



Chilling and gibberellin acids hyperinduce β -1,3-glucanases to reopen transport corridor and break endodormancy in tree peony (*Paeonia suffruticosa*)

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ABSTRACT

Bud endodormancy is accompanied by transport channel apertures blockage through callose deposition, and its resume to growth requires evoking β -1,3-glucanases (BGs) to unchoke the conduit. To understand out its working manner, the statuses of the transport channels were evaluated and candidate BGs were identified during chilling and gibberellin acids (GA) induced dormancy release in tree peony. Calcein reflects plasmodesmata permeability, and no calcein was observed in the bud together with density aniline blue fluorescent around the stem phloem at 0 d chilling. With the increase of chilling accumulation, the contents of glucan declined and the activities of glucanase increased gradually in buds, and the calcein reached the top of flower primordia at 21 d chilled bud. Both GA₃ and GA₄ feedings promoted bud sprouting and growth along with rapidly unchoking the transport channels, and GA₃ was more effective. Several candidate β -1,3-glucanase genes were detected, combining transcriptional profiling and quantitative PCR analysis. *PsBG1*, *PsBG3*, *PsBG6*, *PsBG8* and *PsBG9* were inducible by chilling accumulation and presented laminarin hydrolyzing activities after prokaryotically expression, while *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* responded to GAs application. Subcellular localizations revealed that *PsBG6* and *PsBG9* were plasmodesmata residents. It was concluded that *PsBG6* played a vital role in chilling accumulation response and *PsBG9* was central in GAs-induced dormancy release, and they could be used as marker genes for dormancy release in tree peony. These results were of great value to understand the mechanism of dormancy regulation and as an important fundamental for forcing culture technology in tree peony.

1. Introduction

Bud endodormancy is associated with temporary suspension of visible growth, which is a crucial step in the physiological cycle for survival in cold winter, and complete dormancy release determines the quality of succulent bud break, flowering and fruiting. This process requires sufficient accumulation of non-freezing low temperatures, and exogenous application of gibberellin acid (GA) is also effective in some plants (Saure, 1985). Extensive concerns have been drawn on the regulation mechanism of dormancy. It has been reported that the CONSTANS/FLOWERING LOCUS T (CO/FT) regulatory module controls

seasonal growth cessation (Böhnenius et al., 2006), and TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (PpTCP20) and two AP2 type transcript factors EARLY BUD-BREAK 1 (EBB1) and EBB3 accelerates dormancy release in peach and poplar (Azeez et al., 2021; Wang et al., 2020; Yordanov et al., 2014), while CENTRORADIALIS/TERMINAL FLOWER 1 (CEN/TFL1) is a negative regulator of dormancy release in poplar (Rozi et al., 2010; Ruonala et al., 2008). Additionally, phytohormone ABA and GA play crucial roles in dormancy regulation (Singh et al., 2018, 2019).

As known, the transport channels are blocked at the stage of dormancy in winter (Han et al., 2014), which is caused by callose

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deposition (Rinne et al., 2001; Rinne and van der Schoot, 1998), and the regrowth requires to reopen the transport channels. Symplastic pathways, composed by plasmodesmata (PD) and phloem network, are the main transport channels for intercellular communication and short or long-range molecular motion in vascular plant (Kim, 2018), such as endogenous proteins (transcription factors, TFs), RNA signals (mRNA, siRNA, etc.) and other micro and large molecules (Brunner et al., 2014; Cheval and Faulkner, 2018; Lee and Frank, 2018). It was found that FT/FTL2 (FLOWERING LOCUS T-LIKE 2) enters the phloem and is transported to the top tissues through the intercellular transport, and then the apical meristem begins the transformation of flower, so as to achieve flowering of plants (Yoo et al., 2013).

The permeability of PD and phloem varies under different environmental conditions and growth stages (Knox and Benitez-Alfonso, 2014; Otero et al., 2016), which is regulated through the deposition and hydrolysis of callose (Doxey et al., 2007; Levy et al., 2007; Simpson et al., 2009). Callose synthetase (CALS) is an endogenous enzyme in charge of callose synthesis, conversely, β -1,3-glucanase (glucan endo-1,3-glucosidases, BGs, E.C. 3.2.1.39) specifically degrades callosome by the cleavage of 1,3- β -D-glucosidic linkages in β -1,3-glucans, also named as glycosyl hydrolase GH-17. At the dormant state of poplar, PD in the apical meristem is tightly wrapped by the corpus callosum which is called “dormancy sphincters”, and the intercellular conduits are blocked to restrict the access of growth-promoting signals. It was reported that ABA and SVP-LIKE (SVL) induce the expression of CALS, which promotes PD closure and entering dormancy in hybrid aspen (Singh et al., 2019; Tylewicz et al., 2018). After sufficient low temperature accumulation, callose in the aperture of PD disappears, and PD regains the ability to transfer fluorescent dyes between cells. In this process, the increasing of β -1,3-glucanase results in the hydrolysis of PD callose (Rinne et al., 2001, 2011). Remarkably, it is assumed that β -1,3-D-glucanase is the crucial component in dormancy release and PD transport restoration other than GA (van der Schoot and Rinne, 2011), which is extensively used as a marker of dormancy release, based on the facts that several near-lethal factors can accelerate to break dormancy, and all converge on a glucanase-based damage-repair system as concerns their potential working mechanism (Pérez et al., 2009; Wisniewski et al., 1997). However, dynamic status about the sphincters during dormancy release process is still not clear.

BGs play an important role in the regulation of PD permeability. The interaction between low temperature and long-term sunlight activates β -1,3-glucanase and reopens the sieve hole in spring (Rinne et al., 2001, 2011; Simpson et al., 2009). BGs with a glycosylphosphatidylinositol (GPI) anchor or CBM43/X8 (carbohydrate-binding module 43) domain, and lipid body (LB)-related are assumed to involve in PD permeability regulation (Levy et al., 2007; Rinne et al., 2011; Simpson et al., 2009). In hybrid aspen, chilling induces the transcripts of BGs belonging to GPI-anchored group 1b and LB-associated group. Additionally, GA₃ feeding upregulates all LB-associated GH17s, and GA₄ upregulates most GH17s with a GPI anchor and/or callose binding motif (Rinne et al., 2011). Rinne et al. proposed a fusion model of corpus callosum, cell wall, PD structure regulation and intercellular communication, in which gibberellin signal induces callose hydrolysis in sieves and intercellular plasmas (Rinne et al., 2016).

Tree peony (*Paeonia suffruticosa* Andr.) is a well-known horticultural, oil and medicinal plants native to China, honored as “the King of Flower”. It is a deciduous shrub with numerous stocky fleshy roots, hoarding plenty of assimilation substances in autumn for the coming regrowth under the suitable condition. Forcing is a main content of peony culture, especially for the Spring Festival in China. One major obstacle of tree peony forcing is the deep dormancy at autumn, and the sufficient release from bud dormancy is the basis of normal growth and development. The bud dormancy release of tree peony requires an effective low temperature accumulation, or combining with exogenous application of GA₃, which is widely used in forcing culture practice. In order to improve the forcing technology and the quality of flowering, it

is necessary to understand the mechanism of dormancy release. In the past 10 years, there were several studies on the regulation of dormancy in tree peony, such as transcriptomics, proteomics analysis, and metabolic variation during chilling-induced dormancy release (Gai et al., 2013; Xin et al., 2019; Zhang et al., 2015, 2018). However, its mechanism is still poorly understood now.

From late autumn to the next spring, tree peony must undergo a blockage-reopen cycle of transport channels, to survive in winter and resume growth in warm season. Unfortunately, the variation of transport channel has not been uncovered throughout the dormancy process. As mentioned above, β -1,3-glucanases, which belong to a multigene family, are responsible for reopening the PD channels during dormancy release. Transcriptome survey had explored 95 BG members, and several of them differentially expressed during chilling-induced dormancy release in tree peony (Gai et al., 2013). However, we still don't know which β -1,3-glucanase members are involved in the regulation of dormancy release in tree peony, and how do they work to reopen the transport channels? In this paper, we revealed the reopen mechanism of transport channels during chilling- and GAs- induced dormancy breaking process, which benefits to improve the technology of flower forcing culture in tree peony.

