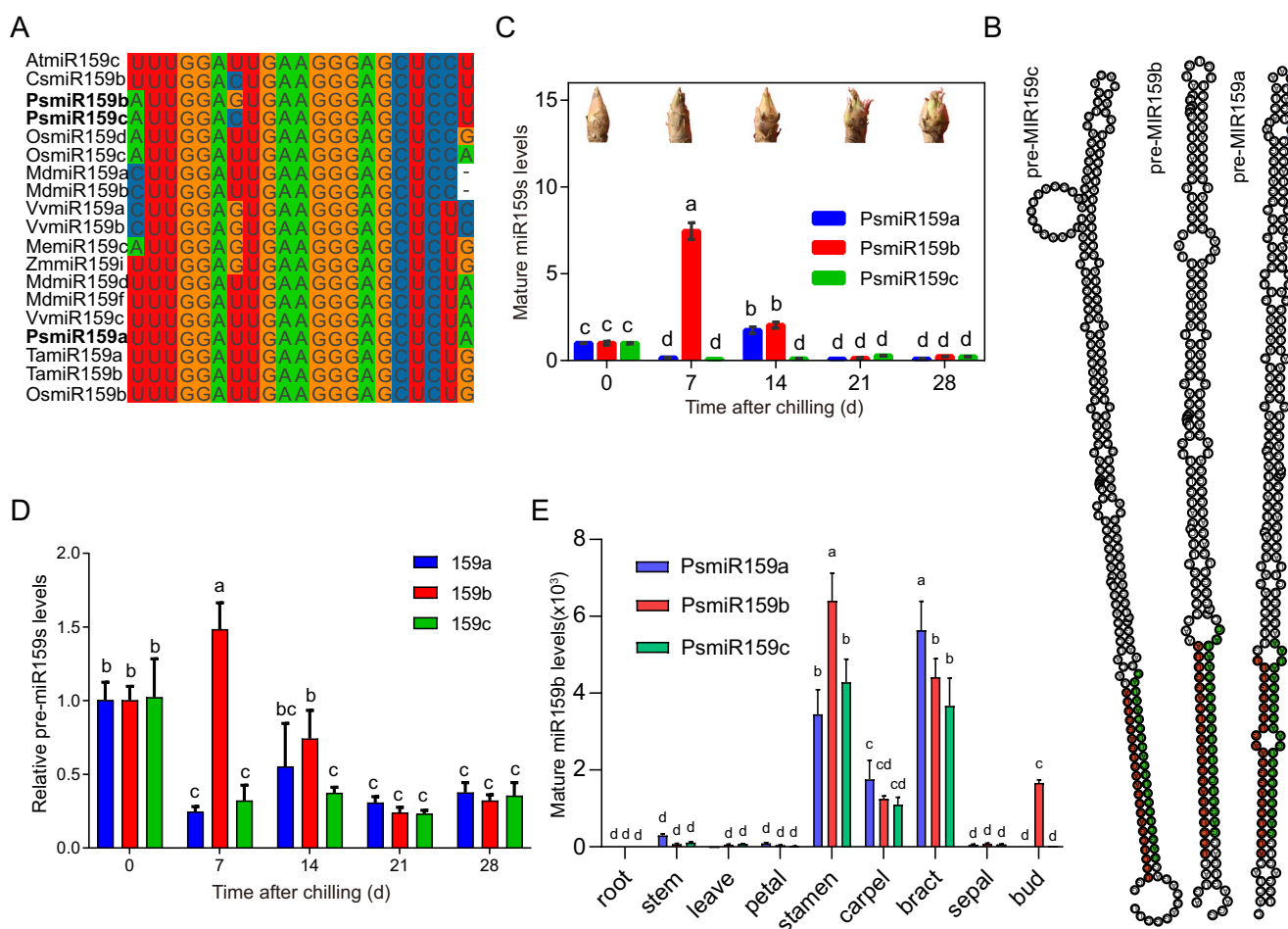
Gibberellins (GAs) and abscisic acid (ABA) play vital roles in the regulation of bud dormancy [6, 9]. Endogenous ABA levels increase during the establishment of endodormancy [10], whereas they decrease and GA levels increase during chilling-induced EDR [11].

Short vegetative phase (SVP)-like (SVL) acts as a regulatory molecule of the phytohormone pathway to hinder EDR [12]. Prolonged LT exposure impairs SVL expression, increases GA levels, and facilitates budbreak [13, 14]. Several studies have demonstrated that GA contributes to EDR and budbreak [4, 14]. External application of GA for accelerating dormancy release and budbreak has been demonstrated in numerous woody plants [15, 16]. The DELLA protein family acts as a negative regulator of the GA signaling pathway [17], and the DELLA gene, PsRGL1, inhibits dormancy release in tree peony [18]. However, GA-regulated downstream pathways in budbreak are poorly described.

In addition, accumulating evidences suggest that microRNAs (miRNAs) play a role in endodormancy regulation [19–21]. PsmiR172 functions in tree peony EDR by repressing EARLY BUD-BREAK 1 (EBB1), which is a positive regulator of plant EDR [13, 22]. Based on small RNA sequencing, it has been reported that miR159s are differentially expressed between dormancy induction and EDR [21, 23]. However, the precise regulatory mechanisms of miR159 during bud dormancy require further investigation. miR159 is conserved in major land plants and targets a class of R2R3 MYB transcriptional factor (TF) named gibberellin MYB (GAMYB) [24]. GAMYB, a component of GA signaling, was first

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**Figure 1.** Identification and expression patterns of PsmiR159 family in tree peony 'Luhehong'. (A) Alignment of mature miR159 sequences of tree peony and other known plants. Gene symbol with bold letters indicated the members of PsmiR159 family in tree peony. (B) Stem-loop structures of PsmiR159s. Red dots indicated the mature sequence of PsmiR159s. (C–D) Relative expression levels of the mature (C) and precursors (D) of PsmiR159s at 0, 7, 14, 21, 28 days of artificial chilling (DAC). Chilling perception phase was determined at 0~14 DAC, 14~21 DAC was the transition phase between endodormancy and endodormancy release (EDR), and 21~28 DAC was in ecodormancy state [29]. Pictures of tree peony buds were captured after being transferred to greenhouse for 10 days (18–22°C, 16/8 h light/dark). Data were represented as the mean ± standard deviation (SD) of three biological replicates. (E) The relative expression levels of mature miR159s in different tissues. Twenty-one-day chilling treated plants were transferred to greenhouse for 45 d, and the tissues were collected. The buds were a mix of 0, 7, 14, and 21 DAC. Data are shown as mean ± SD ( $n=3$ ) from three biological replicates (five buds in each replicate). Letters 'a' to 'd' in this figure indicate significant differences via one-way analysis of variance (ANOVA; Tukey test,  $P < 0.05$ ).

identified in barley (*Hordeum vulgare*). The GAMBY-like family contains three members in Arabidopsis: AtMYB33, AtMYB65, and AtMYB101 [25]. Previous studies have shown that GA induces the transcription of GAMBY, with notable expression in flowers [25, 26]. AtMYB33/65 affects various biological processes, including anther tapetal and pollen development, seed germination, and flowering in response to GA [27].

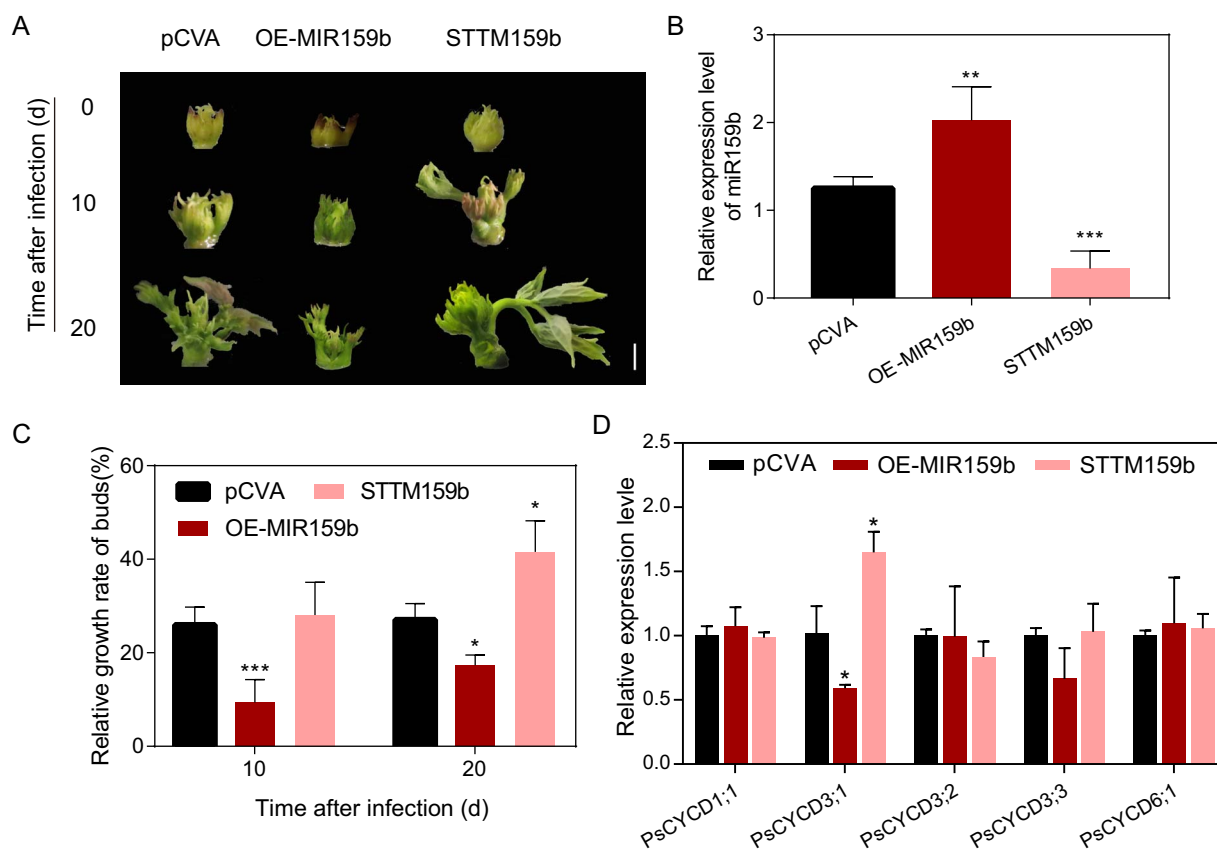
Tree peony (*Paeonia suffruticosa* Andr.), an ancient woody ornamental plant in China, must experience a period of LT to break bud endodormancy. Previous study suggests that EDR is significantly triggered by prolonged chilling in tree peony. However, poor understanding about the molecular mechanisms of budbreak limits its industrial development [28]. Here, we found that a member of the miR159 family, PsmiR159b, and its target gene, PsMYB65, played important roles in tree peony budbreak. PsMYB65 directly bound to the promoter of PsCYCD3;1 and activated its expression, which promotes cell proliferation, thereby facilitating budbreak in tree peony. These findings would provide new insights into the molecular mechanisms of growth resumption during dormancy transition and budbreak.

