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Research article

Morphological, anatomical and DNA methylation changes of tree peony buds during chilling induced dormancy release

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ABSTRACT

Bud endodormancy in tree peony is a growth cessation-like state, and sufficient chilling perception is necessary to break it. In this study, 120 plants were subjected to 0–4 °C climate chamber for 0–28 d with a weekly interval, morphology and structure changes of buds were studied with a scanning electron microscope (SEM) and paraffin sections during the dormancy process. Dormancy status was evaluated after being transferred to greenhouse for 30 d. Results showed that the diameter of the buds gradually expanded, along with continuous elongation of sepals, petals, stamens and carpels in the chilling accumulation process. Notably, dormancy release was marked with the establishment of xylem vessels in lateral vein of the petal. Meanwhile, DNA methylation was detected by HPLC and immunochemical technology, aimed to illuminate the role of DNA methylation in the dormancy release, we found that 5 mC level fell from 39.4% to 24.2% after exposed to 28 d chilling. These results were consistent with the immunochemical analysis, and inversely related to the sprouting rate after being moved to greenhouse for 30 d. Exogenous application of 5azaC (5-azacytidine) decreased DNA methylation level, accompanied by an improved bud sprouting capacity, while the effect of SAM (S-adenosylmethionine) was the opposite. In summary, prolonged chilling was accompanied by further differentiation and development of the compound bud, which resulted in DNA hypomethylation and promoted dormancy release in tree peony.

1. Introduction

Dormancy is an adaptive response of plants to environmental variations, which includes several ways such as seed dormancy, bud dormancy which including endodormancy, ecodormancy and paradormancy, and so on. Ecodormancy refers to that growth is arrested by environmental conditions but resumes in favorable conditions. Paradormancy, also known as apical dominance, is the suppression of lateral bud growth by the actively growing portion. Endodormancy, caused by plant endogenous factors, requires a prolonged chilling duration for regrowth even in growth-promoting conditions (Shim et al., 2014). Bud endodormancy generally exists in deciduous trees (Singh et al., 2016), and is discussed in this paper. Additionally, endodormancy release, which is a prerequisite for forcing culture, is often referred to the transition from endo to ecodormancy (Ding et al., 2016; Singh et al., 2016). According to the organs they will form, buds are divided into leaf buds, floral buds and compound buds. The flower bud of deciduous plants usually completed morphological differentiation before the winter, and the primordium of the pistil and stamen was differentiated. Xie et al. (2018) revealed the rebuilding of vascular connections between leaf buds and canes during bud break in grape, and thought that the xylem development of vascular bundles played an important role in endodormancy release. After the dormancy was released in the following spring, the flower bud continued its stamen differentiation, and then bloomed. Some plants that bloom in early spring, such as the apricot, may begin its anther development of stamens before winter, and the pistil forms a stigma, style, and ovary (Song et al., 2017). Tree peony (*Paeonia suffruticosa*) is a famous

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Abbreviations: **5azaC**, 5-azacytidine; **CTAB**, Cetyl Trimethyl Ammonium Bromide; **DAM**, DORMANCY ASSOCIATED MADS-box; **DAPI**, 4′, 6-diamidino-2-phenylindole; **HPLC**, High Performance Liquid Chromatography; **5 mC**, 5-methylcytosine; **PBS**, Phosphate- Buffered Saline buffer; **SAM**, S- adenosylmethionine; **SEM**, scanning electron microscope

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ornamental plant cultivated worldwide. The bud of tree peony is a typical compound bud, containing both young leaves and one flower. Once formed, a bud may stay in dormant condition in winter, then the bud grows and forms a large flower and some pinnately compound leaves in the next spring. However, the process of flower bud differentiation and development has not been reported in detail.

Forcing culture is a main part of the tree peony industry, but dormancy is a major obstacle to its forcing culture. In recent years, a lot of researches have been focused on the dormancy release of tree peony. The studies showed that a sufficient chilling duration is required to release the bud from dormancy. Temperature with a range of 0–10 °C was effective, with 0–4 °C frequently applied (Huang et al., 2008). We found the requirement of the chilling duration period varied among cultivars, for instance, an 18 d chilling duration with a range of 0–4 °C was effective to break the dormancy of the ‘Fengdan’ cultivar (Gai et al., 2013), but 21 d was required for the ‘Luhehong’ cultivar (Huang et al., 2008). During chilling duration process, anatomical changes continued to occur in the floral bud despite in a dormant status (Huang et al., 2008). However, past studies lacked histological and cytological experiments on the buds during chilling-induced dormancy release in tree peony. Therefore, great value exists in the comprehension of developmental changes during dormancy, and in establishing the mechanism and technology of dormancy regulation in tree peony.

The mechanism regulating dormancy induction, maintenance and release may provide a theoretical basis for solving critical problems in agriculture, especially for forcing culture in some fruit trees and flowers of temperate regions. Recent genomic and transcriptomic-based studies revealed aspects of the molecular mechanisms in dormancy regulation. *DAM* (*DORMANCY ASSOCIATED MADS-box*) genes might be related to dormancy induction in pear, poplar, apricot, etc (Rohde et al., 2011; Sasaki et al., 2011; Niu et al., 2016), while the CO/FT regulatory module controlled seasonal growth cessation in poplar (Böhlenius et al., 2006). In poplar, an AP2 type transcript factor EBB1 (EARLY BUD-BREAK 1) accelerated dormancy release (Yordanov et al., 2014), while CEN/TFL1 (CENTRORADIALIS/TERMINAL FLOWER 1) was a negative regulator of dormancy release (Rozi et al., 2010). However, the mechanism of dormancy release in most perennial plants was still poorly understood, although transcriptomics analysis indicated that epigenetic regulation was involved in the dormancy of aspen, raspberry and poplar (Druart et al., 2007; Mazzitelli et al., 2007; Ruttink et al., 2007; Shim et al., 2014; Conde et al., 2019). Recently, an inverse tendency of acetylation in histone H4 and variation of 5 mC was found between bud set and bud burst in polar and potato (Law et al., 2004; Conde et al., 2013). H3 histone trimethylation was also appears to be involved in dormancy regulation, as H3K27me3 modifies the dormancy regulators in peach (De la Fuente et al., 2015). While genes, proteins and transcripts had been implicated in chilling induced dormancy release in tree peony (Huang et al., 2008; Gai et al., 2012; Zhang et al., 2015), their regulation was less well understood. Therefore, epigenetic studies of dormancy in tree peony would further explain the relationship between DNA methylation and dormancy release.