2. Materials and methods

2.1. Experimental materials

Four-year-old tree peony plants (*Paeonia suffruticosa* ‘Luhehong’) with 8–12 plump and healthy buds were from the Tree Peony Research Institute of Qingdao Agricultural University (Qingdao, Shandong, China). They were planted in single pots and moved to a dark room (0–4 °C, 24-h-dark) for a chilling accumulation from 5 November 2017. Buds were collected after 0, 7, 14, 21 (dormancy release) and 28 d (ecodormancy) chilling exposures, and then were immediately frozen in liquid nitrogen, –80 °C storage until use (Xin et al., 2019). Three replicates (3 plants/replicate) were performed. Additionally, 15 plants per treatment were transferred to a greenhouse (18–22 °C, 8-h-light/16-h-dark cycle) for dormancy status evaluation as described previously (Xin et al., 2019).

500 mg·L^{−1} GA₃ (Sigma) and 500 mg·L^{−1} GA₄ (Sigma) were respectively injected into the inner space of bud from the tip when being transferred to the greenhouse at November 12, with distilled water as the control (mock). Flower buds were harvested 0, 0.5, 1, 2 and 5 d after GA₃ and GA₄ feedings, and then frozen and stored at –80 °C until use. Bud break was monitored under the forcing conditions as described before (Xin et al., 2019). Three biological replicates were set.

2.2. Determination of callose content

About 1.0 g fresh stem was grinded into powder by a micro-crusher and sifted through a 60-mesh sieve prior to callose extraction. Callose content was measured following the methods described by Zhang et al. with minor modification (Zhang et al., 2015). Specifically, callose was extracted from 0.5 g bud tissue or stem powder (1.0 g) and homogenized in 1 mL NaOH (1 M) for 2 min, placed in a water bath at 80 °C for 30 min. After a centrifuge of 12 500 g for 15 min, 200 μ L supernatant was mixed with 1.2 mL aniline blue mixture (3:1 0.1% aniline blue: 1 M glycine, pH 9.5) at 50 °C for 20 min. The callose was determined by fluorescence spectrophotometry with excitation at 400 nm and emission at 500 nm, using laminarin (Sigma) as a standard callose source. Three technical repeats were performed and the mean was used for analysis.

2.3. β -1,3-Glucanase activity measurement

About 2.0 g of tree peony buds was rapidly grinded into powder in a liquid nitrogen, the β -1,3-glucanase was dissolved with a citrate sodium buffer (pH 5.0), and its activity was calculated by measuring the OD₅₅₀

using the 3, 5-dinitro salicylic acid (DNS) method (Zhang et al., 2015). An aliquot of the glucanase extract reacted with laminarin (Sigma) for 1 h, and glucose production was measured using a UV spectrophotometer (U-2900, Hitachi, Tokyo, Japan), after mixing with DNS reagent and incubating for 15 min in boiling water. One unit of β -1,3-glucanase activity was defined as the amount of enzyme that releases 1 μ M of glucose per min from laminarin under the assay conditions. The measurement was run in triple.

2.4. Histological observation of tree peony stem and mixed bud

Calcein is a fluorescent dye that can move only through the symplastic pathway, but cannot move between cells by diffusion and permeation. Therefore, the transport capacity of calcein to the bud apex was used to evaluate the PD permeability (Rinne et al., 2011). The buds were cut at 1.0 cm below their base, and immersed into a 0.1% calcein solution for 24 h just with their bottom in contact with the solution to make sure fluorescent dye moving to the apex only via the vasculature. Frozen section was then performed with a Lycra frozen slicer, and photographed with a blue light of a fluorescence microscope. Three plants (15–20 buds) were used per test solution.

The stems of annual shoot (about 1.0–1.5 cm below the bud) with different chilling duration were fixed in a FAA solution, and callose deposition in the phloem sieve tube sap was detected by aniline blue staining (Aloni and Peterson, 1991). Fixed materials were dehydrated, embedded in paraffin, and cut into 8 μ m sections on a rotary microtome Leica R-133 (Leica, Germany). After staining for 30 min in a 10% aniline blue staining solution and subsequent decolorization, the observation and photographing were carried out under ultraviolet light excitation using a fluorescence microscope.

2.5. Screening of BGs genes from tree peony transcriptome databases

In order to obtain putative β -1,3-glucanases encoding sequences in tree peony, *Arabidopsis* β -1,3-glucanase sequences (Doxey et al., 2007) were used to search orthologs in tree peony bud full-length transcriptome database (accession to the SRA data: PRJNA720276). Additionally, PD-related GH17s in *Populus*, like PtGH17_44, PtGH17_61, PtGH17_65, PtGH17_101, etc. (Rinne et al., 2011), were also used as reference sequences. The protein domain of hit sequences were predicted using ExPasy InterProScan program (<http://www.ebi.ac.uk/Tools/InterProScan/>). Phylogenetic analysis was performed with Clustal W and the MEGA 6.0 software (<http://www.megasoftware.net/>), and a phylogenetic tree was constructed with the neighbor-joining method on 1000 bootstrap replications.

The 79 putative BGs from full-length transcriptome were aligned to our previous 454 database (TSA project ID 65217) (Gai et al., 2012), and their transcriptional profiling during chilling duration were evaluated according to the GEO data (GSE4004) (Gai et al., 2013). Differentially expressed BGs with up-regulation based on hybridization signal intensity were regarded as candidate chilling-induced BGs, and their contained protein domains were also considered.

GAs-induced PsBGs were screened from transcriptomes treated by GA₃ and GA₄ for 2 d, differentially expressed unigenes with p -value < 0.05 were candidate. To assess the differences between chilling and GAs response, the chilling-induced PsBGs were also included.

2.6. Cloning and expression analysis of tree peony PsBGs

Total RNA was isolated from 0.5 g homogenized buds of different treatments using RNA isolation kit (TIANGEN, China) according to the manual. DNase (TIANGEN, China) was used to remove the genomic DNA contaminant, then the extracts were quantified by photometry and electrophoresis, and stored at -80 °C until use.

The first-stand cDNA was synthesized using PrimerScript™ RT reagent Kit (TaKaRa, Dalian). RACE amplification was performed to

obtain full length cDNA of PsBGs according to the protocol of SMART™ RACE cDNA Amplification Kit (Clontech, USA). The quantitative real-time PCR (qPCR) was utilized to analyze the expression patterns of PsBGs during chilling- and exogenous GAs- induced dormancy release, using SYBR® Premix Ex Taq™ II Kit (TaKaRa, Dalian) and run in a LightCycler® 480 II (Roche, USA). The specific primers for RACE and qPCR were designed by Primer Premier 5.0 and listed in Supplemental Table S1. The reactions were run in triplicate as described before (Gai et al., 2013), and the transcriptional abundances were normalized by *Psbeta-tubulin*. The relative expression levels were quantified using $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).

2.7. Subcellular localization of PsBGs

The open reading frames (ORF) of tree peony *PsBG6* and *PsBG9* without terminal codons were amplified using primers in Supplemental Table S1. The obtained fragments were inserted into pSuper1300-GFP to create *PsBG6:GFP* and *PsBG9:GFP* fusions, respectively. TMV movement protein (MP) fragment was amplified from TMV RNA with the primers in Supplemental Table S1. MP fragment was then inserted into pGDR vector with an RFP marker. The fusion of pGDR-RFP:TMV-MP was used as a marker of plasmodesmata localization. Afterwards, the recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method, and identified by the colony PCR and restriction analysis.

Agrobacteria with pSuper1300-*PsBG6:GFP*, pSuper1300-*PsBG9:GFP* and pGDR-RFP:TMV-MP were grown to an OD₆₀₀ value of 0.8, respectively. The bacteria were precipitated by centrifugation at 3000 g, then resuspended in 200 μ M acetosyringone, 10 mM MgCl₂ and 10 mM 2-(N-Morpholino) ethanesulfonic acid hydrate (MES) to a final cell density of OD₆₀₀ 0.8, and prepared for transformation. *Nicotiana benthamiana* plants with 2- to 4-week-old were cultured and used for transient expression analysis. For colocalization, bacteria carrying TMV-MP and PsBGs were equally mixed and infiltrated into the tobacco leaves. The tobacco plants were cultured in darkness for 1 d, then in light for 2–4 d before confocal laser scanning microscope observation by Leica TCS-SP2. The fluorescence signals of GFP and RFP were detected with excitation wavelength of 488 nm and 561 nm, and the emission wavelength were 505–550 nm and 580–620 nm, respectively.