## Results

### Identification and expression analysis of PsmiR159 in tree peony

We previously identified some differentially expressed miRNAs during EDR, including three PsmiR159s (Fig. S1, see online supplementary material), through small RNA sequencing. PsmiR159a, b, and c were identified in the tree peony genome. Here, we found that the lengths of mature PsmiR159a–c were highly conserved with 21 nucleotides (nt) (Fig. 1A). PsmiR159a–c shared the same 18 nt, differing only in the first, seventh, 20th, and 21st nucleotides at the 5'-ends (Fig. 1A). Then, the precursors of PsmiR159s were cloned, and the mature sequences were located in the 3'-stem arm of their stem-loop structures (Fig. 1B; File SS1, see online supplementary material).

The expression patterns of PsmiR159s in tree peony buds were analysed during chilling-induced EDR (0~28 days of artificial chilling, DAC) using quantitative reverse transcription-polymerase chain reaction (RT-qPCR). PsmiR159a was inhibited by short-term chilling treatment (7 DAC), and reached a peak



**Figure 2.** PsmiR159b inhibited tree peony budbreak. (A) Morphology of PsMIR159b-overexpressing (OE-MIR159b) and PsMIR159b-silenced (STTM159b) buds. pCVA, transgenic buds with empty pCVA vector. Buds were pictured at 10 and 20 d after infection (DAI). Scale bar, 5 mm. (B) Relative level of PsmiR159b at 10 DAI. Data were shown as mean  $\pm$  SD ( $n=3$ ). (C) Relative growth rate of OE-MIR159b and STTM159b buds at 10 and 20 DAI. Data were shown as mean  $\pm$  SD ( $n=6$ ). (D) Expression levels of D-type cyclin (CYCDs) in OE-MIR159b, STTM159b buds and control at 10 DAI. Data were shown as mean  $\pm$  SD ( $n=3$ ). Asterisk (\*) indicated statistically significant differences via two-tailed Student's *t* test (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ).

after 14 DAC, and then decreased until 28 DAC. PsmiR159b was induced by chilling treated for 7 days, followed by a sharp decrease with the prolonged chilling. PsmiR159c exhibited low expression levels throughout all stages (Fig. 1C). To confirm these results, we also detected the expression patterns of their precursors during chilling-induced EDR. The results showed that the precursor of PsmiR159s were concomitant with mature PsmiR159s (Fig. 1C and D). Overall, the RT-qPCR data revealed expression patterns that were similar to those obtained by high-throughput sequencing. In term of spatial distribution, tissues other than the buds were isolated at the early stages of flowering, and the buds were mixed at 0, 7, 14, and 21 DAC. The results showed that all PsmiR159s were mainly expressed in flower organs, especially the stamen and bract, whereas only PsmiR159b showed higher expression levels in mixed buds (0–21 DAC) than others (Fig. 1E).

### PsmiR159b represses budbreak in tree peony

Similar to previous observations [29], over half of apical buds burst for 14 DAC plant, while almost all of the apical buds burst for the 21 DAC treatment, which indicated that 14~21 DAC is the transition from endodormancy to EDR in tree peony 'Luhehong'. Because effective low-temperature accumulation is necessary for tree peony bud dormancy release, only PsmiR159b was relatively highly expressed in the bud, and its expression level was down-regulated with the extension of chilling accumulation (14 and 21 DAC), suggesting that it might play

an important role during bud dormancy release. To further explore the function of PsmiR159b in budbreak, PsMIR159b was overexpressed in tree peony buds via virus-based miRNA expression system (Fig. 2A). Compared to the control, miR159b levels in PsMIR159b-overexpressing (OE-MIR159b) buds increased by approximately 1.7-fold (Fig. 2B). In contrast, OE-MIR159b buds delayed bud sprouting (Fig. 2A), and the relative growth rate of buds significantly decreased at 10 days after infection (DAI) (Fig. 2C). Using the short tandem target mimic (STTM) approach, PsMIR159b-silenced (STTM159b) buds were generated [30] (Fig. 2A; Fig. S2, see online supplementary material). In STTM159b buds, miR159b expression levels were approximately 73% lower than those in the control (Fig. 2B). STTM159b transgenic buds accelerated bud sprouting with a higher relative growth rate compared with the pCVA control, especially at 20 DAI (Fig. 2A and C). These results indicated that PsmiR159b inhibits budbreak in tree peony.

Bud endodormancy is accompanied by the reactivation of the cell cycle, with CYCD genes showing increased expression during EDR and budbreak in tree peony and poplar [8, 15, 28]. Gene family analysis of CYCD was performed using the CYCD HMM profile (Pfam: 00134) in tree peony, and five members of CYCD family were obtained (Fig. S3, see online supplementary material). The expression levels of PsCYCDs in STTM159b and OE-MIR159b buds were evaluated. Among them, the transcripts of PsCYCD3:1 significantly increased in STTM159b transgenic buds and decreased in OE-MIR159b buds (Fig. 2D).

## PsMYB65 is a target of PsmiR159b in tree peony

To identify the target gene of *PsmiR159b* in tree peony, we performed a target gene prediction analysis using PsRobot [31], and a MYB homolog with target site at 950–970 nt was obtained (Table S1, see online supplementary material). Phylogenetic analysis indicated that the predicted MYB was closely homologous to AtMYB65 (Figs S4 and S5A, see online supplementary material), and thus it was named PsMYB65. Alignment of amino acids showed that PsMYB65 contained an R2R3 domain and three conserved motifs (Fig. S5B, see online supplementary material). Sub-cellular localization analysis revealed that PsMYB65 was localized in the nucleus (Fig. S5C, see online supplementary material). Moreover, yeast assays demonstrated the transcriptional activation activity of PsMYB65, and its putative transcription regulatory domain (TRD) was essential for this activity (Fig. 3A).

Temporal-spatial expression patterns of PsMYB65 were analysed using RT-qPCR. The transcripts of PsMYB65 were upregulated during chilling-induced EDR and peaked at 21 DAC (Fig. 3B). PsMYB65 also responded to GA<sub>3</sub> treatment, with a significant increase after 24 h of treatment (Fig. 3C). Moreover, PsMYB65 levels were higher in buds than other tissues (Fig. S5D, see online supplementary material).

The expression levels of *PsmiR159b* and PsMYB65 exhibited an inverse pattern from 7 to 21 DAC (Figs 1D and 3 B). Additionally, we detected the expression levels of PsMYB65 in OE-MIR159b and STTM159b buds. As expected, the transcripts of PsMYB65 decreased by 52% in OE-PsmiR159b buds, while increased by approximately 1.6-fold in STTM159b transgenic buds (Fig. 3D). This led us to hypothesize that PsMYB65 was a target of *PsmiR159b* during endodormancy transition. To verify this hypothesis, we generated the miR159b-insensitized PsMYB65 (*mPsMYB65*) by synonymous mutation at the miR159 target site of PsMYB65.  $\beta$ -glucuronidase (GUS) reporter gene was used to construct a fusion protein with PsMYB65/*mPsMYB65* under the control of the CaMV35S promoter, with 35S::GUS as a control. Meanwhile, the precursor of *PsmiR159b* was driven by CaMV35S promoter (35S::PsmiR159b) to express *PsmiR159b*. Histochemical staining and GUS activity were used to evaluate GUS signals. The leaves transformed with 35S::GUS and 35S::PsMYB65-GUS exhibited strong GUS signals, whereas those co-expressing 35S::PsMYB65-GUS and 35S::PsmiR159b exhibited weaker GUS signals (Fig. 3E; Fig. S5E, see online supplementary material). Notably, GUS signals in leaves co-expressing 35S::*mPsMYB65*-GUS and 35S::PsmiR159b were not significantly different from those with only 35S::*mPsMYB65*-GUS (Fig. 3E; Fig. S5E, see online supplementary material).

Next, PsMYB65 mRNAs in buds at 7 DAC were used to examine the cleavage site by RLM-5'-RACE. The results showed that the cleavage site was located between the 10th and 11th bases from the 5'-end of the target site (Fig. 3F). Taken together, these results confirmed that PsMYB65 was the target gene of *PsmiR159b* during EDR in tree peony.

## PsMYB65 promotes budbreak in tree peony

To further investigate the role of PsMYB65 in bud endodormancy and/or budbreak regulation, PsMYB65 was silenced via VIGS in tree peony buds. Expression levels of PsMYB65 were determined at 10 DAI, and the average expression levels of PsMYB65 decrease by 56% in TRV2-PsMYB65 buds (Fig. 4A). Compared to TRV2 buds, TRV2-PsMYB65 buds delayed sprouting (Fig. 4B), and the relative growth rate decreased by 48% after 10 DAI (Fig. 4C). As markers of the EDR state, five PsCYCDs transcripts were examined using

RT-qPCR. We found that PsCYCD3s levels significantly decreased in PsMYB65-silenced buds (Fig. 4D).