To illuminate the role of DNA methylation, 5-methylcytosine (5 mC) levels were artificially regulated and analyzed in dormant bud of tree peony. 5-azacytidine (5azaC) is a demethylating agent that lowers 5 mC levels leading to an early flowering phenotype in *Arabidopsis* and wheat (Burn et al., 1993; Brock et al., 1994). On the contrary, S-adenosylmethionine (SAM), a DNA methylating agent, can stimulate DNA methyltransferase reactions and trigger hypermethylation (Chiang et al., 1996). In this paper, we described the histological and cytological characteristics of tree peony buds during chilling induced dormancy release, and we intended to illuminate the role of DNA methylation in the dormant bud.

2. Materials and methods

Four-year-old tree peony plants (*Paeonia suffruticosa* cv. ‘Luhehong’) were selected. They each had 8-12 healthy buds, and came from the same mother plant with grafting, thus had high genetic uniformity. Totally, 201 plants were planted in pots and moved to a dark, 0–4 °C room for 7, 14, 21 and 28 d, then they were moved to the greenhouse (18–22 °C, 8-h-light/16-h-dark cycle). The control group was placed in the greenhouse without chilling treatment. Buds were sampled from three plants for each treatment and set in triplicate, and three buds were harvested per plant for three technical replicates. The buds of different treatments were immediately frozen in liquid nitrogen and stored at −80 °C until use, except for FAA fixation. Additionally, the sampled plants were excluded from the subsequent sprouting observation. Buds sprouting rates for different treatments were calculated after one month in the greenhouse with five plants per treatment in triplicate. The status that bud sprouting rate doesn't increase significantly even if more chilling duration exposure was defined as dormancy release. Flow diagram of experimental design was list in Supplementary Fig. S1.

2.1. Microscope observation

The buds treated with 0, 7, 14, 21 and 28 d chilling were separately fixed in FAA (Formalin, acetic acid and alcohol). A scanning electron microscope (SEM, JSM-7500F) was used to observe the diameter, the features of bracts, sepals, petals, stamens and pistils of the buds. Paraffin sections were cut at 8–10 μm thickness. Image J software was used to measure the length of each structure. Ferroalumen hematoxylin staining was used to observe the change of cell development, and safranine-fast green staining was used for vascular bundle observation. After sealed with neutral balsam, the sections were observed under a microscope (OLYMPUS BH-2).

2.2. 5azaC and SAM treatments

5azaC and S- adenosylmethionine (SAM) were purchased from Sigma-Aldrich. For plants with 0, 7, 14 and 21 d chilling treatments, 50 μmol L^{-1} 5azaC or 10 μmol L^{-1} SAM was applied by injecting when being transferred to the greenhouse, with double-distilled water as the control. The 5 mC level was measured 5 d after treatment, and the sampled buds were stored at −80 °C before use.

2.3. Immunohistochemical analysis

Immunochemical analysis was performed according to Valledor et al. (2010) with some modifications. The buds were fixed with 4% paraformaldehyde in 0.1 mol L^{-1} Phosphate-Buffered Saline buffer (PBS, pH 7.3) overnight at 4 °C, and subsequently stored in 0.1% paraformaldehyde. After dehydration in an ascending ethanol series and paraffin embedding, samples were sectioned at 5–7 μm and deparaffined in xylol. The prepared sections were incubated in anti-5mC mouse antibody (Eurogentec, BI-MECY-0100) at a 50-fold dilution, after being blocked with 5% bovine serum albumin in PBS for 10 min. Alexa Fluor 488-labelled anti-mouse polyclonal antibody (Molecular Probes, Cat. no. A-11001) with a 25-folds dilution was the secondary antibody for the 5 mC detection. DAPI (4’, 6-diamidino-2-phenylindole, Solarbio) was used as a counterstain and the negative controls were obtained by replacing the primary antibody with PBS. Fluorescence was observed using a confocal microscope (Leica TCS SP5 II). Image J was used to quantify the fluorescence levels of DAPI and anti-5mC.

2.4. DNA methylation evaluation

Genomic DNA was extracted from 100 mg frozen buds using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method as described previously (Gai et al., 2012). Residual RNA was degraded with 10 U·mL^{-1} RNase A (Qiagen) at 37 °C for 1 h. The DNA was precipitated, dissolved in ddH_2O to a concentration of 1 μg μL^{-1} and stored at 4 °C.

DNA hydrolysis was performed as described by Hasbún et al. (2008). About 5 μg DNA was denatured by heat at 100 °C for 2 min and chilled rapidly on ice. Subsequently, the denatured DNA was subjected to nuclease P1 (Sigma) and alkaline phosphatase (Sigma) at 37 °C. Hydrolyzed solutions were centrifuged for 20 min at 15 000 × *g*, the supernatant was dialyzed on 0.025 μm filter paper (Millipore, Bedford, MA), and then injected to an Eclipse XDB-C18 column (5 μm, 150 × 4.6 nm, Agilent) for HPLC analysis (Agilent 1100 Series). The isocratic mobile phase was KH_2PO_3/methanol (98: 2, v/v) for about 20 min. Levels of cytosine (C) and 5 mC were assessed using co-migration under the same chromatograph conditions according to the commercial standards from Sigma. The DNA methylation level was calculated as: mC/(C + mC) × 100%.

2.5. Observation of bud sprouting

After moved to the greenhouse, the bud sprouting of plants with 0, 7, 14, 21 and 28 d chilling treatments were observed after one month (Gai et al., 2013). Those with chilling and 5azaC treatment or chilling and SAM treatment were observed every 2–5 d until one month, respectively.