2.8. Prokaryotic expression, purification and characterization of candidate PsBGs

The ORFs of PsBGs were obtained by PCR using the primers listed in Supplemental Table S1. After digested by the corresponding enzyme combination, the PCR fragments were inserted into the multiple cloning sites of prokaryotic expression vector to obtain pET28a⁺-PsBGs. The recombinant pET28a⁺-PsBGs and pET28a⁺ were transformed into *E. coli* BL21 and verified by colony PCR. The identified clones were subsequently used to express glucanase.

The positive clone of pET28a⁺-PsBGs were cultivated at 37 °C to an OD₆₀₀ 0.6–0.8 in Luria-Bertani (LB) liquid medium with 50 μ g·mL⁻¹ kanamycin, then added 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and cultured at 30 °C for 4 h. After ultrasonic decomposition, the supernatant was loaded onto Ni-NTA affinity resin (Qiagen) for glucanases separation and purification. Cell lysates and the purified recombinant proteins were subjected to SDS-PAGE using 10% gel and stained with 0.1% Coomassie brilliant blue R250.

The recombinant enzyme activity and their laminarin hydrolysis pattern were then characterized, which was determined by DNS method described above and High-performance liquid chromatography (HPLC) analysis. The purified β -1,3-glucanase was incubated with 0.5% of laminarin in 50 mM Na₂HPO₄-citric acid buffer (pH 6.0) for 3 h at 50 °C. The reaction was then stopped by boiling for 10 min, and the hydrolysates were performed HPLC with laminarin and glucose as the standard (Li et al., 2007).

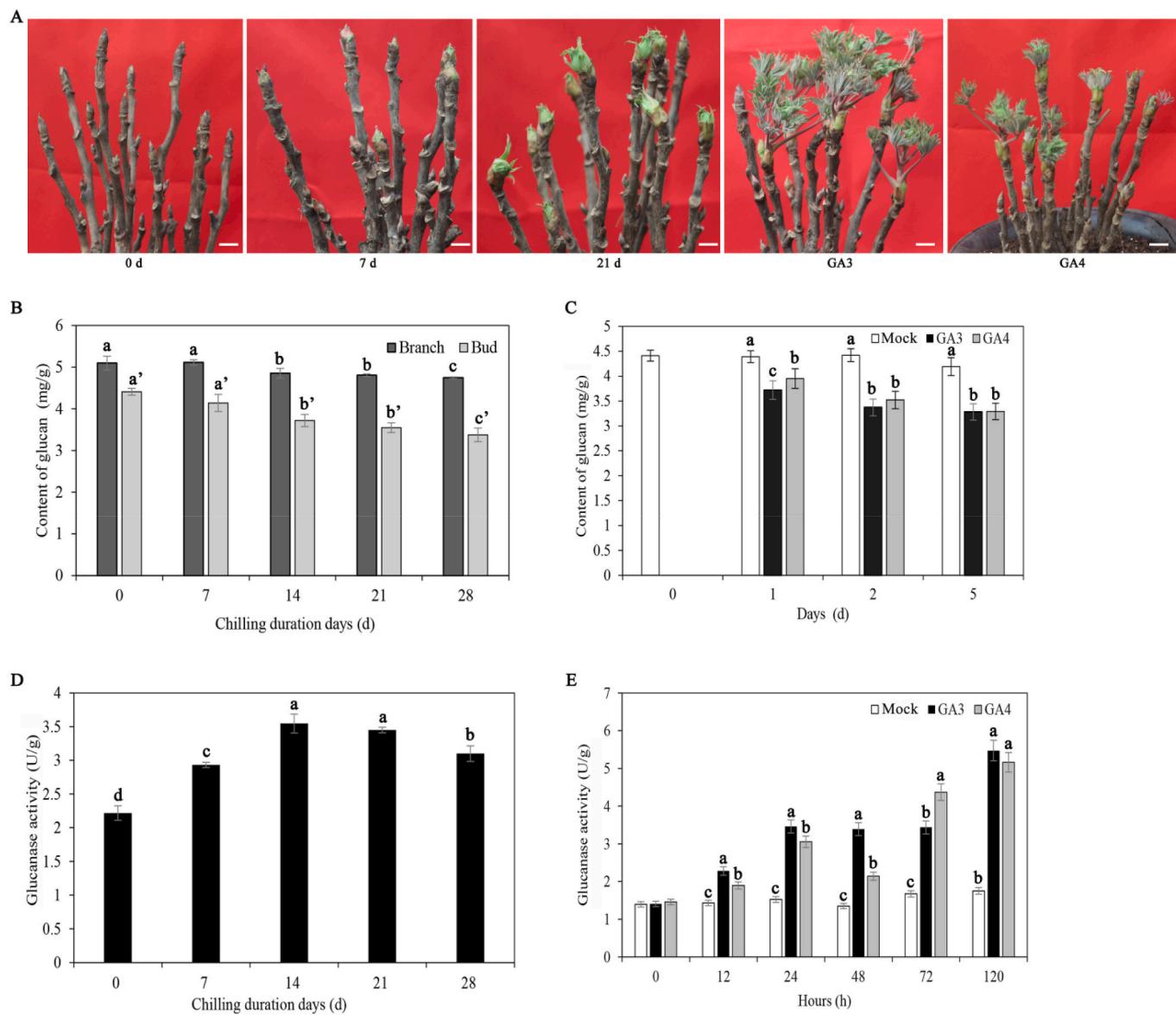


Fig. 1. The morphology, glucan content and glucanase activity in tree peony during dormancy release after chilling exposure and GAs applications. A Evaluation of dormancy status. Plants were moved to greenhouse for 15 d after continuous chilling duration and GAs feedings. Bars = 2 cm. B The glucan contents in the buds and annual branches during chilling-induced dormancy release. C The glucan contents in the buds after GA₃ and GA₄ treatments, that with distilled water as the control (mock). D Glucanase activity during chilling-induced dormancy release in tree peony. E Glucanase activity after GA₃ and GA₄ treatments, that with distilled water as the control (mock). Data were mean ± SE of three biological replicates, each with three technical repeats. Different letters show significance (Duncan's test, $p < 0.05$).

3. Results

3.1. Variation of the glucan contents and glucanase activities during bud dormancy release in tree peony

To evaluate the dormancy stage, the sprouting status of plants exposed to continuous chilling was observed after being transferred into greenhouse for 15 d. Bud sprouting was not observed for non-chilling treated plants, and only a small part of bud scales burst after 7 d treatment, but nearly 100 percent of the apical buds sprouted with more than 21 d chilling exposure (Fig. 1A). Thus, the stage of 21 d chilling duration was defined as dormancy release, in accordance with the previous histological observation (Xin et al., 2019). GA₃ and GA₄ application for 5 d significantly promoted bud burst with almost all the treated buds sprouting, and the shoots were about 10 cm length in GA₃ treatment, but only 5.0 cm in GA₄ after transferred into greenhouse for 15 d, indicating

GA₃ was superior to GA₄ in promoting shoot elongation (Fig. 1A).

In dormant status, callose deposits in sieve cells and PD of the bud, which shuts down material transport and signal transduction pathway for growth cessation and environment adaption. Thus, glucan contents were measured to evaluate the status of transport channel both in the buds and annual branches during dormancy release. The results revealed that the glucan contents were steady at the early stage of dormancy (0–7 d), and dramatically declined at 14 d chilling exposure, a transition stage to dormancy release, and followed by a slight decrease at 28 d. There was no significant difference between 14 d and 21 d (Fig. 1B). Specifically, the glucan content variation trends of branches and buds were similar, indicating the regulation of intercellular and long-distance channels was synchronous. Additionally, the glucan contents rapidly and dramatically declined just 1 d after GAs feeding, and maintained steady state after 2 d until 5 d, and GA₃ was more effective than GA₄ to induce the degradation of callose (Fig. 1C).