## PsMYB65 affects cell cycle and cell division in tree peony buds

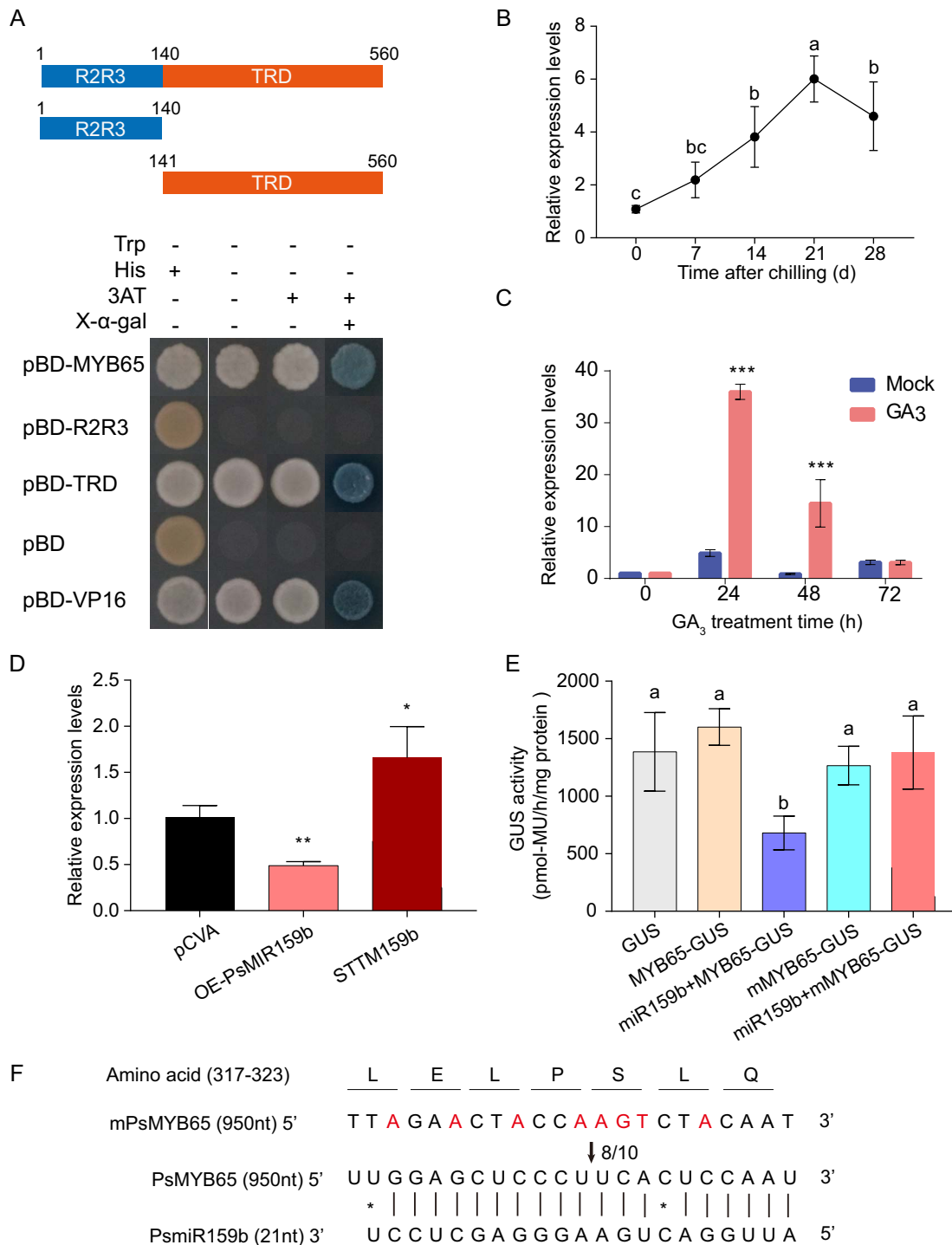
To identify the downstream genes of PsMYB65, we performed RNA-seq of TRV2-PsMYB65 buds, using TRV2 buds as a control (Table S2, see online supplementary material). In total, 1352 differentially expressed genes (DEGs) were obtained including 373 downregulated and 979 upregulated genes (Fig. 5A). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that these DEGs were enriched in several pathways, including ATP-dependent chromatin remodeling, DNA replication, and plant hormone signal transduction (Fig. 5B). Moreover, we performed Gene Ontology (GO) enrichment analysis to obtain further insight into the functions of these DEGs during budbreak. Among the top 20 GO terms, at least 10 GO terms were related to cell cycle and cell division (Fig. 5C). Moreover, the heatmap indicated that the expression levels of several known genes participated in cell cycle were down-regulated in TRV2-PsMYB65 buds, such as Ribonuclease H2 subunit A (RNASEH2A) and PsCYCD3;1, whereas several genes known to negatively regulate cell cycle were upregulated in TRV2-PsMYB65 buds, such as Mitotic arrest-deficient 2 (MAD2) and Cyclin-dependent protein kinase inhibitor (SMR3) (Fig. 5D). These findings suggested a potential connection between PsMYB65 and cell cycle regulation.

## PsMYB65 directly activates the expressions of PsCYCD3;1

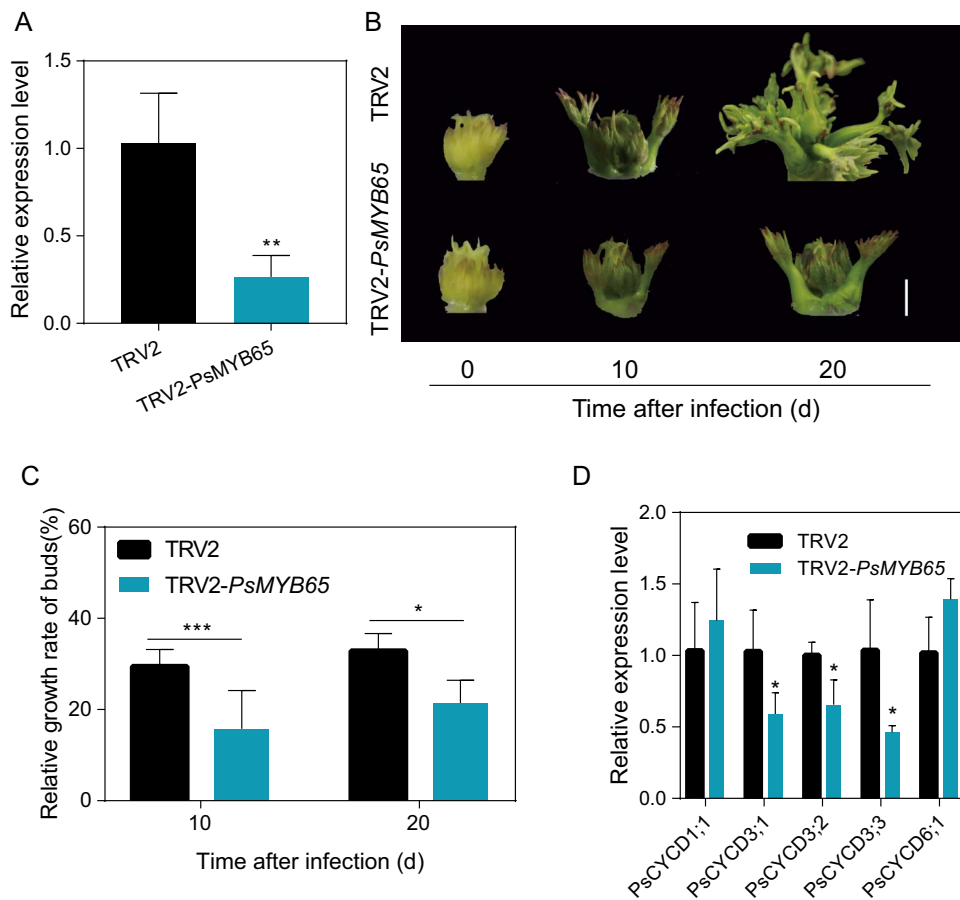
As known, EDR and budbreak accompanied by reactivation of cell cycle, and CYCD plays a critical role in EDR and budbreak [13, 28]. Among down-regulated DEGs enriched in cell cycle term, PsCYCD3;1 was hindered in TRV2-PsMYB65 buds but increased in STTM159b buds. Moreover, PsCYCD3;1 was significantly induced by GA<sub>3</sub> treatment (Fig. S6, see online supplementary material). Therefore, we explored whether PsMYB65 regulated the expression of PsCYCD3;1. Firstly, a 1700 bp promoter fragment of PsCYCD3;1 was isolated and analysed using PlantCARE tool [32]. The analysis revealed two putative MYB-binding sites in PsCYCD3;1 promoter (Fig. 5B), suggesting that PsMYB65 might regulate PsCYCD3;1. Furthermore, a dual-luciferase (LUC) reporter assay was performed to verify the relationship between PsMYB65 and PsCYCD3;1. LUC reporter gene, under the control of the promoter of PsCYCD3;1 (*proPsCYCD3;1::LUC*), served as the reporter. *pro35S::PsMYB65* was used as the effector and empty vector SK as the control. The relative LUC activity in samples co-transformed *proPsCYCD3;1::LUC* and *pro35S::PsMYB65* was 3.5-fold higher than that in samples co-transformed with *proPsCYCD3;1::LUC* and empty vector SK (Fig. 6A).

Then, to confirm whether PsMYB65 directly bound to the promoter of PsCYCD3;1, a yeast one-hybrid (Y1H) assay was employed. The promoter of PsCYCD3;1 was truncated into two parts based on the distribution of MYB-binding sites 'TGTTTA' (Fig. 6B). As shown in Fig. 6C, yeast cells harboring *proPsCYCD3;1-P2* and AD-PsMYB65 could grow on synthetic defined (SD)-Trp-Leu-His medium with 30 mM of 3-amino-1,2,4-triazole (3-AT), whereas yeast cells with *proPsCYCD3;1-P1/pHIS2* empty vector and AD-PsMYB65 could not grow. To further test the direct binding of PsMYB65 to the PsCYCD3;1 promoter *in vitro*, we synthesized a probe containing this binding site, and labeled with biotin. Electrophoretic mobility shift assays (EMSA) showed that the binding signal was observed when mixing recombinant PsMYB65-glutathione S-transferase (GST) and the labeled probe.