2.6. Statistical analysis

A randomized design with three replicates was used and subjected to analysis of variance (ANOVA) using IBM SPSS Statistics software 22.0. Differences were considered significant at the $P < 0.05$ level.

3. Results

Tree peonies naturally form buds in summer, fall leaves in late autumn and enter dormant period concomitantly. They have compound buds for adult plants, that is, there are flower buds and young leaves in the whole buds. Their compound buds gradually differentiate and develop in winter. The next February or March, compound buds sprout, and the flower buds are revealed. They expand progressively and bloom in April naturally. We can see that an alabastrum of tree peony 'Luhehong' is composed of bracts (mostly 5), sepals (mostly 3), petals (pink, many), stamens (many) and pistils (mostly five) (Fig. 1).

3.1. Morphological changes of flower buds of tree peony during chilling duration

During chilling treatments, the flower buds gradually expanded (Fig. 1), and significant difference was observed (Table 1). Compared to buds without chilling treatment, the average diameter of buds enduring 7, 14, 21 and 28 d chilling increased by 0.24, 0.69, 0.92 and 1.37 mm, respectively. A similar tendency was also observed in sepals, petals and carpels, respectively. The most obvious change was observed in the length of carpels, as lengths increased by 73.15, 311.68, 454.45 and 735.31 μm, respectively.

3.2. Stamens and carpels variation during chilling duration

In a non-chilling treated bud, many oval small stamen primordia and five separated carpels could be seen. With the extension of chilling duration, stamen primordia gradually became longer and larger. They turned into the four prism-like anthers after 21 d chilling treatment, and from the cross section view, the sporogonium began to develop, and no meiosis phase was observed. During the chilling treatment, the top of carpel gradually formed protrusions, which would develop to the stigma in the future (Fig. 2).

3.3. Variation of bracts, sepals and petals during chilling duration

Low temperature treatment promoted epidermis growth and development. At the beginning of dormancy (0–7 d), the epidermal cells of bract, sepal and petal were irregularly shaped with few cuticles and stomata. Epidermal cells gradually matured with more regular shapes, a thicker cuticle, and increased stomata frequency along with chilling duration (Fig. 3).

The bracts, sepals and petals of tree peony buds were similar in anatomical structure with upper and lower epidermis, parenchyma and vascular bundles (Fig. 4). Prior to chilling, parenchyma cells in bracts, sepals and petals had thick cytoplasm and small intercellular space. After 21 d chilling treatment, the parenchyma cells became oval or nearly circular, and they had thin cytoplasm and big intercellular space. As the outermost layer of flower buds, the bract differentiated early, and the main vein and lateral veins had differentiated vascular bundles at the beginning of dormancy. Sepal differentiated later than bracts, as vascular bundles were only found in the main vein prior to chilling. Differentiation of vascular bundles was the latest in petal, which were rare prior to chilling. Vascular bundles increased after chilling accumulation, and they also differentiated in the lateral veins of petals after 21 d of chilling (Fig. 4).

3.4. DNA methylation level and sprouting rate

DNA methylation levels were examined in buds throughout the chilling period. The sprouting rate was monitored 30 d after being moved to greenhouse (Fig. 5). The results showed that slight increase in methylation occurred at 7 d chilling, but afterwards significantly declined with an increased chilling duration. Sprouting rate increased, but no significant difference was found between 21 and 28 d, which indicated dormancy release at 21 d chilling exposure. Thus, there was an inverse relationship between the methylation level and flower buds sprouting rate, indicating that a prolonged chilling treatment reduced DNA methylation level.

3.5. Intensity of 5 mC fluorescence in dormant buds

As a relatively well-developed tissue, young leaves in dormant buds were used to detect 5 mC fluorescence in nuclei during dormancy release. In general, the fluorescence was evenly distributed. The average fluorescence intensity indicated that 5 mC increased at 7 d, but continuously decreased thereafter (Fig. 6 and Fig. 7), consistent with the HPLC result (Fig. 5).

3.6. Effect of 5azaC and SAM on DNA methylation level and dormancy release

As shown in Fig. 8, 5azaC treatment efficiently reduced DNA methylation level, and significantly promoted bud sprouting rates from 13 to 23 d after plants being moved to greenhouse, and no significant difference was found after 25 d (Fig. 8). The results indicated that application of 5azaC resulted in DNA hypomethylation and promoted bud sprouting, an index parameter of dormancy release. However, SAM treatment dramatically reduced bud sprouting percentages after 16 d. Thus, DNA methylation level was inversely corelated with bud sprouting rate, and hypomethylation promoting dormancy release in tree peony.

4. Discussion

4.1. The anatomical changes of the tree peony mixed bud

Previous studies indicated that dormancy regulation mechanisms were different between vegetative buds and flower buds (Walker, 1970). Vegetative buds usually complete differentiation at the