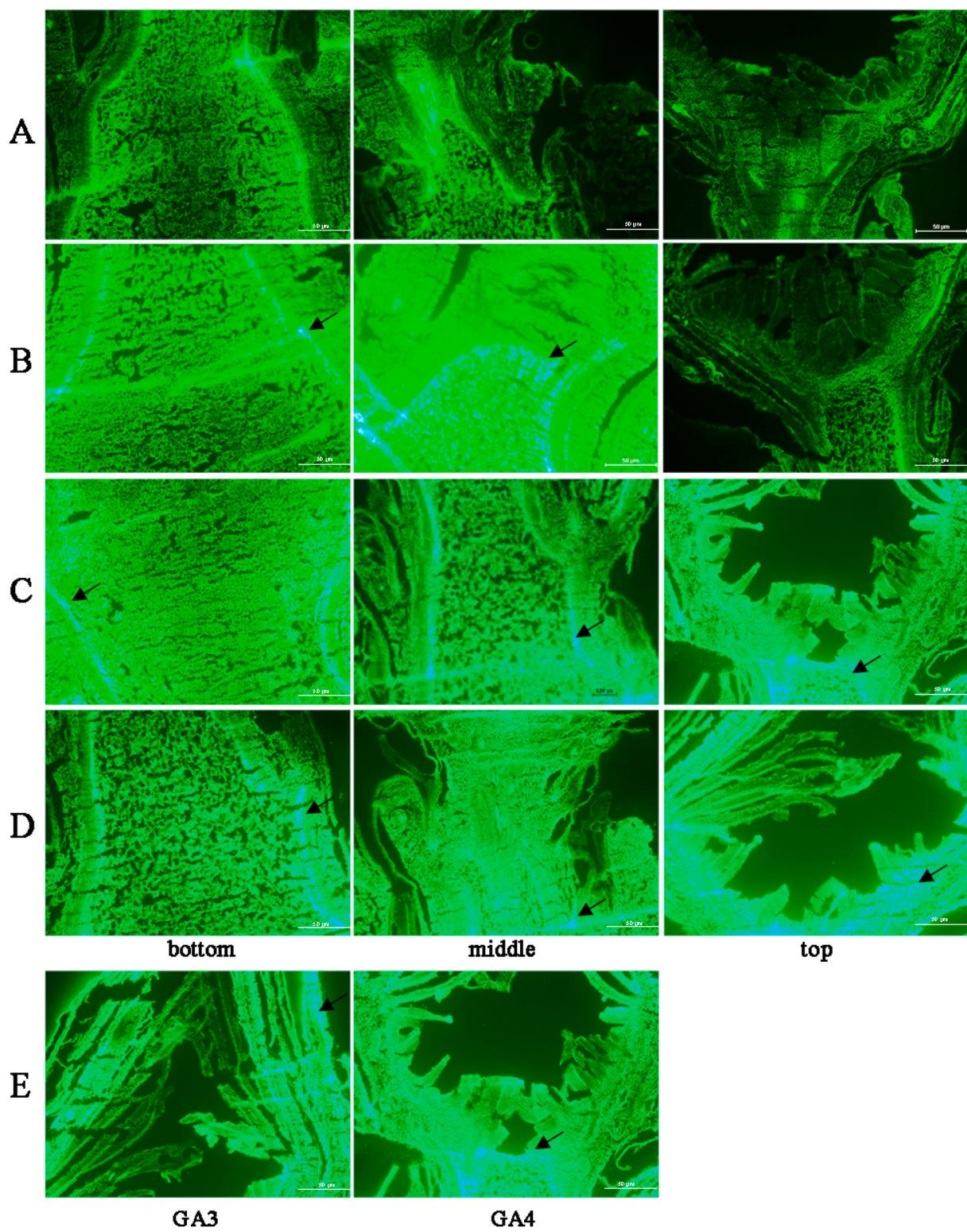


Fig. 2. The fluorescent of calcein during dormancy release in buds of tree peony. **A** The fluorescent of calcein in 0 d chilling bud. **B** 7 d chilling. **C** 14 d chilling. **D** 21 d chilling. **E** The fluorescent of calcein at the top of bud after GA₃ and GA₄ feedings for 24 h. Bottom, middle and top presented the bottom, middle and top part of the bud, respectively. Bars = 50 μ m. Arrow marked the fluorescence of calcein. The imaging was repeated three times with similar results. The photos were representative of three independent experiments.

In addition, the glucanase enzyme activity were determined in the mixed bud, and the results showed that the enzyme activity increased gradually with the prolongation of the low temperature exposure, and reached the highest level at 14 d and decreased after 28 d (Fig. 1D). The changes of glucanase activity were in accord with the variations of

glucan contents during chilling accumulation process. Similarly, glucanase activity increased persistently after GAs application. Consistent with glucan content, GA₃ was more effective to promote the activity of glucanases (Fig. 1E).

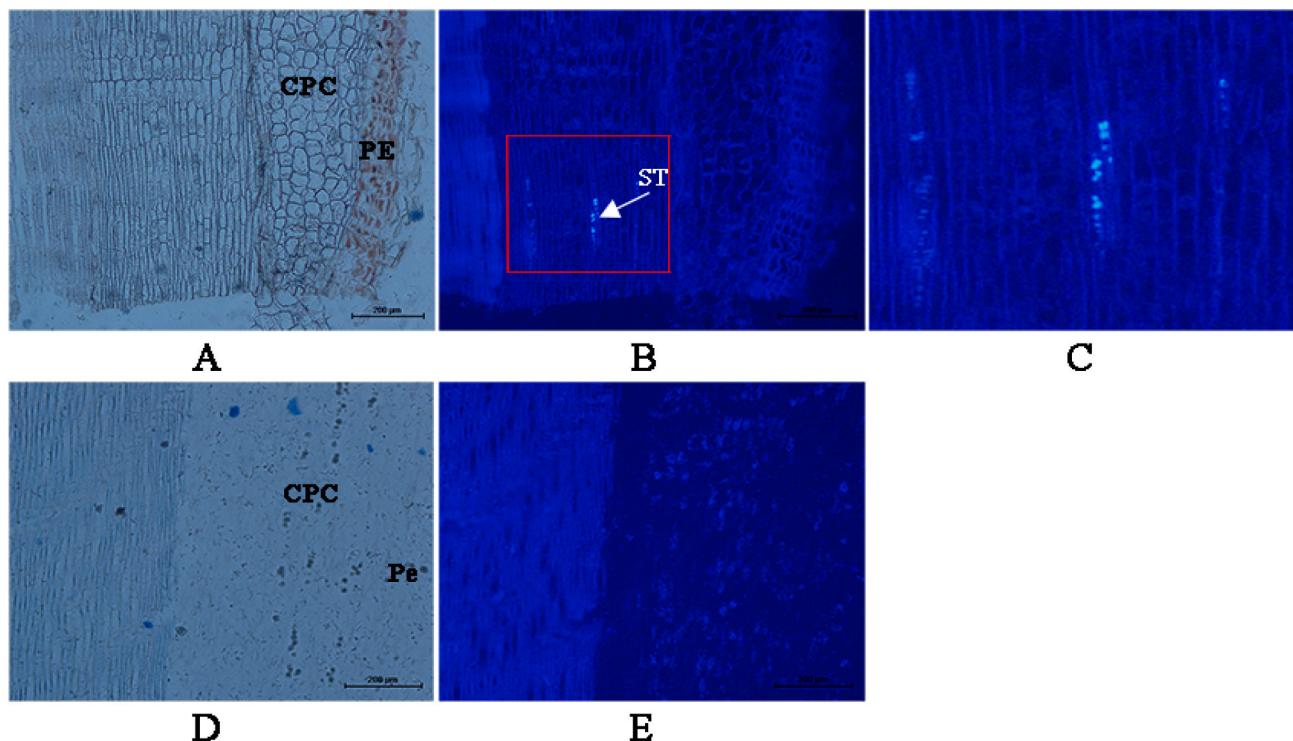


Fig. 3. Vertical section view of stem by aniline blue staining in tree peony. A-C 0 d chilling treated stem. C Enlarged image of B in red rectangular box. D and E 21 d chilling. A and D Bright field. B, C and E ultraviolet light. Bars = 200 μ m. Pe, Periderm. CPC, cortex parenchyma cells. ST, sieve tube. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. State of substance transport channel during chilling or GAs-induced dormancy release

The bud of tree peony is a compound one, which will develop to flower and branch with leaves (Xin et al., 2019). The vertical section of whole buds was observed under a fluorescence microscope after dipped into a calcein solution. At non-chilling treatment, almost no fluorescence was observed in the whole buds (Fig. 2A). After 7 d, the fluorescence presented at the basal and middle part of the bud, but not at the apex (Fig. 2B). Small amount of fluorescence was detected at the top of the bud after 14 d (Fig. 2C). After 21 d chilling durations, calcein reached the apex of the bud, and the fluorescence gathered at stamens and petals (Fig. 2D), indicating an unimpeded state of the symplastic transport channels thereafter. The results implied that the material transport channel of the bud was gradually opened up along with the prolongation of chilling accumulation. After GAs feedings, dense calcein fluorescence was detected at the apex of petal primordium just 1 d after GA₃ feeding, while only reached the bottom of receptacle for GA₄ (Fig. 2 E), indicating GA₃ was more effective and rapidly to reopen the symplastic channel in tree peony.