**Figure 3.** PsmiR159b targeted PsMYB65 in tree peony. (A) The TRD domain was necessary for transcriptional activation activity of PsMYB65. R2R3, R2R3 domain, TRD, the putative transcription regulatory domain. The transformants were selected on the SD/-Trp, SD/-Trp/-His, SD/-Trp/-His/ with 30 mM 3-amino-1,2,4-triazole (3-AT) and SD/-Trp/-His/X-α-Gal. The pBD-VP16 was as a positive control and pBD as a negative control. (B-C) Expression of PsMYB65 during chilling-induced EDR (B), and after GA<sub>3</sub> treatment (C). Data were represented as the mean ± SD (*n* = 5). Letters indicated the significant differences (one-way ANOVA, Duncan's multiple range test). (D) Relative expression levels of PsMYB65 in OE-MIR159b and STTM159b buds. pCVA, empty vector control. Values were represented as the mean ± SD of three biological replicates. Asterisk (\*) indicated the significant differences via two-tailed Student's *t*-test (\**P* < 0.05 and \*\**P* < 0.01). (E) Enzyme activity of GUS in transformed tobacco leaves with 35S::GUS, 35S::PsMYB65-GUS, 35S::mPsMYB65-GUS, 35S::PsMYB65-GUS + 35S::PsMIR159b, and 35S::mPsMYB65-GUS + 35S::PsMIR159b. mPsMYB65 indicated the synonymously mutated PsMYB65 in the miR159b target region. Values were represented as the mean ± SD of six biological replicates, and letters over columns indicated the significant differences (one-way ANOVA, Duncan's multiple range test). (F) Cleavage sites are identified via RNA ligase-mediated (RLM) 5'-rapid amplification of cDNA ends (RACE). A black arrow indicates the cleavage sites, and the numbers next to the arrow indicate the frequency of the cleavage fragments.



**Figure 4.** PsMYB65 accelerated budbreak in tree peony buds. (A) Expression levels of PsMYB65 in TRV2-PsMYB65 and control buds at 10 DAI. Data were represented as the mean  $\pm$  SD ( $n = 5$ ). (B) Morphology of TRV2-PsMYB65 buds at 10 and 20 DAI. TRV2, pTRV2 empty vector control. TRV2-PsMYB65, PsMYB65-silenced buds. Scale bar, 5 mm. (C) Relative growth rate of TRV2-PsMYB65 buds at 10 and 20 DAI. Data were represented as the mean  $\pm$  SD ( $n = 5$ ). (D) Expression levels of PsCYCDs in TRV2-PsMYB65 and control buds at 10 DAI. Asterisks (\*) in this figure indicated the statistically significant differences via two-tailed Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ).

The binding signal was enlarged in a dosage-dependent manner, whereas the binding signal was diminished with the addition of a competitive probe (Fig. 6D). These findings demonstrated that PsMYB65 directly bound to the promoter of PsCYCD3;1 to regulate its expression.

### PsmiR159b–PsMYB65 regulates cell division during budbreak

Considering that PsMYB65 was the target of PsmiR159b, and PsMYB65 directly activated PsCYCD3;1 expression (Figs 3 and 5), then we investigated the function of PsCYCD3;1 during bud EDR and budbreak by VIGS (Fig. 7A). When compared with the control, the expression level of PsCYCD3;1 was reduced by 57% in TRV2-PsCYCD3;1 buds, and PsCYCD3;1-silenced buds exhibited slower sprouting with a lower relative growth rate after 10 and 20 DAI (Fig. 7C).

It is well known that CYCD play an important role in the cell cycle. Therefore, we assumed that PsmiR159b–PsMYB65 affected cell division during EDR and budbreak in tree peony buds. To confirm this hypothesis, we observed and quantified the cell number and size in OE-PsMIR159b and TRV2-PsMYB65 buds by paraffin sections. The number of cells significantly decreased in OE-PsMIR159b and TRV2-PsMYB65 buds (Fig. 7D and E), whereas the average cell sizes were bigger than the control buds (Fig. 7F). The reason might be that the overexpression of PsmiR159b and silencing of PsMYB65 inhibited cell divisions. Overall, these results

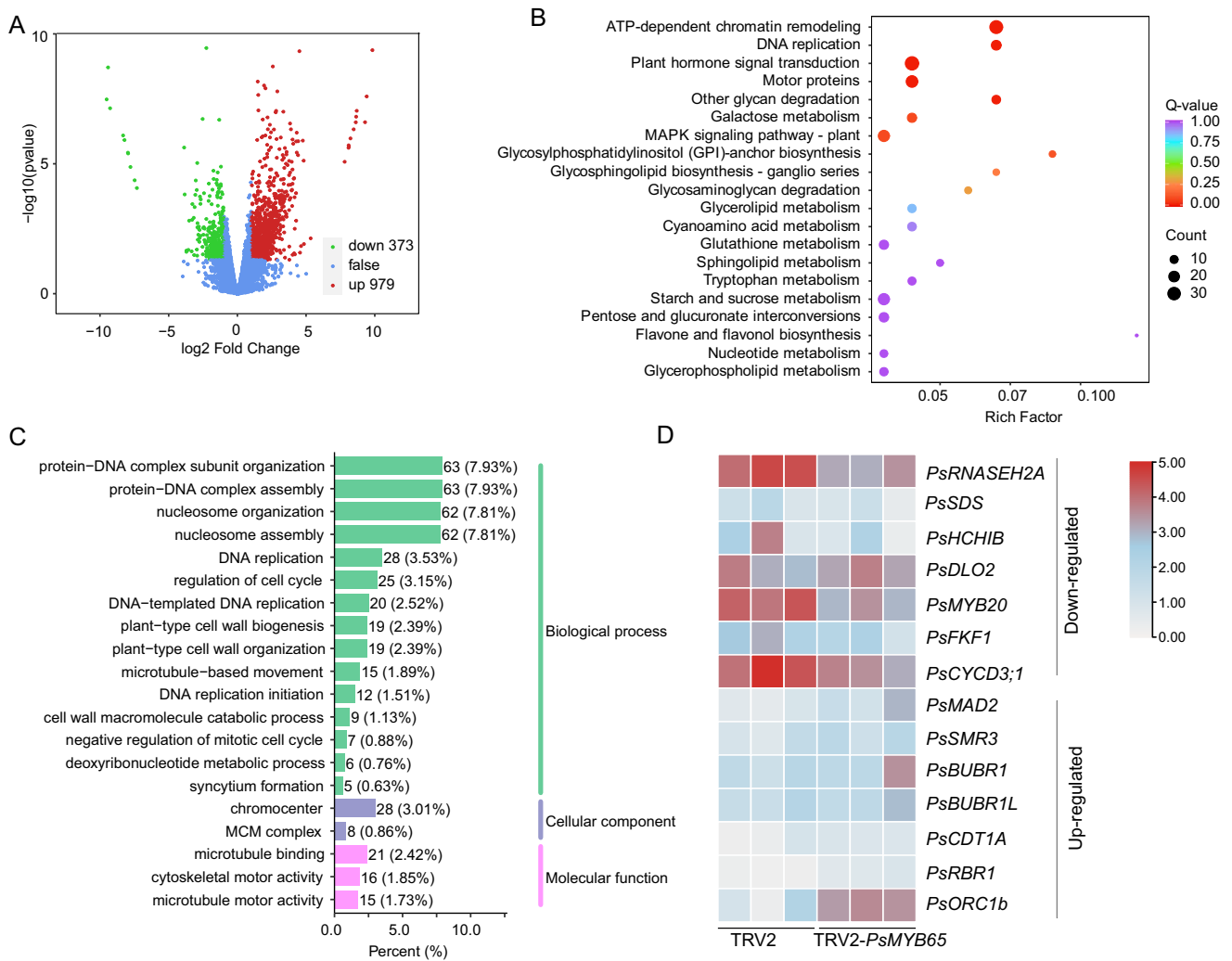
indicated that PsmiR159b–PsMYB65 could modulate cell division, finally promoting budbreak in tree peony.

## Discussion

### miR159b targets PsMYB65 to regulate budbreak

miR159–GAMYBs play important roles in plant growth, flower organ development, flowering, fruit set, and defense response [25, 33–36]. However, their roles in EDR and budbreak remain unknown. Here, we reported for the first time that a miR159–MYB65 module was involved in budbreak in tree peony by influencing the cell cycle. Genetic analyses revealed that PsmiR159b acted as a repressor in budbreak (Fig. 2). GUS signal and RLM-5'-RACE results demonstrated that PsmiR159b targeted PsMYB65 (Fig. 3), a GAMYB gene, which promoted budbreak (Fig. 4). Furthermore, PsMYB65 was found to directly bind to the TGGTTA motif in PsCYCD3;1 promoter to activate its expression (Fig. 5). These findings indicated that miR159b–PsMYB65 module functioned in budbreak in tree peony.

To screen for EDR- or budbreak-associated genes, transcriptional profiling comparisons between the prolonged chilling and early chilling stages, other than no chilling, are recommended [28, 37]. In this study, PsmiR159b peaked at 7 DAC, but was significantly downregulated by prolonged chilling (Fig. 1), suggesting that PsmiR159b is a candidate EDR-associated gene. Subsequent results verified that PsmiR159b acts as a potential cell cycle



**Figure 5.** PsMYB65 affected cell cycle and cell division. **(A)** A volcano map of differentially expressed genes (DEGs) in RNA-seq of TRV2-PsMYB65 and control buds. **(B)** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs. **(C)** The top 20 GO terms of DEGs. **(D)** A heatmap of DEGs enriched into the term of cell cycle. DLO2, Downy mildew resistant 6 (DMR6)-LIKE OXYGENASE 2. FKF, flavin-binding, kelch repeat, F box. RNASEH2A, Ribonuclease H2 subunit A. SDS, SOLO DANCERS. HCHIB, basic chitinase. MAD2, Mitotic arrest-deficient 2. SMR3, Cyclin-dependent protein kinase inhibitor. BUBR1, BUB1-related 1. BUBR1L, BUBR1-LIKE. CDT1A, Arabidopsis homolog of yeast CDT1. RBR1, Retinoblastoma-related 1. ORC1b, Origin of replication complex subunit 1b.