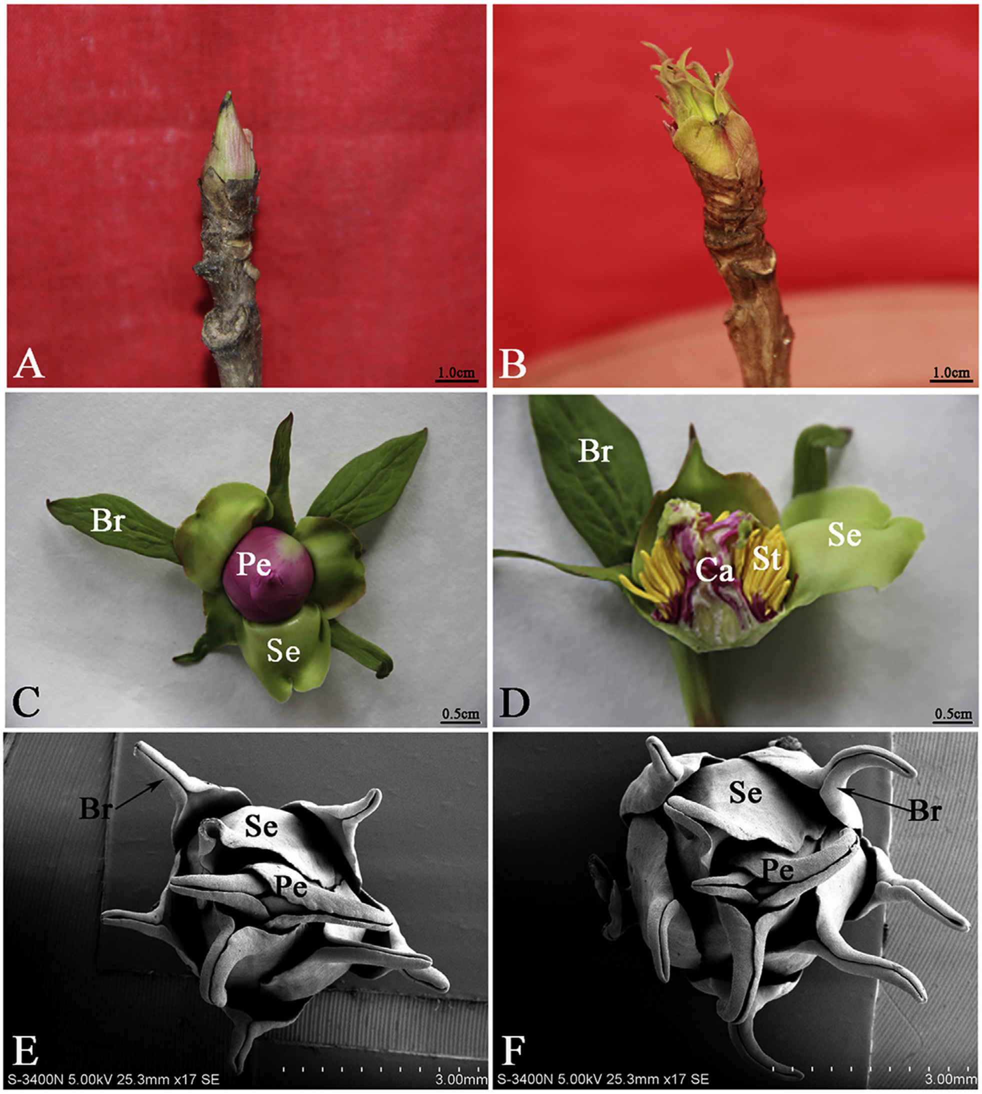

Fig. 1. Flower bud of *Paeonia suffruticosa* cv. 'Luhehong' and morphological structure under SEM. (A) A compound bud in dormant period. (B) A sprouting compound bud. (C) Surface view of an alabastrum. (D) Vertical section of an alabastrum (with petals removed), showing stamens and carpel. (E) SEM observation of 0 d chilling treated bud. (F) SEM observation of 28 d chilling treated bud. Br. Bract; Se. Sepal; Pe. Petal; St. Stamen; Ca: Carpel.

beginning of dormancy in some tree species (Fonti et al., 2007; Begum et al., 2008). Conversely, cell division, enlargement, and differentiation were found in androecium and gynoecium of flower buds during the whole dormant period (Luna et al., 1991). However, little attention had been directed at the anatomical changes of mixed buds of woody plants during dormancy release. In this study, the anatomical changes that occur in tree peony buds during dormancy release were assessed using light and scanning electron microscope. Morphologically, all components of the flower had differentiated completely prior to dormancy. After chilling, the diameter of buds and their sepals, petals and carpels increased continuously (Fig. 1 and Table 1), indicating the ongoing development of compound buds during dormancy release. In apricot (*Armeniaca vulgaris*), dormancy release marked a boundary between the development of sporogenous tissue and the occurrence of meiosis (Julian et al., 2011). Tree peony usually blooms about a month later than apricot in spring. We found that the stamens in their flower buds had not yet entered a period of differentiation, and they were cylindrical when peonies entered dormancy in winter (Fig. 2). But as the chilling treatment went on, the stamens began to differentiate, indicating that low temperature could promote the differentiation of the stamens. The anther entered a four prism-like stage with 21 d chilling, which indicated the need for a longer chilling and differentiation period in tree peony than in apricot. Additionally, meiosis was not detected both for the male and female even after the buds entering ecodormancy

Table 1
Morphological changes of flower buds of *Paeonia suffruticosa* cv. 'Luhehong'.

Size	0 d	7 d	14 d	21 d	28 d
Diameter of bud (mm)	3.23 ± 0.01a	3.47 ± 0.04b	3.92 ± 0.11c	4.15 ± 0.21d	4.60 ± 0.31e
Length of sepal (mm)	3.75 ± 0.02a	4.14 ± 0.05b	4.48 ± 0.11c	4.68 ± 0.31d	5.02 ± 0.33e
Length of petal (mm)	2.50 ± 0.03a	2.74 ± 0.08b	3.31 ± 0.10c	3.67 ± 0.12d	4.02 ± 0.21e
Length of carpel (μm)	423.46 ± 2.36a	496.61 ± 3.64b	735.14 ± 5.01c	877.91 ± 7.52d	1158.77 ± 7.60e

Note: Different letters a, b, c, d and e represent significant difference ($P < 0.05$).