Callose can be specifically stained by aniline blue, and the fluorescence intensity under ultraviolet light can reflect the callose accumulation in the sieve tube. Therefore, the state of phloem tube in the stem of annual branch during dormancy was detected by aniline blue staining. At 0 d, high density fluorescence presented at the phloem, indicating the long-distance transport was badly blocked. After a prolonged chilling for 21 d, the fluorescence could hardly be detected (Fig. 3), which meant that the channels were unchoked after bud dormancy release.

3.3. Acquisition of candidate PsBGs in tree peony transcriptome database

GH17s are widely found in plants, and are involved in different biological processes. Based on the sequence alignment correlation algorithm, putative PsBGs sequences were screened and identified in the

full-length transcriptome of dormant bud in tree peony (accession number: PRJNA720276), using GH17 members in *Arabidopsis* and in *Populus* (candidates involving in dormancy release) as references (Rinne et al., 2011). Finally, 79 target sequences were identified after removing redundancy. The multiple sequence homology was aligned using Clustal W, and the phylogenetic tree was constructed by MEGA 6.0 (Fig. 4A). Amino acid sequence alignment identified 68 of them possessing GH17 domains with an 86.1 percent in number. It is believed that BGs containing GPI domain and/or CBM43/X8 domain might function at PD. After domain analysis, we found 32 of them possessed GPI domain with a percentage of 40.5, and 73 individuals with CBM43 domain occupied by 92.4%. As known, there are three clades of BGs both in *Arabidopsis* and *Populus*, i. e. α , β and γ clades (Doxey et al., 2007). Totally, 15 of them were grouped to β clade, and the others to α clade. Interestingly, the member of γ clade was not detected in the dormant bud transcriptome of tree peony, which might reside in endomembrane, vacuolar, and/or cell wall (Bayer et al., 2006; Gunl et al., 2011; Le Roy et al., 2007).

Based on structural organization and expression patterns during chilling duration, putative *PsBGs* involved in dormancy release were filtered. According to the microarray data, most of the putative BGs were down-regulated or steady during chilling accumulation except that 9 individuals were up-regulated, which were named *PsBG1* to *PsBG9*, and their expression patterns were listed in Fig. 5A.

Full-length cDNAs were obtained by RACE amplification, which ranged from 1642 to 1941 bp encoding 388 to 520 amino acids, and the detailed information were listed in Table 1 and supplemental file 1. BLAST analysis and multiple sequence homology comparison showed that the proteins encoded by *PsBGs* gene were similar to that of other plants with identities of 78%–85%, indicating that the *PsBGs* were highly conserved in evolution. These nine putative *PsBGs* proteins were divided to α clade except *PsBG4* and *PsBG8* belonging to β clade. Additionally, they all contained the conservative region glyco-hydro-17, and CBM43/X8 domain except *PsBG7*. GPI domain was also found in

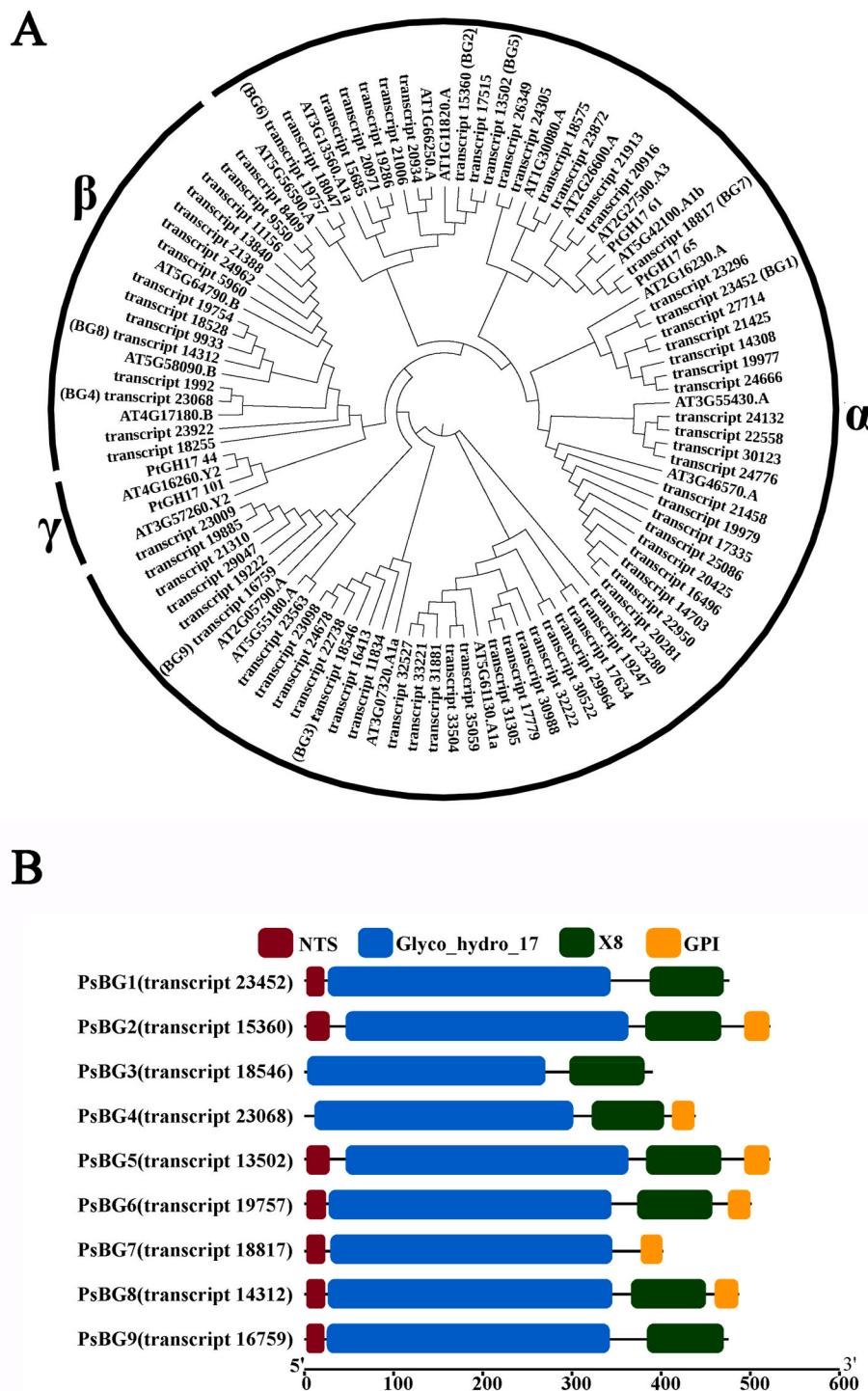


Fig. 4. Phylogenetic tree of β-1,3-glucanases and characteristics of candidate PsBGs in buds of tree peony. A Phylogenetic tree of β-1,3-glucanases in tree peony with several in *Arabidopsis* and *Populus* as reference. The candidates PsBGs were marked in brackets. None of the tree peony BGs were grouped into **γ**-clade. B Protein domain architectures of candidate PsBGs. N-terminal signal peptide (NTS), the core glycosyl hydrolase family 17 domain (GH17), X8, also known as carbohydrate binding module family 43 (CBM43), and a hydrophobic C-terminal sequence that may encode a transient transmembrane domain involved in GPI anchor attachment (GPI) were indicated by red, blue, green and orange rectangle, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

several members, such as PsBG2, PsBG4, PsBG5, PsBG6, PsBG7 and PsBG8 (Fig. 4B).