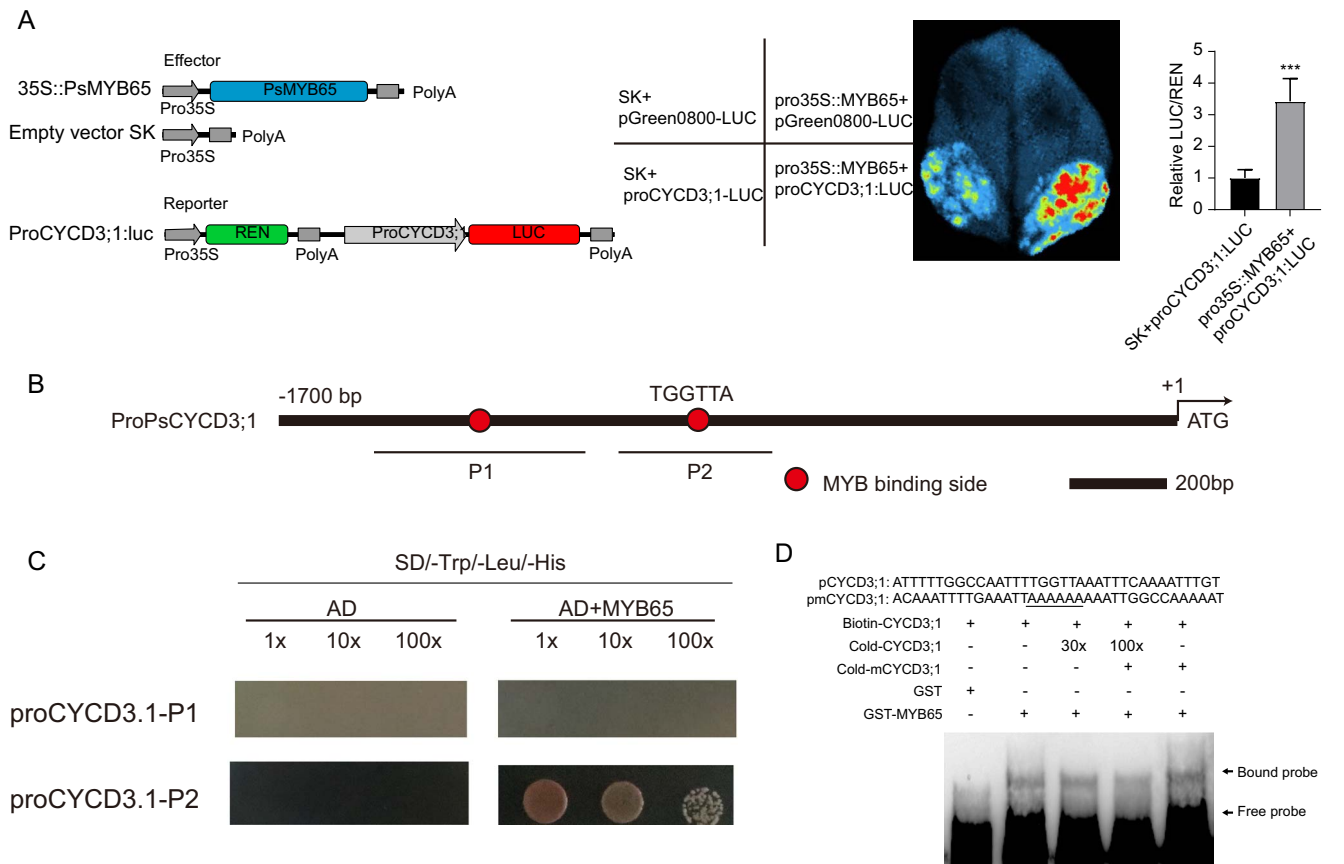
inhibitor in the budbreak of tree peony. Interestingly, PsmiR159b was also low in abundance at 0 DAC (Fig. 1), indicating a dormant state. It is well known that the perception of SD is the main trigger for the establishment of bud dormancy, with low temperatures also playing a significant role in this process [4]. For PsmiR159b, SDs alone may not be sufficient to increase expression to levels that arrest cell division. MiRNAs usually target multiple transcripts and regulate several biological processes [38]. We speculated that there might be other unknown genes targeted by PsmiR159b in the dormancy processes in tree peony, and that the low expression of PsmiR159b was required to work well in the system. Another explanation is that factors other than PsmiR159b hinder cell division. In poplar, a reduction in FT2 expression plays a central role in bud formation and dormancy induction [5], probably caused by repressing CYCD expression [39]. Thus, the regulation and function of PsmiR159b during the entire dormancy process require further investigation.

In addition, PsmiR159s have been shown to have high expression levels in some floral organs such as stamens, bracts, and carpels during the early flowering stage (Fig. 1D), suggesting

their potential role in the development of these organs. The involvement of miR159s in anther development has been reported in Arabidopsis, grapes, and *Brassica campestris* [33, 40, 41]. Thus, high expression of PsmiR159 in stamens may further influence anther development. Currently, there are not many reports on the relationship of miR159 with sepals and carpels. Based on the public database (TAIR), we found that AtmiR159b exhibited high expression levels in the sepals and carpels of Arabidopsis (Fig. S7, see online supplementary material). We speculated that the higher expression of miR159s in sepals and carpels was unrelated to the MYB65-CYCD pathway because flower buds still require cell growth and expansion to full bloom. Other targets of miR159b or biological processes may be involved in the early flowering stage, such as the miR159-CKX6 module, which regulates petal size through cytokinin catabolism in *Rosa hybrida* [42].

### PsMYB65 participates in GA-mediated budbreak

GAs are growth-promoting hormones in plants that play a major role in EDR and budbreak [4, 11, 14]. Activation of GA signaling is



**Figure 6.** PsMYB65 directly bound to the promoter of PsCYCD3;1 and activated its expression. **(A)** PsMYB65 activated the expression level of PsCYCD3;1 using a dual-LUC reporter system. A schematic representation of the constructed dual LUC reporter system is shown on the left. Statistics are shown on the right-hand side. Data were represented as the mean  $\pm$  SD ( $n = 8$ ). Asterisks (\*) indicated the significant differences between ProPsCYCD3;1::LUC + Pro35S::PsMYB65 and empty vector (SK) + ProPsCYCD3;1::LUC via two-tailed Student's t-test (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ ). **(B)** Analysis of MYB-binding sites in the promoter PsCYCD3;1. PsCYCD3;1 promoter was truncated into two parts according to the distribution of potential MYB-binding sites (TGGTTA). **(C)** Y1H assay for the binding of PsMYB65 to PsCYCD3;1 promoter. Fragments (P1 and P2 in B) of PsCYCD3;1 promoter were inserted into the pHis2 vector. Co-expressing yeast cells were screened on SD/-Trp/-Leu/-His with 30 mM 3-AT. **(D)** EMSA assay of PsMYB65 binding to the TGGTTA motif in PsCYCD3;1 promoter. pCYCD3;1 indicated the PsCYCD3;1 promoter (-1139 to -1175 bp). pmCYCD3;1 indicated the mutation probes, and the mutation sites are underlined.

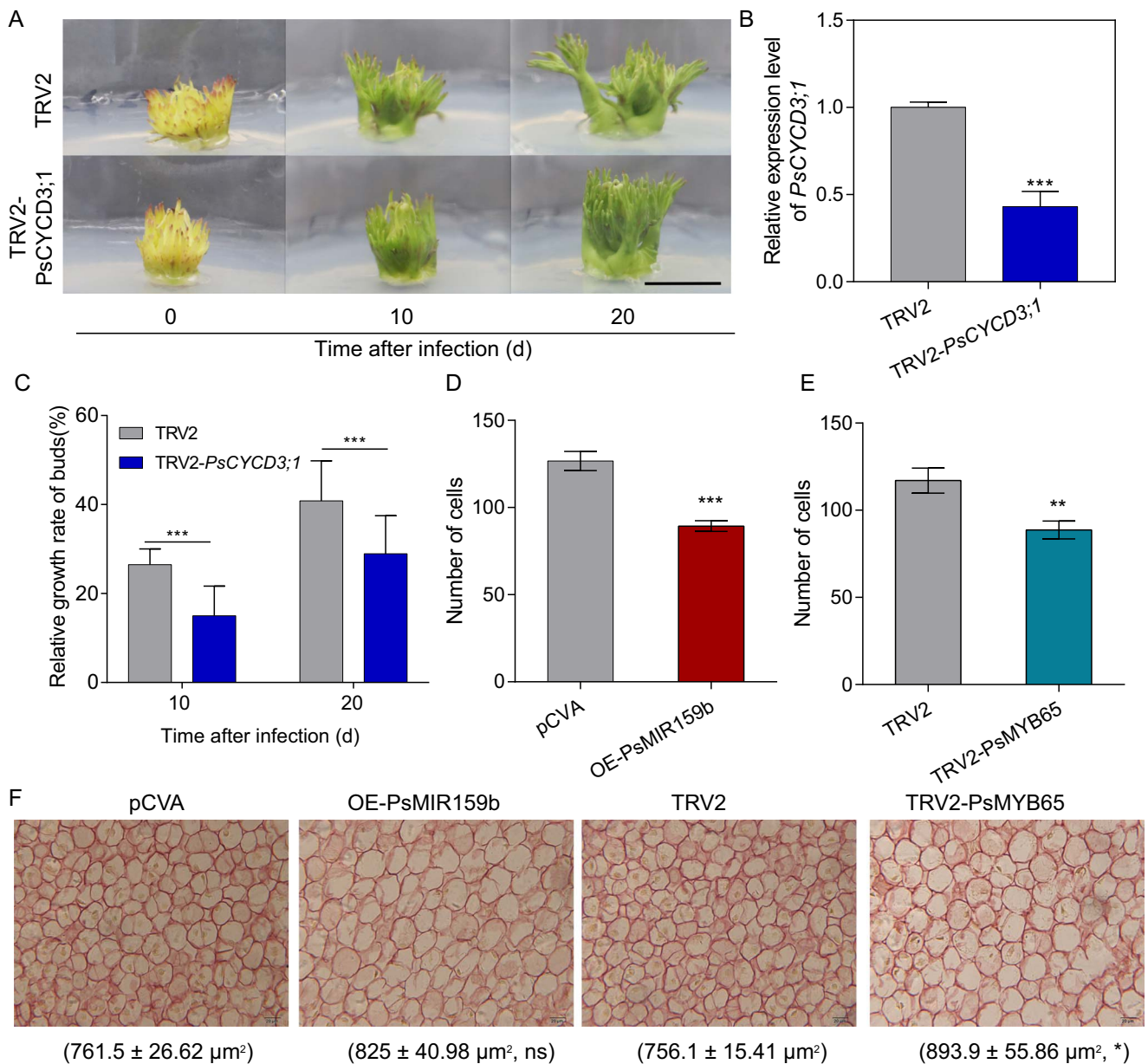
essential during EDR and budbreak in perennial woody plants [4]. Endogenous active GA levels increase from the dormant state to EDR stage in poplar and tree peony [3, 11]. The key genes involved in active GA biosynthesis, including GA20ox and GA3ox, are significantly up-regulated during EDR [28]. However, the mechanism by which the GA pathway influences the EDR remains unclear. Here, we identified that PsMYB65, a downstream GAMYB TF of GA signal transduction, was upregulated during EDR in tree peony (Fig. 3). Recent study has also reported that the expression of GAMYB was upregulated during EDR in *Culinary rhubarb* [43]. Our genetics and molecular biology findings indicated that PsMYB65 positively regulated EDR and budbreak (Fig. 3). PsMYB65 promoted cell proliferation during EDR and budbreak by directly activating PsCYCD3;1 expression (Fig. 5). Therefore, GA-regulated EDR and budbreak might be partially mediated by GAMYB.