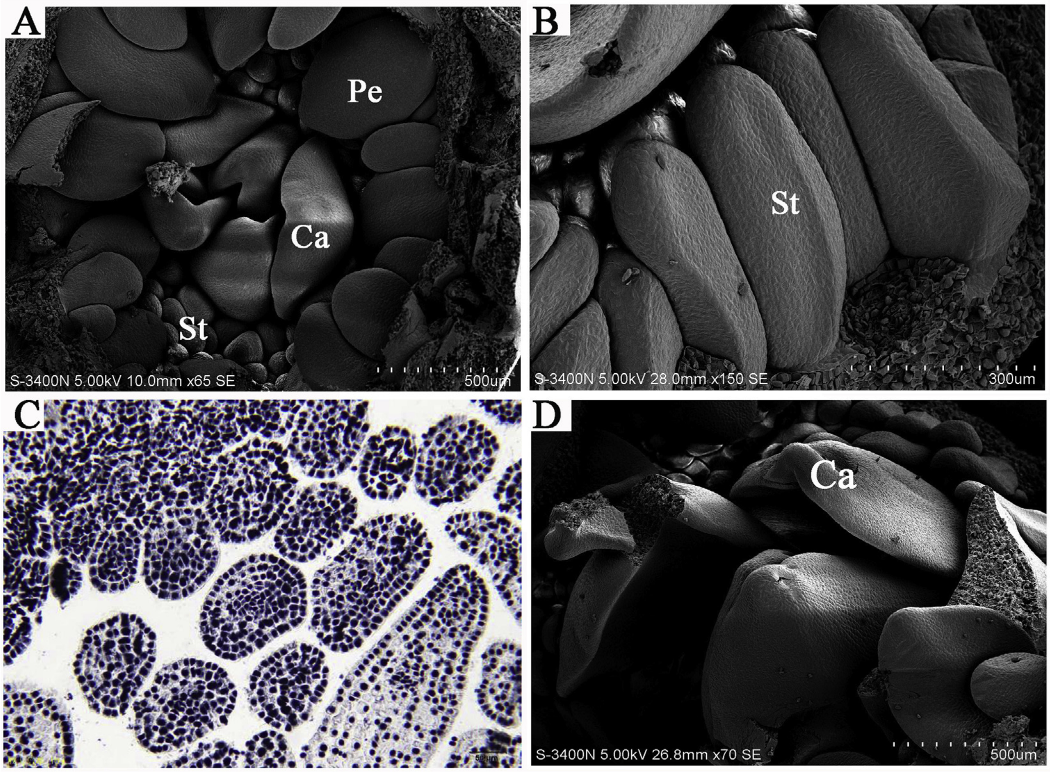

Fig. 2. Microscope observation of petals, stamens and carpels in flower buds of 'Luhehong' during chilling treatments. (A, B and D) Under SEM. (A) Non-chilling bud, showing petals, stamens and carpels. (B, D) 21 d chilling treatment, showing four prism-like stamens and growing stigma and style, respectively. (C) Cross section view of stamen endured 21 d chilling, showing the structure of young anthers. Pe. Petal; St. Stamen; Ca. Carpel.

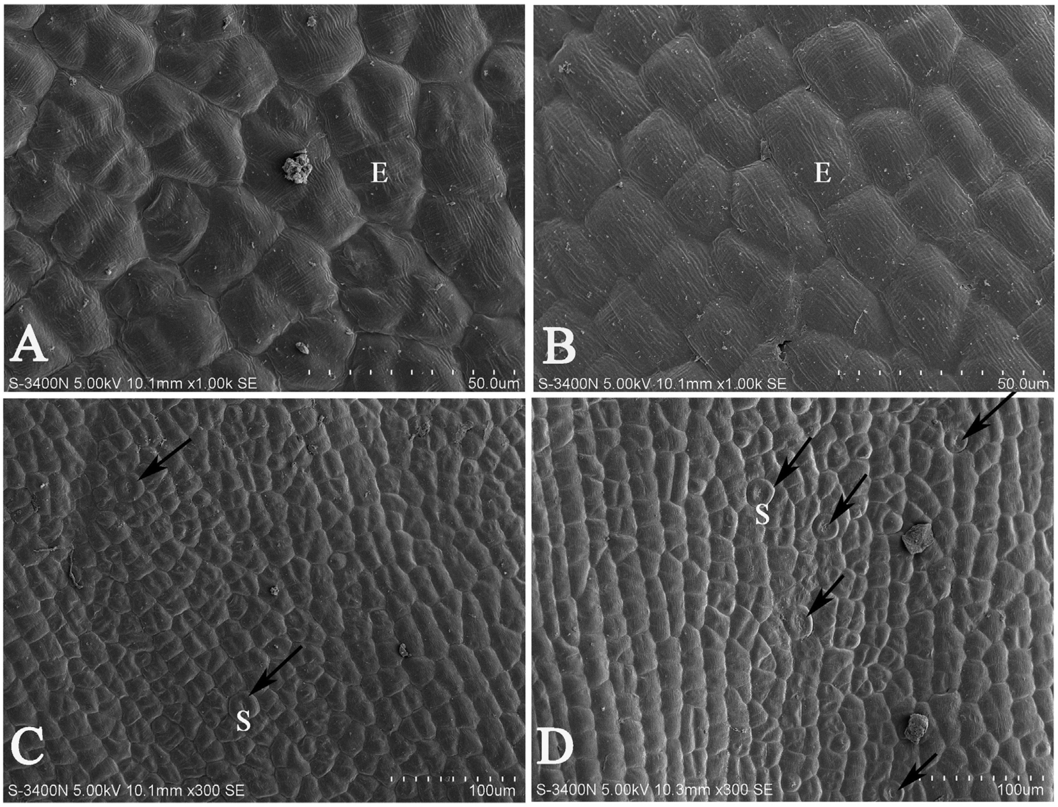

Fig. 3. Morphological structure of epidermis on sepal in flower buds of 'Luhehong' during chilling treatments under SEM. (A, C) Sepals with non-chilling treatment. (B) Sepals with 21 d chilling treatment, showing regular epidermal cells and their thick cuticle. (D) Sepals with 21 d chilling treatment, arrows show stomata. E. Epidermis; S. Stomata.