3.4. Expression patterns of candidate PsBGs during chilling- and GAs-induced dormancy release

As an important parameter to recognize putative participants of β-1,3-glucanase involved in the reopening transport channel of dormant bud and the recovering bud growth ability, the expression patterns of the candidate PsBGs genes during dormancy release process were validated by qPCR. According to the microarray data, almost all the

candidates were up-regulated at the end of endodormancy except PsBG6, which was activated at the early stage of chilling accumulation (Fig. 5A). The qPCR results showed that PsBG2 and PsBG5 were relatively steady, while PsBG1, PsBG3, PsBG4, PsBG6, PsBG7, PsBG8 and PsBG9 were all up-regulated inordinately after chilling duration (Fig. 5B). Globally, most of the increase occurred at 21 d and reached maximum at 28 d except PsBG4 and PsBG6. Conspicuously, the transcripts of PsBG9 elevated with 15.1 folds at 28 d compared to the control. The expression of PsBG4 slightly increased from 7 to 21 d chilling duration. Remarkably, the transcripts of PsBG6 were abundant and up-regulated all along after chilling exposure with a 71 folds increase at 7

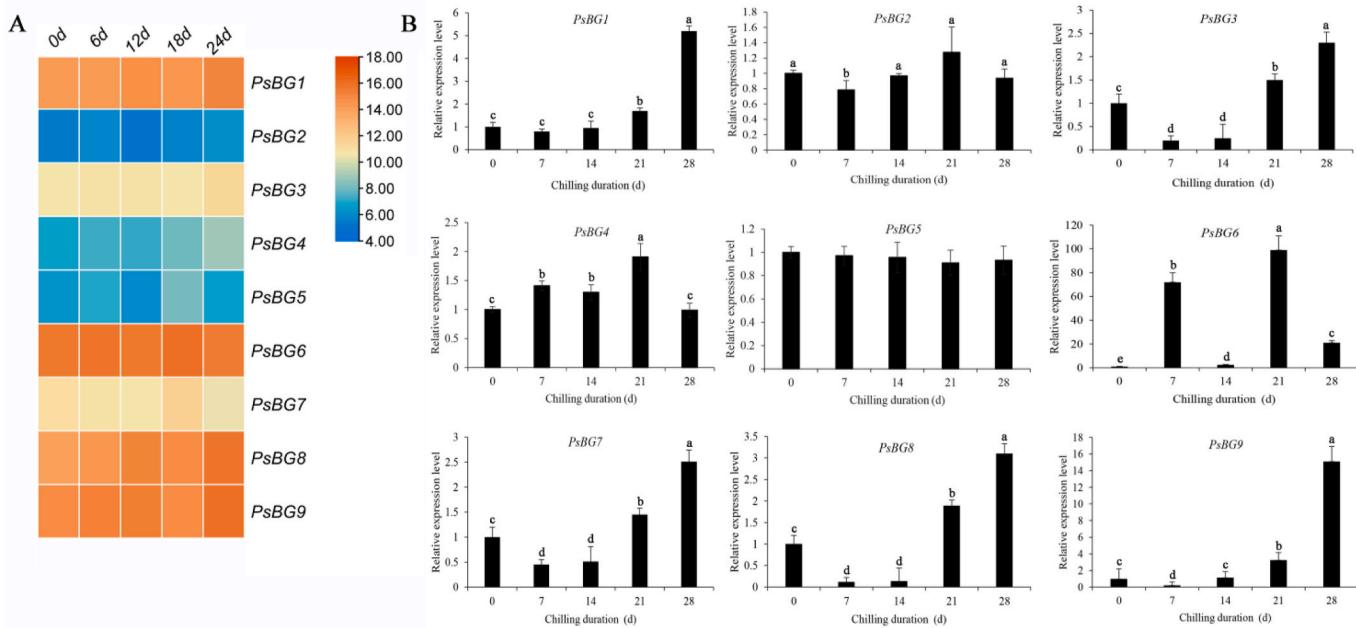


Fig. 5. Expression patterns of *PsBGs* during chilling-induced bud dormancy release. A Heatmap of differentially expressed *PsBGs* after chilling treatments in 'Fengdanbai' based on the cDNA microarray data. The colors from blue to red in heatmap represented the relative expression levels of DEGs from low to high, and the blue-red colors corresponded to the 4.0–18.0 values of normalized hybridization signal intensities. B The expression patterns of *PsBGs* after chilling treatments in 'Luhehong' by qPCR. Psbeta-Tubulin as a reference. Values were representative of three biological replicates and three technical replicates, and error bar showed \pm SE. Different letters showed significance (Duncan's test, $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Information of candidate *PsBGs* involving in dormancy release in tree peony. Similarity represents the highest similarity between putative *PsBG* proteins and the other known plant BG protein. Pt: *Populus trichocarpa*; Sl: *Solanum lycopersicum*; Ac: *Amygdalus communis*; Nn: *Nelumbo nucifera*; Tc: *Theobroma cacao*; St: *Solanum tuberosum*; Vv: *Vitis vinifera*; Cs: *Citrus sinensis*. +: positive; -: negative; N: not detected.

Genes	<i>PsBG1</i>	<i>PsBG2</i>	<i>PsBG3</i>	<i>PsBG4</i>	<i>PsBG5</i>	<i>PsBG6</i>	<i>PsBG7</i>	<i>PsBG8</i>	<i>PsBG9</i>
Full-length (bp)	1763	1842	1885	1802	1725	1941	1642	1909	1802
ORF (bp)	1422	1560	1164	1164	1560	1497	1200	1445	1419
Coding amino acid	474	520	388	388	520	499	499	485	473
Similarity	Pt 83%	Sl 84%	Ac 84%	Nn 83%	Sl 84%	Tc 85%	St 78%	Vv 80%	Cs 83%
Enzyme activity of recombinant protein	+	N	+	–	+	+	–	+	+

d chilling duration, and with a 98 folds increase at 21 d chilling period. Taken together, we assumed that *PsBG1*, *PsBG3*, *PsBG4*, *PsBG6*, *PsBG7*, *PsBG8* and *PsBG9* might involve in the hydrolysis of the callose and reopening of symplastic pathway during chilling-induced dormancy release. However, *PsBG6* might be the most important according to its expression abundant and patterns during chilling-induced dormancy release.

As known, exogenous GA₃ and GA₄ also play an important role in the release of bud dormancy, GAs-induced *PsBGs* were screened and identified. According to the transcriptomic data, six differentially expressed genes, *PsBG1*, *PsBG3*, *PsBG4*, *PsBG6*, *PsBG7*, *PsBG8* and *PsBG9* were screened ($p < 0.05$), and their responses to GA₃ and GA₄ were also analyzed (Fig. 6A). However, the transcripts of *PsBG6* and *PsBG7* decreased after GAs treated for 2 d. The results indicated that *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* were inducible to GA₃ and GA₄.

qPCR analysis showed that the transcripts of *PsBG1*, *PsBG3*, *PsBG4*, *PsBG5*, *PsBG8* and *PsBG9* were all induced by both GA₃ and GA₄, but those of *PsBG2*, *PsBG6* and *PsBG7* were inhibited or no significant difference after exogenous GAs applications (Fig. 6B). Among them, *PsBG1*, *PsBG3*, *PsBG4* and *PsBG8* were more sensitive to GA₄ than GA₃. For example, the transcript of *PsBG8* was about 34 folds higher than the mock after 2 d GA₄ feeding, while the highest increase was only 6.13 folds for GA₃ treatment. Remarkably, *PsBG9* was the most sensitive one

responding to both GA₃ and GA₄, with about 2530 and 2130 folds up-regulations at 0.5 d and 2 d respectively, and responded to GA₃ faster. Thus, *PsBG9* was the only member that was more sensitive to GA₃ than to GA₄. Additionally, the upregulation of *PsBG5* was only presented after GAs applications for 5 d, a stage of bud scale burst, and it might be due to rapid development of the bud or other reasons.

Taken together, *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* might participate in GAs-mediated callose hydrolysis, symplastic pathway reopening, and promoting dormancy release finally, of which *PsBG9* might play a central role.