### PsmiR159b-PsMYB65 module regulates budbreak by affecting cell cycle

The cell cycle is fundamental to eukaryotic growth and also organ development and comprises a DNA synthesis phase (S), a mitotic phase (M), and two gap phases (G1 and G2). Each stage is tightly controlled by cyclin-dependent kinases (CDKs) and cyclin com-

plexes [44]. Plant CDK proteins are divided into eight subgroups, of which A-type CDK (CDKA) and the plant-specific B-type CDK (CDKB) play a major role in cell cycle regulation [45]. type-A (CYCA), type-B (CYCB), and type-D (CYCD) are also crucial for controlling the cell cycle [44]. GAs have been shown to promote the expression of several CDKs and cyclins (CDKA;2, CYCB2;1, CYCB2;2, and CDKD;1), thus accelerating cell cycle progression [44], GAs application reduces the expression of the CDK inhibitors (CKIs) [46]. Xue et al. [47] finds that GAMYBs accelerate the cell cycle via the miR159 pathway in *Arabidopsis*. Although CYCD has been identified to play a positive role in growth of dormant buds [8], the transcriptional regulatory mechanisms of CYCD via GA signaling during budbreak after EDR still remain unclear. A few numbers of TFs have been found to regulate the expressions of CYCDs in EDR and budbreak, including AIL1 (AINTEGUMENTA-LIKE 1) and EBB3 (EARLY BUD-BREAK 3) [8, 13]. In this work, we identified PsMYB65 as a novel regulator of CYCD, directly promoting PsCYCD3;1 expression during budbreak after dormancy (Fig. 5). Overexpression of CYCD3;1 promotes cell proliferation and growth in poplar [48]. In tree peony, cell cycle was slowed down in OE-PsMIR159b and PsMYB65-silenced buds, suggested that miR159b-PsMYB65 module regulated cell replication in budbreak after dormancy.





**Figure 7.** PsmiR159b–PsMYB65 module affected cell cycle and cell division during budbreak. (A) Morphology of PsCYCD3;1-silenced buds at 10 and 20 DAI. TRV2, pTRV2 empty vector control. TRV2-PsCYCD3;1, PsCYCD3;1-silenced buds. OE-PsMIR159b, PsMIR159b-overexpressed buds. Scale bar, 5 mm. (B) Expression levels of PsCYCD3;1 in TRV2-PsCYCD3;1 and control buds. Data were represented as the mean  $\pm$  SD ( $n=3$ ). (C) Relative growth rate of TRV2-PsCYCD3;1 bud. Data were represented as the mean  $\pm$  SD ( $n=5$ ). (D, E) Cell number of OE-MIR159b (D) and TRV2-PsMYB65 buds (E). Data were represented as the mean of three different visual field of three buds with SD error bars. (F) Images of cell size in OE-MIR159b (D) and TRV2-PsMYB65 buds (E). Cell area is given below the image ( $n=5$ ). Asterisks (\*) in this figure indicate the statistically significant differences via two-tailed Student's *t*-test (\* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ).

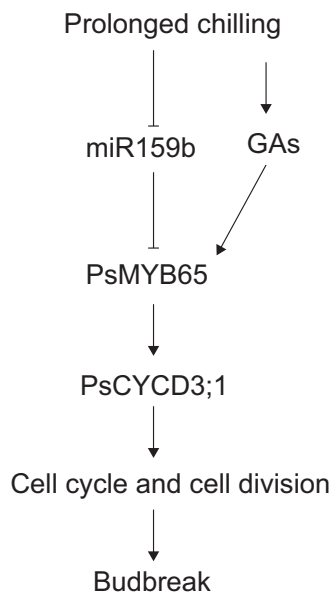
In conclusion, we proposed a working model of PsmiR159b–PsMYB65 regulating budbreak in tree peony (Fig. 8). Short-term chilling induced PsmiR159b, which targeted to PsMYB65 and suppressed its transcripts, resulting in blockage of the downstream signaling. With the extension of chilling accumulation (14 and 21 DAC), the transcript of PsmiR159b decreased, and the expression of PsMYB65 was up-regulated along with the increase of endogenous GAs. High levels of PsMYB65 enhanced cell replication by directly activating PsCYCD3;1 expression, and finally accelerated budbreak. Our findings provided novel insights into the miR159 function and GA signaling pathway in the regulation of tree peony budbreak after dormancy.

## Materials and methods

### Plant materials, growth conditions, and treatment

Four-year-old tree peony (*P. suffruticosa* cv. 'Luhehong') plants from Qingdao Agricultural University (Qingdao, China) were potted in late October 2018, which were moved to a refrigerator for artificial chilling treatment (0–4°C, 24 h/dark) as described by Zhang et al. [22]. Apical buds of uniform size were picked at 0, 7, 14, 21, and 28 days of artificial chilling (DAC), immediately frozen in liquid nitrogen, and stored at –80°C until use.

Plants after 7 DAC were transferred into a greenhouse (18–22°C, 16/8 h light/dark). The buds were then treated with 500 mg·L<sup>-1</sup>



**Figure 8.** PsmiR159b–PsMYB65 module regulates cell cycle during budbreak. A model of the PsmiR159b–PsMYB65 module regulates budbreak in tree peony. After a prolonged chilling treatment, PsmiR159b level decreases, and hence, it could not sufficiently block PsMYB65, while the increasing endogenous GA promotes the expression of PsMYB65. Accumulated PsMYB65 accelerates cell cycle by promoting the expression of PsCYCD3;1, thereby accelerating bud burst.

gibberellic acid (48 880, Merck, Germany). Three plants were used for biological duplication, and at least three biological replicates were set.

To evaluate dormancy status, an equal number of unsampled plants after different DAC were transferred to a greenhouse, as previously described [49].

### Sequence processing and phylogenetic analysis

The sequences of PsMYB65 and PsCYCDs were obtained from the tree peony transcriptome database [15], and their homologs in *Arabidopsis thaliana* were downloaded from the Arabidopsis Information Resource (<https://www.arabidopsis.org/>). Sequences were aligned using ClusterX (<http://www.cluster-x.org/>). Phylogenetic trees were constructed using MEGA 7.0 with the neighbor-joining method [50]. The stem-loop structures of PsMIR159s were elucidated using TBtools [51]. A schematic representation of PsMYB65 was drawn using IBS1.0.2 [52].

### Gene expression analysis

A total of 100 mg buds after different treatments including chilling and GA<sub>3</sub> were used to extract total RNA using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China), and genomic DNA was removed using DNase I. Total RNA (1 µg) was used for cDNA synthesis by HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed to determine the relative expression levels using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). PsActin was used as an internal control [22].

For miRNA analysis, HiScript III 1st Strand cDNA Synthesis Kit was used for cDNA synthesis with a reaction solution containing PsmiR159bF/stem-loop universal-R primers, 1 µg RNA, RT Mix, and HiScript III Enzyme Mix. The mixture was incubated at 25°C for 5 min, 37°C for 45 min, and 85°C for 5 s. Then, SYBR RT-qPCR

Kits (TaKaRa, Dalian, China) were used to determine the miRNA expression levels. RT-qPCR was performed at 95°C for 1 min, followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. U6 was used as an internal control [21].

Relative expression levels of all genes and miRNAs were calculated using the  $2^{-\Delta\Delta CT}$  method [53]. All primers used for RT-qPCR are listed in Table S3 (see online supplementary material).

### Transient transformation in *Nicotiana benthamiana* leaves

To investigate the subcellular localization of PsMYB65, its coding sequences (CDS) without a termination codon were cloned into pSuper1300 harboring a green fluorescent protein (GFP) to generate the PsMYB65-GFP fusion vector. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). All primers used for subcellular localization are listed in Table S3 (see online supplementary material).

To generate miR159b-insensitized PsMYB65, the target sequence of PsmiR159b in PsMYB65, GGAGCTCCCTTCACTC (951–966 bp), was synonymously mutated to AGAACTACCAAGTCTA via overlapping PCR. Mutated PsMYB65 was named mPsMYB65. All primers used for PsMYB65 mutation are listed in Table S3 (see online supplementary material).