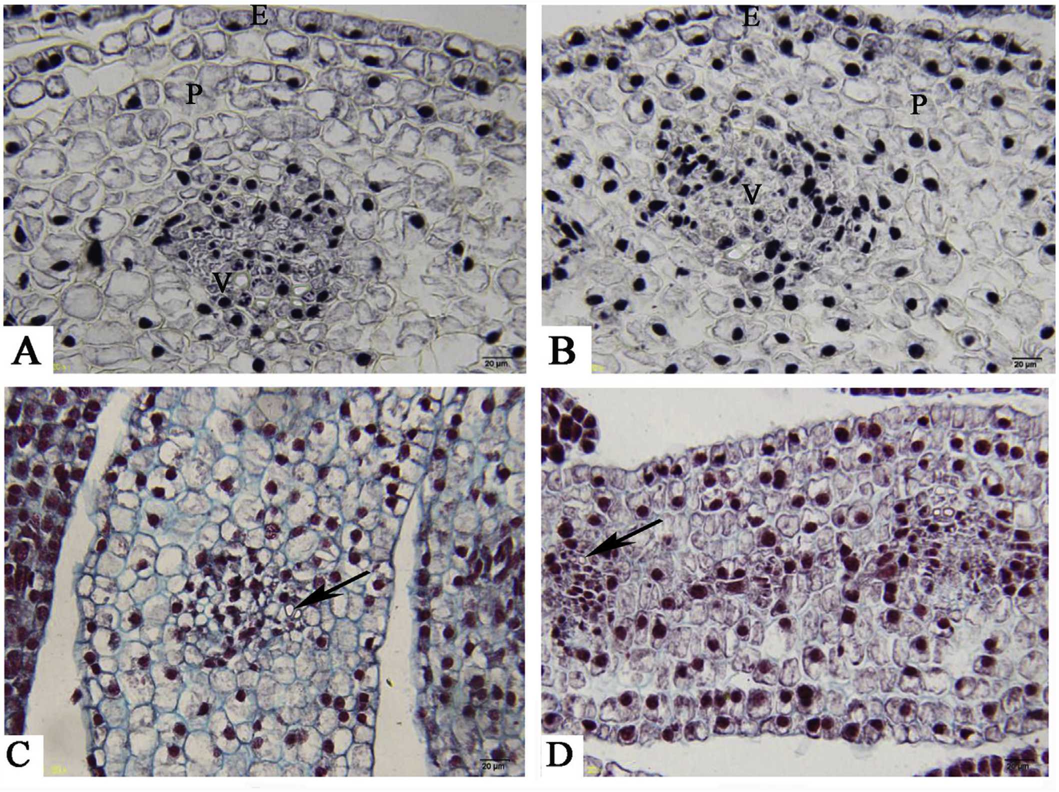

Fig. 4. Anatomical structure of sepals and petals in flower buds of 'Luhehong' during chilling treatments. (A and B) Cross section view of sepals, staining by ferroalumen hematoxylin. (C and D) Cross section view of petals, staining by safranine-fast green. (A and C) 0 d chilling. (B) 21 d chilling treatment, showing parenchyma cells with obvious cellular space. (D) 21 d chilling treatment, showing vascular bundles. Arrows show the xylem vessel, indicating differentiation of vascular bundle. E. Epidermis; P. Parenchyma; V. Vascular bundle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

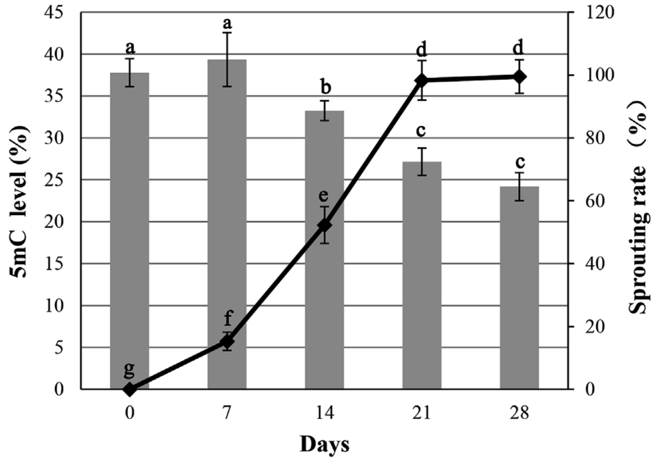

Fig. 5. Variation of 5 mC methylation levels and sprouting rates during chilling-induced dormancy release. Column shows 5 mC level and line chart shows the sprouting rate. The sprouting rate was monitored 30 d after being moved to greenhouse (18–22 °C, 8-h-light/16-h-dark cycle). Letters a to c show significance of 5 mC levels, and d to g is significance of sprouting rate.

period in tree peony.

Bract, sepal and petal of a peony floral bud developed an upper and lower epidermis, parenchyma and vascular bundles at the early stage of dormancy. Chilling treatment increased stomatal frequency and parenchyma developed a more regular arrangement. Xylem vessels were presented in both the main and lateral veins of bracts prior to entering dormancy, and occurred in the lateral vein of sepal before dormancy release. However, xylem vessels were not presented in lateral vessels until 21 d of chilling, a status of dormancy release (Fig. 4). It had been reported that xylem vessel differentiation in the filament of stamen was correlated with dormancy release (Andreini et al., 2012; Bartolini et al., 2006). In the compound bud of tree peony, our results illumined that dormancy release was characterized by the formation of the xylem vessel in the lateral vein of the petal and four prism-like stage anther stage. Taken together, we speculated the establishment of vascular connections be correlated with the breaking of dormancy.

4.2. *DNA hypomethylation accelerated dormancy release of tree peony*

Recent studies indicated that the genome-wide epigenetic modification was related to dormancy events, and increasing evidences revealed the involvement of chromatin modification in the regulation of seasonal dormancy cycles in perennial plants, including DNA methylation and histone modification (Ríos et al., 2014; Kumar et al., 2016). DAM played a vital role in the regulation of endodormancy in several perennial plants (Mazzitelli et al., 2007; Sasaki et al., 2011; Wu et al., 2012). Recently, the modifications of histone including acetylation and trimethylation were detected in the *DAMs* promoter, coding region and introns, coinciding with repression of *DAM6* and was related to dormancy release (Leida et al., 2012).