3.5. Subcellular localization of *PsBG6* and *PsBG9*

As *PsBG6* and *PsBG9* were suspected to play central roles during dormancy release, their subcellular localizations were further verified as representative BG with or without GPI domain, respectively. The tobacco mosaic virus mobile protein fusing with RFP was selected as a specific marker for PD localization, and the detected proteins were recombined with GFP marker. The result indicated that the fluorescence of pGDR-MP-RFP was distributed dot-like around the cells, consistent with the reported localization of intercellular filaments. The fluorescence of *PsBG6*-GFP and *PsBG9*-GFP were basically merged with the red fluorescent, indicating that *PsBG6* and *PsBG9* were PD localization

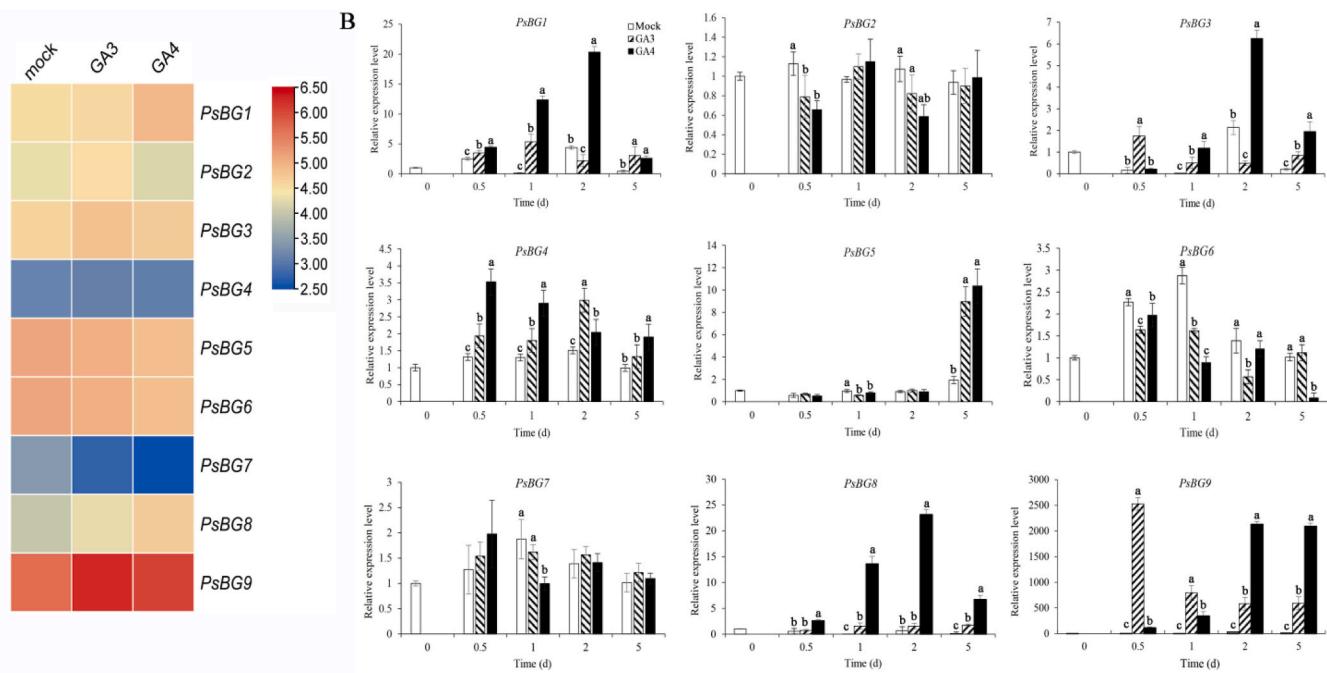


Fig. 6. Expression patterns after GA₃ and GA₄ feedings for 0, 0.5, 1, 2 and 5 days, respectively. **A** Heatmap of differentially expressed PsBGs after GA₃ and GA₄ applications for 2 d in ‘Luhehong’ based on the RNAseq data. The colors from blue to red in heatmap represented the relative expression level of DEGs from low to high, and the blue-red colors corresponded to the 2.5–6.5 values of log₂ (FPKM). **B** The expression patterns of PsBGs after GA₃ and GA₄ applications in ‘Luhehong’ by qPCR, that with distilled water as the control (mock). Values were representative of three biological replicates and three technical replicates. Different letters showed significance (Duncan’s test, $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

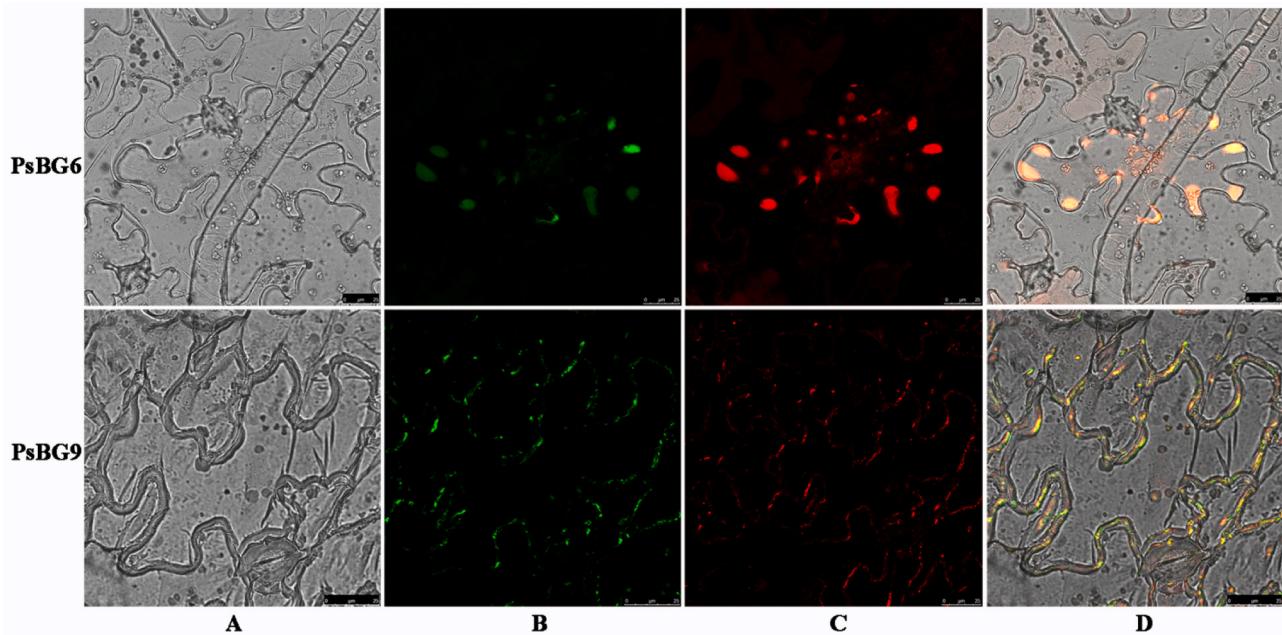


Fig. 7. Subcellular localization of PsBG6 and PsBG9 in *N. benthamiana* epidermal cells. **A** Bright field, **B** GFP, **C** TMV-MP-RFP, **D** Merge. Transient expression of PsBG6, PsBG9 and TMV-MP-RFP fusion proteins in *N. benthamiana* epidermal cells. Bars = 25 μ m. The photos were representative of three independent experiments. These experiments were repeated three times with similar results.

(Fig. 7). Specifically, PsBG6 with a GPI anchor, preferentially accumulated inside the PD channel, resembling the MP of TMV, and PsBG9 without GPI domain, tended to cover the PD channel in small patches. The localization of PsBG9 was similar to the LB-associated GH17 in hybrid aspen (Rinne et al., 2011).