To determine whether PsmiR159b targeted PsMYB65, a 182 bp PsmiR159b stem-loop sequence (File S51, see online supplementary material), the CDS sequences of PsMYB65 and mPsMYB65 were ligated into the pBI121 vector (harboring a GUS tag) to generate overexpression (OE) vectors 35S::PsmiR159b, 35S::PsMYB65-GUS, and 35S::mPsMYB65-GUS. All primers used for vector construction are listed in Table S3 (see online supplementary material).

These constructed vectors were then transformed into *Agrobacterium tumefaciens* strain GV3101, which was further used to transform *N. benthamiana* leaves. Tobacco transformation was performed as previously described [22]. After infection for 3 d, GFP and DAPI fluorescence were observed via confocal microscopy (Leica SP8; Germany) at 488 and 360 nm, respectively. GUS staining and GUS protein activity analysis were performed using the method described by Jefferson *et al.* [54].

### RLM-5'-RACE

To determine the cleavage site of PsMYB65 transcripts by PsmiR159b, an RNA ligase-mediated 5'-rapid amplification of cDNA ends (RLM-5'-RACE) assay was performed using a FirstChoice RLM-RACE kit (Invitrogen, San Diego, CA, USA). First, total RNA extracted from buds of 7 DAC was ligated to the 5'-adapter using T<sub>4</sub> RNA ligase. cDNA was then synthesized using M-MLV reverse transcriptase at 42°C for 1 h. PCR product was cloned into the pMD18-T vector. Ten randomly selected recombinant single colonies were sequenced. All primers used in this assay are listed in Table S3 (see online supplementary material).

### Transformation of tree peony buds

A virus-based miRNA expression system was used for overexpressing of PsmiR159b as previously described with some modifications [22]. Dormant buds exposed to 7 DAC were used and hardly burst *in vivo* under growth conditions. Briefly, 182 bp PsmiR159b precursor (File S51, see online supplementary material) was ligated into pCVA vector to generate PsMIR159b-overexpressed vector (OE-PsmiR159b). For PsMIR159b silencing, the short tandem target mimic (STTM) of PsmiR159b (STTM159b; Figure S2, see online supplementary material) was constructed

and then ligated into pCVA vector. Then, these recombinant vectors were transformed into *A. tumefaciens* strain EHA105.

For PsMYB65 and PsCYCD silencing, VIGS was performed as previously described, with minor modifications [22]. Briefly, a specific 450 bp fragment of PsMYB65 cDNA and a 367 bp fragment of PsCYCD3;1 was introduced into pTRV2 vector (pTRV2-PsMYB65 and pTRV2-CYCD3;1), respectively. Then, they were transformed into *A. tumefaciens* strain EHA105.

*A. tumefaciens* strains harboring the recombinant plasmids (OE-PsMIR159b, STTM159b, TRV-PsCYCD3;1, and TRV2-PsMYB65), empty plasmids (pCVA and pTRV2), and companion plasmids (pCVB and pTRV1) were cultured overnight in Luria–Bertani (LB) medium with 50 mg·L<sup>-1</sup> rifampicin and 50 mg·L<sup>-1</sup> kanamycin. After centrifugation (5000 rpm for 10 min), the precipitate was re-suspended in an infection buffer (10 mM MgCl<sub>2</sub>, 100 mM acetosyringone, and 10 mM MES, pH 5.6) to an OD<sub>600</sub> of 0.8–1.0. Then, the infection buffers containing transformed *A. tumefaciens* were mixed in a 1:1 (v/v) ratio as follows: pCVA + pCVB, pCVA-PsMIR159b + pCVB, STTM159b + pCVB, TRV1 + TRV2, and TRV1 + TRV2-PsMYB65. They were left in the dark for approximately 3 h before infection.

For the transformation, sterilized buds were pre-cultured on 1/2 MS medium for 2 days prior to infection. Then, they were immersed in *A. tumefaciens* and subjected to vacuum infiltration at -0.9 kg cm<sup>-2</sup> for 5 min, and then slowly deflated. After rinsing three times with sterile distilled water, the buds were then re-cultured on 1/2 MS medium, and maintained at 20°C (16/8 h light/dark). All primers used for the transient expression of flower buds are listed in Table S3 (see online supplementary material).

To determine the morphological changes in the transformed buds, images of buds were taken every 10 days using a digital camera (Canon EOS 400D; Japan). Bud height was measured using the ImageJ2 software (<https://imagej.net/>). Heights at 0, 10, and 20 DAI were recorded as H0, H1, and H2, respectively. Relative growth of the flower buds was calculated using the following formula: (H1 or H2 - H0)/H0 × 100%. At least three biological replicates (> 10 buds per replicate) were used.

## RNA-seq analysis

Ten independent TRV2-PsMYB65 and TRV2 control buds were evaluated by RT-qPCR, and three lines with silencing efficiency >50% were also selected for RNA-seq analysis. RNA concentration and integrity were determined using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and 2100 Bioanalyzer (Agilent Technologies, USA), and 1 µg of total RNA from each sample was used for cDNA library construction. Three biological replicates were performed using an Illumina HiSeq4000 platform. Approximately 6.0 Gb of clean data was generated per sample after low-quality read filtration (Table S2, see online supplementary material). For transcript assembly and annotation, Trinity 2.6.6 and Blast were used. DEGs were filtered based on |log<sub>2</sub> (fold-change)| > 1 and adjusted *P* (q-value) < 0.05, using DESeq2 1.22.2. KEGG and Gene Ontology analyses of DEGs were performed using the 'ggplot' package.

## Yeast assays

The transcriptional activation activity of PsMYB65 was assessed as previously described [55]. The CDS sequences of PsMYB65, VP16, R2R3, and TRD were cloned into the pGBKT7 vector and transformed into Y2H yeast strain. The pBD-VP16 and pBD empty vectors served as positive and negative controls, respectively. Transformants were screened on SD/-Trp/-His, SD/-Trp/-His/3-amino-1,2,4-triazole (3-AT), and SD/-Trp/-His/X-α-Gal.

For Y1H assay, CDS of PsMYB65 was ligated into the pGADT7 vector (AD-PsMYB65), and the promoter fragments of PsCYCD3;1 were inserted into the pHIS2 vector. These constructs were then transformed into Y187 yeast strain, and 3-AT was used to suppress the background expression of the pHIS2 vector. Transformants were selected on SD/-Trp, SD/-Trp/-His and SD/-Trp/-His/-Leu media. All primers used for yeast assays are listed in Table S3 (see online supplementary material).

## Dual-luciferase (LUC) assay

In order to determine the regulatory effects of PsMYB65 on its downstream candidate gene (PsCYCD3;1), the CDS of PsMYB65 was cloned into the pGreenII0029 62-SK vector to construct the effector. The promoter fragments of PsCYCD3;1 were inserted into the pGreenII 0800-LUC vector in order to generate reporters. These effectors and reporters were co-transfected into *N. benthamiana* leaves. The dual-LUC assay was performed as described previously [22]. All primers used are listed in Table S3 (see online supplementary material).

## Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described by Zhang et al. [22]. Briefly, The CDS of PsMYB65 was ligated into the pGEX-4 T-2 vector and then transformed into *Escherichia coli* strain BL21. The fusion protein was induced by adding isopropylthio-β-galactoside (0.5 mM) overnight at 16°C. The recombinant protein was then purified using a GST-Trap column (GE Healthcare, Pittsburgh, PA, USA). EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. All probes used in EMSA are listed in Table S3 (see online supplementary material).

## Analysis of cell number and size

ImageJ was used to analyse cell number and size. Briefly, sections from TRV2-PsMYB65, TRV2 control, pCVA, and OE-PsMIR159 transgenic buds after 20 DAI were imaged by Leica TCS-SP2 microscope. Then, three sections from each of three transgenic buds were used to calculate cell number and size.

## Statistical analyses

Student's t-test was used to compare two groups, and one-way analysis of variance was used for multi-group comparisons. All statistical analyses were conducted using GraphPad Prism 7 (CA, USA).

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## Author contributions

TZ, XW, and YY: Writing—original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. SZ and CL: Writing—original draft, Formal analysis, Investigation, Data curation. YZ: Writing—review & editing, Validation, Methodology, Conceptualization. SG: Writing—review & editing, Supervision, Project administration, Conceptualization.



## Data availability

The RNA-seq data have been deposited National Center for Biotechnology Information (NCBI) under accession number PRJNA813336. Sequence data used in this article can be found in the Arabidopsis Genome TAIR database or GenBank database under the following accession numbers: PsCYCD1;1 (OP142277), PsCYCD3;1 (OP142273), PsCYCD3;2 (OP142275), PsCYCD3;3 (OP142274), PsCYCD6;1 (OP142276), AtCYCD3 (AT4G34160), PtCYCD3;2 (CAN88857), AtCYCD3;3 (AT3G50070), AtCYCD3;2 (AT5G67260), PtCYCD6;1 (CAN88865), AtCYCD1;1 (AT1G70210), AtCYCD4;1 (AT5G65420), AtCYCD7;1 (AT5G02110), AtCYCD2;1 (AT2G22490), AtMYB97 (AT4G26930), AtMYB120 (AT5G55020), AtMYB101 (AT2G32460), PsMYB65 (MT211968), AtMYB65 (AT3G11440), AtMYB33 (AT5G06100), AtMYB104 (AT2G26950), AtMYB81 (AT2G26960).

## Conflict of interest statement

The authors declare no competing interests.

## Supplementary data

Supplementary data is available at Horticulture Research online.