DNA cytosine methylation is also an important epigenetic modification manner affecting gene expression during various developmental and adaptive processes. More and more evidences indicated that DNA methylation modification participated in endodormancy process (Law et al., 2004; Santamaría et al., 2011). Prolonged chilling decreased total methylation in apple, from 27.2% in dormant bud to 21.0% in fruit set stage (Kumar et al., 2016). In tree peony, the global 5 mC level by HPLC detection went up mildly at 7 d point in the dormant buds, and then reduced constantly from 39.4% to 24.2%. Totally,

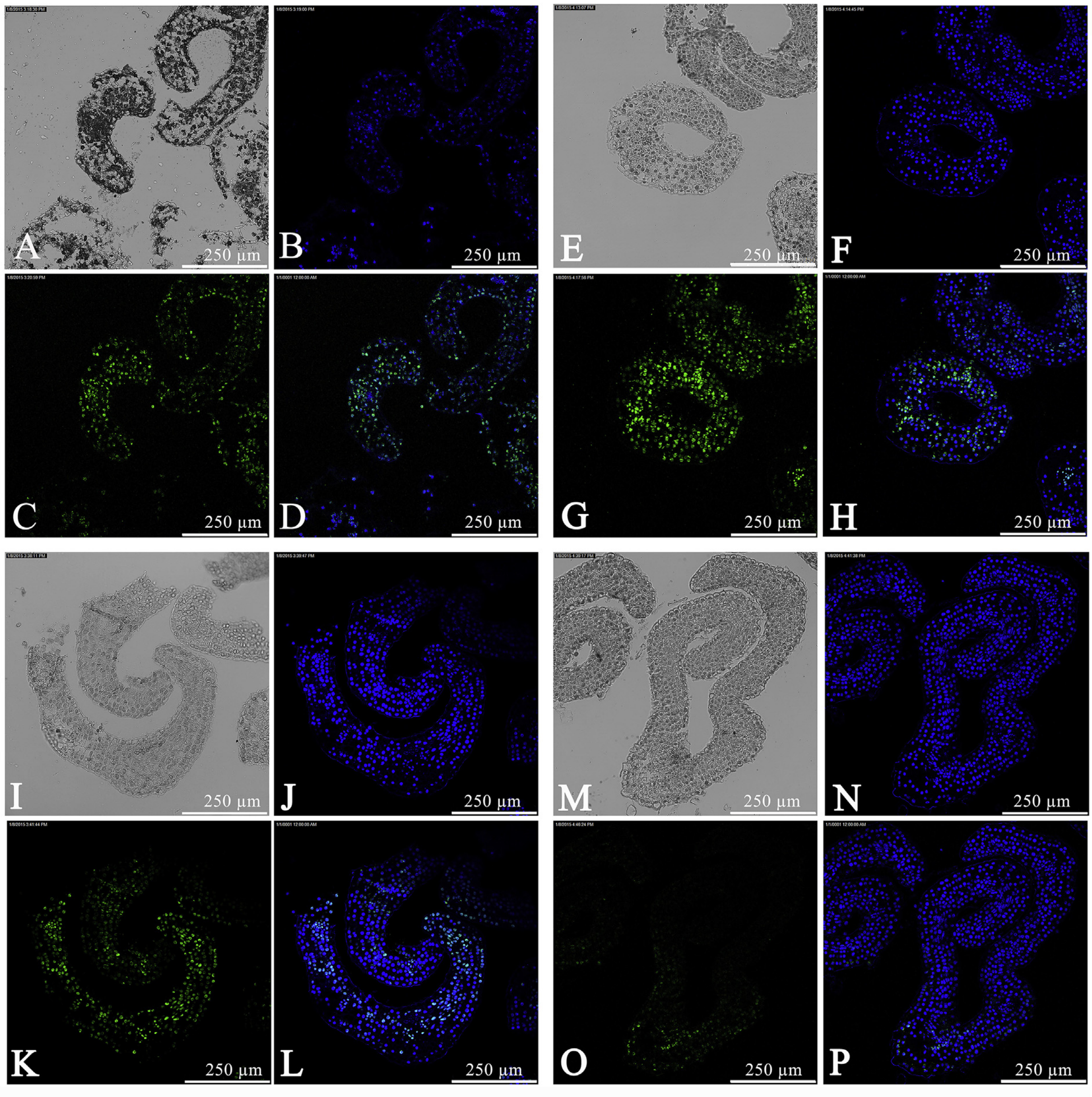

Fig. 6. Immunochemical detection of 5 mC in sections young leaf of buds through dormant period in horizontal axis using a confocal microscope. (A–D) non-chilling. (E–H) 7 d chilling. (I–L) 21 d chilling. (M–P) 28 d chilling. (A, E, I and M) Bright-field differential interference contrast (DIC). (B, F, J and N) DAPI (blue signals) labelling. (C, G, K and O) 5 mC (green signals) labelling. (D, H, L and P) Merged images of 5 mC and DAPI signals.

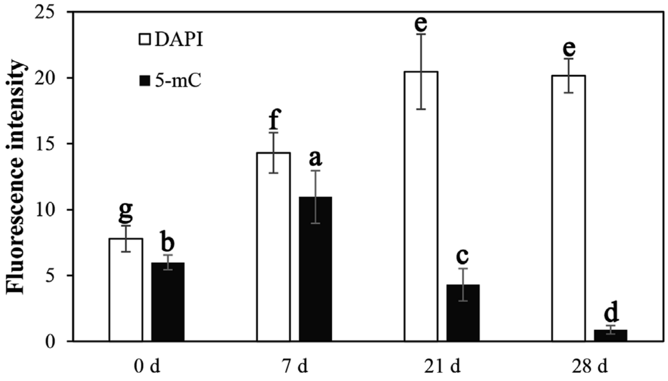

Fig. 7. Average fluorescence intensity of 5 mC and DAPI. Letters a to d show significance of fluorescence intensity of anti-5mC, and e to g show that of DAPI.

a decline tendency of the DNA methylation appeared during chilling-induced dormancy release. The results were accordance with that by immunochemical analysis, and also similar to other reports (Law et al., 2004; Santamaría et al., 2011; Kumar et al., 2016; Prudencio et al., 2018). Concomitantly with the reduction of DNA methylation, the sprouting rate and speed elevated gradually during chilling duration, which suggested a negative correlation between them. Moreover, low temperature associated with changes in DNA methylation had been reported previously (Sherman et al., 2002; Kumar et al., 2016). Thus, prolonged chilling resulted in DNA hypomethylation, which might be related to the induction of the mechanism of an active DNA demethylation trigged by plant specific DNA demethylases. Several DNA demethylases have been found induced in dormancy release in buds (Shim et al., 2014; Kumar et al., 2016; Conde et al., 2019). Moreover, in *Populus*, one DNA demethylase, is involved in active DNA demethylation prior bud break in spring(Conde et al., 2017). Our previous work also indicated a PsROS1 was induced by chilling treatment (Gai et al.,

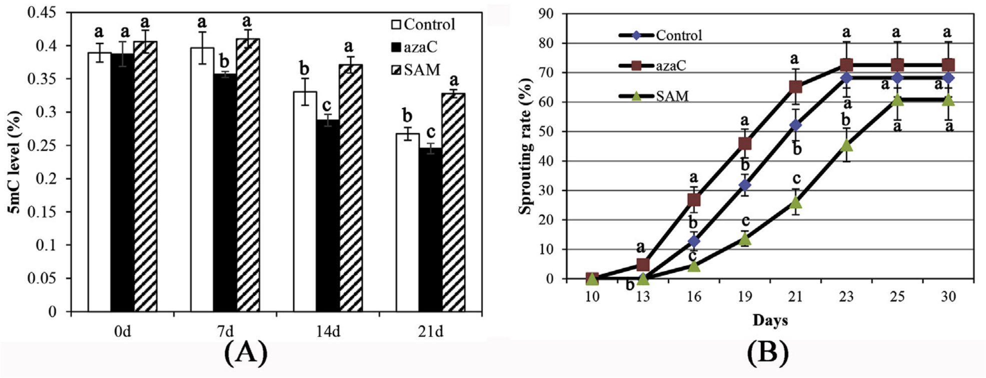

Fig. 8. DNA methylation level and sprouting rate of 5azaC and SAM treated buds after cultured in greenhouse (18–22 °C, 8-h-light/16-h-dark cycle). The plants were exposed to 0–4 °C for 14 d, then 50 μmol L^{-1} 5azaC and 10 μmol L^{-1} SAM were respectively applied and double distilled water as control. Bud break was checked per 2–5 d after being moved to greenhouse. (A) Variation of 5 mC levels. (B) The sprouting rate after being transferred into greenhouse. Letters show significance.

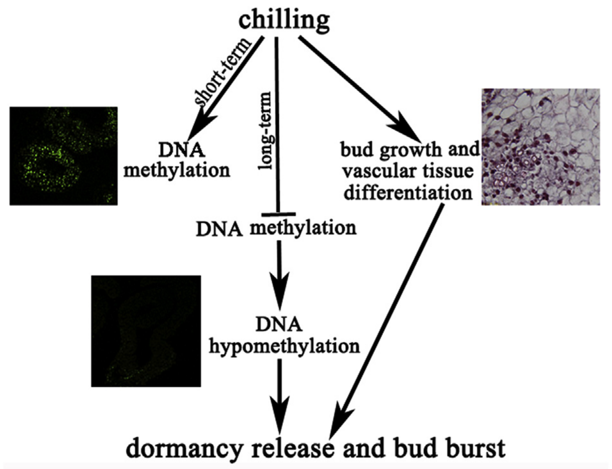

Fig. 9. Work model of the relation between chilling, DNA methylation and dormancy release.

2013).

It is still unknown whether DNA hypomethylation is an important cause of dormancy release in tree peony. Because it was difficult to regulate the methylation level by genetic transformation in tree peony, so, to answer this question, 5azaC and SAM were applied to artificially change the bud DNA methylation status, and investigate the corresponding morphological variation. The results showed that they really functioned in regulating DNA methylation conditions. Along with the lessening of DNA methylation caused by 5azaC application, bud sprouting rate also accelerated significantly, which is an important parameter of dormancy status. On the contrary, SAM treatment elevated methylation and reduced bud sprouting rate and ratio, thus hindered dormancy release. Taken together, it summarized that prolonged chilling reduced DNA methylation and DNA hypomethylation promoted endodormancy release in tree peony.

Variation of DNA methylation changes chromatin configuration and subsequently regulates gene transcription. Usually, high methylation level generates “closed” chromatin configurations and represses gene expression (Hashida et al., 2006; Zhu et al., 2007). As a subsequence of DNA hypomethylation, large amounts of genes related to dormancy release and bud growth awakened and accelerated dormancy release. We presumed a model between DNA methylation and development of tree peony buds after chilling exposure. Chilling treatment promoted DNA hypomethylation, which activated expression in growth-related genes and probably facilitated the development of flower primordia and the differentiation of vascular bundles. Finally, at 21 d an intact xylem vessel system formed, indicating the development of a substantial and signal transport channel thereby promoting dormancy release (Fig. 9).

5. Conclusions

The differentiation and development of the compound bud continued throughout the endodormancy process in tree peony by scanning electron microscope and paraffin sections observations. Chilling-induced dormancy release was accompanied by the establishment of xylem vessels in lateral vein of the petal and DNA hypomethylation. Exogenous application of 5azaC decreased DNA methylation level and promoted dormancy release, while artificial SAM treatment increased DNA methylation level and delayed dormancy release. Taken together, it was assumed that DNA methylation involved in dormancy regulation, and DNA hypomethylation promoted dormancy release. The results indicated sufficient chilling benefit not only to dormancy release, but also to the high quality of flower bud differentiation. Thus, sufficient chilling exposure was recommended in forcing culture practice of tree peony. Additionally, technical measures to decrease DNA methylation level would be potential ways in tree peony forcing culture.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.017.

Author contributions

GS conceived and designed the experiments. ZY, WX, LC and FW performed the experiments. XH, GS and ZY analyzed the data. XH, ZY and GS wrote and revised the paper. All authors read and approved the final version of the manuscript.

Declarations of interest

The authors declare no conflict of interests.

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