## References

- Busov VB. Plant development: dual roles of poplar SVL in vegetative bud dormancy. *Curr Biol.* 2019;**29**:R68–70
- Yang Q, Gao Y, Wu X. et al. Bud endodormancy in deciduous fruit trees: advances and prospects. *Hortic Res.* 2021;**8**:139
- Maurya JP, Bhalerao RP. Photoperiod- and temperature-mediated control of growth cessation and dormancy in trees: a molecular perspective. *Ann Bot.* 2017;**120**:351–60
- Singh RK, Svystun T, AlDahmash B. et al. Photoperiod- and temperature-mediated control of phenology in trees – a molecular perspective. *New Phytol.* 2017;**213**:511–24
- Böhlenius H, Huang T, Charbonnel-Campaa L. et al. CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science.* 2006;**312**:1040–3
- Ruttink T, Arend M, Morreel K. et al. A molecular timetable for apical bud formation and dormancy induction in poplar. *Plant Cell.* 2007;**19**:2370–90
- Tylewicz S, Tsuji H, Miskolczi P. et al. Dual role of tree florigen activation complex component FD in photoperiodic growth control and adaptive response pathways. *Proc Natl Acad Sci USA.* 2015;**112**:3140–5
- Karlberg A, Bako L, Bhalerao RP. Short day-mediated cessation of growth requires the downregulation of AINTEGUMENTA-LIKE1 transcription factor in hybrid aspen. *PLoS Genet.* 2011;**7**:e1002361
- Tylewicz S, Petterle A, Marttila S. et al. Photoperiodic control of seasonal growth is mediated by ABA acting on cell-cell communication. *Science.* 2018;**360**:212–5
- Zheng C, Halaly T, Acheampong AK. et al. Abscisic acid (ABA) regulates grape bud dormancy, and dormancy release stimuli may act through modification of ABA metabolism. *J Exp Bot.* 2015;**66**:1527–42
- Zhang T, Yuan Y, Zhan Y. et al. Metabolomics analysis reveals Embden Meyerhof Parnas pathway activation and flavonoids accumulation during dormancy transition in tree peony. *BMC Plant Biol.* 2020;**20**:1–17
- Singh RK, Miskolczi P, Maurya JP. et al. A tree ortholog of SHORT VEGETATIVE PHASE floral repressor mediates photoperiodic control of bud dormancy. *Curr Biol.* 2019;**29**:128–133.e2
- Azeez A, Zhao YC, Singh RK. et al. EARLY BUD-BREAK 1 and EARLY BUD-BREAK 3 control resumption of poplar growth after winter dormancy. *Nat Commun.* 2021;**12**:1123
- Singh RK, Maurya JP, Azeez A. et al. A genetic network mediating the control of bud break in hybrid aspen. *Nat Commun.* 2018;**9**:4173
- Zhang Y, Yuan Y, Liu Z. et al. GA<sub>3</sub> is superior to GA<sub>4</sub> in promoting bud endodormancy release in tree peony (*Paeonia suffruticosa*) and their potential working mechanism. *BMC Plant Biol.* 2021;**65**:323–32
- Zheng C, Kwame Acheampong A, Shi Z. et al. Distinct gibberellin functions during and after grapevine bud dormancy release. *J Exp Bot.* 2018;**69**:1635–48
- Liu Q, Wu K, Harberd NP. et al. Green revolution DELLAs: from translational reinitiation to future sustainable agriculture. *Mol Plant.* 2021;**14**:547–9
- Gao L, Niu D, Chi T. et al. PsRGL1 negatively regulates chilling- and gibberellin-induced dormancy release by PsF-box1-mediated targeting for proteolytic degradation in tree peony. *Hortic Res.* 2023;**10**:uhad044
- Ding Q, Zeng J, He XQ. Deep sequencing on a genome-wide scale reveals diverse stage-specific microRNAs in cambium during dormancy-release induced by chilling in poplar. *BMC Plant Biol.* 2014;**14**:267
- Liu J, Guo X, Zhai T. et al. Genome-wide identification and characterization of microRNAs responding to ABA and GA in maize embryos during seed germination. *Plant Biol.* 2020;**22**:949–57
- Zhang Y, Wang Y, Gao X. et al. Identification and characterization of microRNAs in tree peony during chilling induced dormancy release by high-throughput sequencing. *Sci Rep.* 2018;**8**:1–14
- Zhang Y, Gao L, Wang Y. et al. Dual functions of PsmiR172b-PsTOE3 module in dormancy release and flowering in tree peony (*Paeonia suffruticosa*). *Hortic Res.* 2023;**10**:uhad033
- Yu J, Bennett D, Dardick C. et al. Genome-wide changes of regulatory non-coding RNAs reveal pollen development initiated at ecodormancy in peach. *Front Mol Biosci.* 2021;**8**:612881
- Guo C, Xu Y, Shi M. et al. Repression of miR156 by miR159 regulates the timing of the juvenile-to-adult transition in Arabidopsis. *Plant Cell.* 2017;**29**:1293–304
- Gocal GFW, Sheldon CC, Gubler F. et al. GAMBY-like genes, flowering, and gibberellin signaling in Arabidopsis. *Plant Physiol.* 2001;**127**:1682–93
- Millar AA, Gubler F. The Arabidopsis GAMBY-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell.* 2005;**17**:705–11
- Ko SS, Li MJ, Ho YC. et al. Rice transcription factor GAMBY modulates bHLH142 and is homeostatically regulated by TDR during anther tapetal and pollen development. *J Exp Bot.* 2021;**72**:4888–903
- Gai S, Zhang Y, Liu C. et al. Transcript profiling of *Paeonia ostii* during artificial chilling induced dormancy release identifies activation of GA pathway and carbohydrate metabolism. *PLoS One.* 2013;**8**:e55297
- Huang X, Zhu W, Dai S. et al. The involvement of mitochondrial phosphate transporter in accelerating bud dormancy release during chilling treatment of tree peony (*Paeonia suffruticosa*). *Planta.* 2008;**228**:545–52



30. Yan J, Gu Y, Jia X. et al. Effective small RNA destruction by the expression of a short tandem target mimic in Arabidopsis. *Plant Cell*. 2012;**24**:415–27
31. Wu HJ, Ma YK, Chen T. et al. PsRobot: a web-based plant small RNA meta-analysis toolbox. *Nucleic Acids Res*. 2012;**40**:W22–8
32. Lescot M, Déhais P, Thijs G. et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res*. 2002;**30**:325–7
33. Achard P, Herr A, Baulcombe DC. et al. Modulation of floral development by a gibberellin-regulated microRNA. *Development*. 2004;**131**:3357–65
34. da Silva EM, Silva GFF, Bidoia DB. et al. microRNA159-targeted SlGAMYB transcription factors are required for fruit set in tomato. *Plant J*. 2017;**92**:95–109
35. Gao J, Chen H, Yang H. et al. A brassinosteroid responsive miRNA-target module regulates gibberellin biosynthesis and plant development. *New Phytol*. 2018;**220**:488–501
36. Zheng Z, Wang N, Jalajakumari M. et al. MiR159 represses a constitutive pathogen defense response in tobacco. *Plant Physiol*. 2020;**182**:2182–98
37. Mathiason K, He D, Grimplet J. et al. Transcript profiling in *Vitis riparia* during chilling requirement fulfillment reveals coordination of gene expression patterns with optimized bud break. *Funct Integr Genomics*. 2009;**9**:81–96
38. Dong QK, Hu BB, Zhang C. microRNAs and their roles in plant development. *Front Plant Sci*. 2022;**13**:824240
39. Azeez A, Miskolczi P, Tylewicz S. et al. A tree ortholog of APETALA1 mediates photoperiodic control of seasonal growth. *Curr Biol*. 2014;**24**:717–24
40. Hu Z, Shen X, Xiang X. et al. Evolution of MIR159/319 genes in *Brassica campestris* and their function in pollen development. *Plant Mol Biol*. 2019;**101**:537–50
41. Wang C, Jogaiah S, Zhang W. et al. Spatio-temporal expression of miRNA159 family members and their GAMYB target gene during the modulation of gibberellin-induced grapevine parthenocarpy. *J Exp Bot*. 2018;**69**:3639–50
42. Jing WK, Gong FF, Liu GQ. et al. Petal size is controlled by the MYB73/TPL/HDA19-miR159-CKX6 module regulating cytokinin catabolism in *Rosa hybrida*. *Nat Commun*. 2023;**14**:7106
43. Wojtania A, Markiewicz M, Waligórski P. Regulation of the bud dormancy development and release in micropropagated rhubarb 'Malinowy'. *Int J Mol Sci*. 2022;**23**:1480
44. Shimotohno A, Aki SS, Takahashi N. et al. Regulation of the plant cell cycle in response to hormones and the environment. *Annu Rev Plant Biol*. 2021;**72**:273–96
45. Vandepoele K, Raes J, De Veylder L. et al. Genome-wide analysis of core cell cycle genes in Arabidopsis. *Plant Cell*. 2002;**14**:903–16
46. Achard P, Gusti A, Cheminant S. et al. Gibberellin signaling controls cell proliferation rate in Arabidopsis. *Curr Biol*. 2009;**19**:1188–93
47. Xue T, Liu Z, Dai X. et al. Primary root growth in Arabidopsis thaliana is inhibited by the miR159 mediated repression of MYB33, MYB65 and MYB101. *Plant Sci*. 2017;**262**:182–9
48. Guan C, Xue Y, Jiang P. et al. Overexpression of ptoycyd3;3 promotes growth and causes leaf wrinkle and branch appearance in Populus. *Int J Mol Sci*. 2021;**22**:1–21
49. Zhang Y, Yu D, Liu C. et al. Dynamic of carbohydrate metabolism and the related genes highlights PPP pathway activation during chilling induced bud dormancy release in tree peony (*Paeonia suffruticosa*). *Sci Hortic (Amsterdam)*. 2018;**242**:36–43
50. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;**33**:1870–4
51. Chen C, Chen H, Zhang Y. et al. TBtools: an integrative toolkit developed for interactive analyses of big biological data. *Mol Plant*. 2020;**13**:1194–202
52. Liu W, Xie Y, Ma J. et al. IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics*. 2015;**31**:3359–61
53. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔCT</sup> method. *Methods*. 2001;**25**:402–8
54. Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 1987;**6**:3901–7
55. Chen J, Li Y, Li Y. et al. Auxin response factor 18–histone deacetylase 6 module regulates floral organ identity in rose (*Rosa hybrida*). *Plant Physiol*. 2021;**186**:1074–87