3.6. Expression and purification of recombinant proteins in *E. coli*

To analysis the function of candidate PsBGs, we constructed in vitro prokaryotic expression vectors, and the recombinant plasmids pET28a⁺-PsBGs were screened and identified in *E. coli* strain BL21. PsBG2 was excluded because its expression was insensitive to chilling duration and

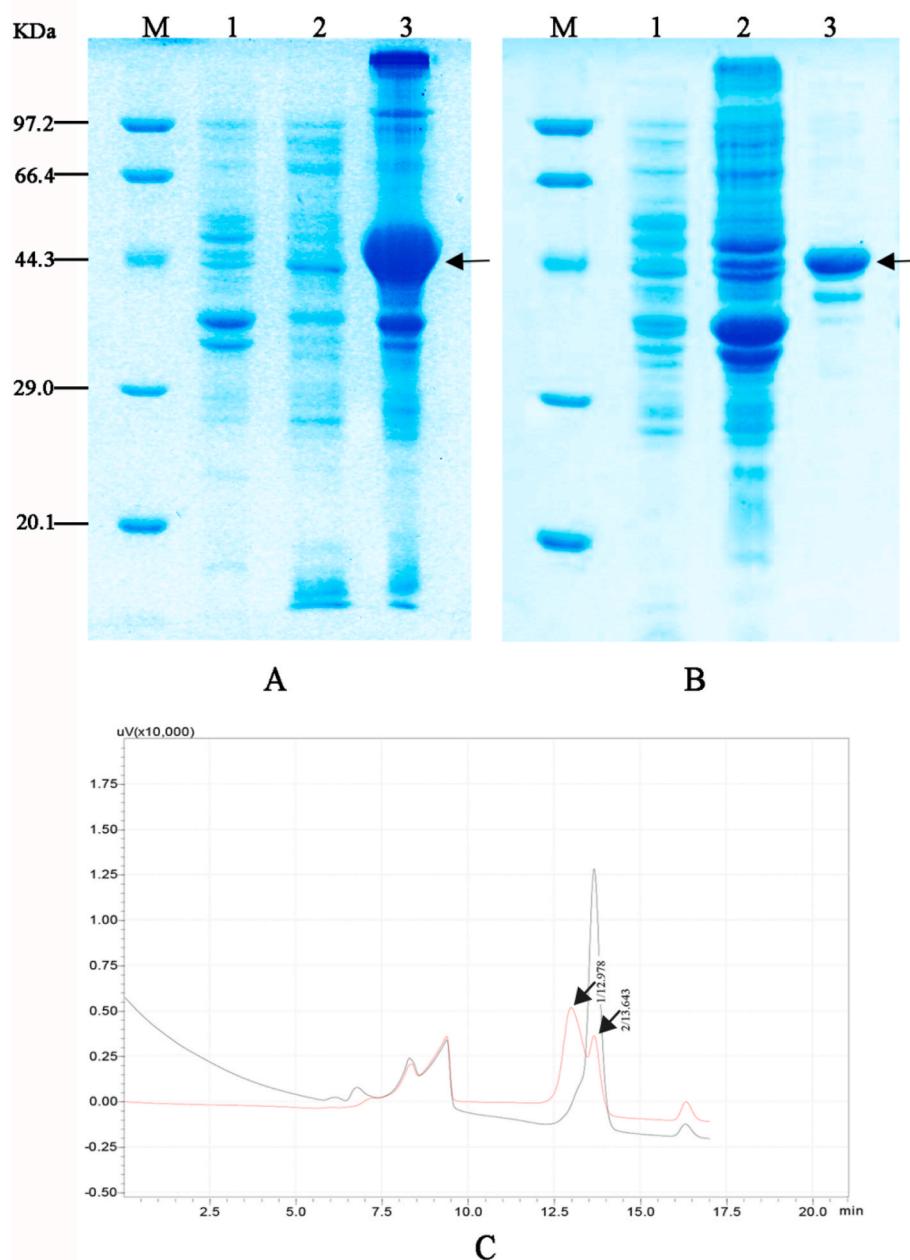


Fig. 8. Characterization of the glucanase enzyme expressed in *E. coli* by SDS-PAGE and the β -1,3-glucanases activities of PsBGs by HPLC. Proteins were separated in 10% acrylamide gels and stained with Coomassie brilliant blue R250. A and B SDS-PAGE electrophoresis of the recombinant PsBG6 and PsBG9. Lane M, protein molecular mass marker with sizes given in kDa; Lane 1, control cells harboring no glucanase gene; Lane 2, Lysate from PET-28a + -PsBGs transformed cells without IPTG; Lane 3, The expressed of protein with IPTG. Arrows showed the recombinant PsBGs. C The HPLC profile of laminarin and its reaction products with the recombinant PsBGs. Reds, the standard substance; Black, after mixed with the purified recombinant PsBGs. Two main peaks of the standard substance (1 and 2, laminarin and glucose) appeared at 12.978 and 13.643 min respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

depressed by GAs applications. Recombinant PsBGs proteins were induced by IPTG, and cell lysates were separated by SDS-PAGE. The expressed proteins were in accordance with the expected sizes, indicating the candidate glucanase genes were successfully expressed (Fig. 8A and Table 1). The recombinant proteins were then purified by Ni-NTA affinity resin.

The purified recombinant proteins were mixed with laminarin, and the products were then subjected to HPLC analysis. Two main peaks of the standard substance (laminarin and glucose) were monitored at 12.978 and 13.643 min respectively. When the standard substances laminarin was mixed with the purified recombinant proteins, the 12.978-min peak disappeared in the reaction system except PsBG4 and PsBG7, given enough reaction time (Fig. 8B), suggesting that the recombinant PsBG1, PsBG3, PsBG5, PsBG6, PsBG8 and PsBG9 possessed the activities of β -1,3-glucanases in vitro.

4. Discussion

Endodormancy is a sophisticated strategy which plants evolve to survive in tough environments with an important impact on the survival and production of plants. During the dormancy process, the symplastic transport channels, mainly including PD and sieve tube, are wrapped by callose, and they are reopened by β -1,3-glucanases, which are activated by prolonged chilling and other breaking dormancy factors, prior to dormancy release (Rinne et al., 2011; Ruonala et al., 2008). More and more evidences suggested that β -1,3-glucanases play crucial role in breaking bud dormancy (van der Schoot and Rinne, 2011), which are generally used as the marker genes to evaluated dormancy status (Rinne et al., 2016, 2018). But it is still not clear how the dormancy sphincter is unchoked and which members of β -1,3-glucanases involve in chilling or GAs-induced dormancy release in tree peony. In this study, prolonged chilling and exogenous GAs were applied to the dormant tree peony, and the status of bud transport channel was assessed. We found the content of glucan declined gradually and the symplastic channels were

progressively opened up along with chilling accumulation, but GAs applications rapidly unchoked the channels. Finally, we identified that *PsBG6* and *PsBG9* played crucial roles during dormancy release of tree peony.

4.1. Symplastic channels were opened up gradually during chilling-induced dormancy release, but rapidly by GAs application

During the whole growing seasons before dormancy, the photosynthesis of the peony leaves produces a large amount of nutrients, and they are stored in the developed fleshy roots and flower buds, which will be invoked for the buds bursting, leafing and flowering in the next growth season. Zhang et al. indicated that a considerable amount of carbohydrate is transported from other parts to the dormancy released buds before bud burst in tree peony (Zhang et al., 2020). Beside the nutrients, regrowth and flowering of buds also require a large number of signal molecules participating in this process, such as FT need to be transported to the apex of the bud prior to dormancy release (Böhlenius et al., 2006; Ruonala et al., 2008).

In deep autumn and winter, deciduous plant develops buds and enters into dormancy with net deposition of callose around and inside the PD channel obstructing further symplastic transport and signaling (Rinne and van der Schoot, 1998; Ruonala et al., 2008). In addition, sieve tubes become occluded with callose deposition (Aloni and Peterson, 1997). PD narrowing may substantially constrain the capacity of the primordia as a sink, and the import of nutrients and growth-promotive signals from the reserves. Therefore, the regulation of the material transport channel during dormancy transition is of great significance for the dormancy induction and release, spreading leaves and flowering.

It was reported that dormancy break factors, such as chilling accumulation, GAs treatments and HC application, could hyper-induce the expressions of β -1,3-glucanase genes and activities of β -1,3-glucanases, degrade the callose and finally reopen the transport conduits (Pérez et al., 2009; Rinne et al., 2001, 2011). To better understand how the glucanases work during the whole process, we measured the glucan contents, the enzyme activities of glucanases, and evaluated the status and ability of transport channels during chilling- and GAs- induced dormancy release in tree peony. The results revealed that calcein fluorescence was not detectable in the buds when enter into dormancy (0 d chilling), and intense fluorescence was observed at the phloem of annual shoot at the same period, indicating that dormancy induction was also accompanied by the closure of the material transport channels including the short-distance PDs and the long-distance sieve tubes in tree peony.

Along with chilling accumulation, the contents of glucan slowly decreased to a minimum at 28 d, and the enzyme activities slightly elevated in the bud (Fig. 1). Accordingly, the transport channels were thoroughly reopened up only after endodormancy release (21 d chilling), based on the absorption and distribution of calcein fluorescent in the bud and the aniline blue fluorescent in the stem (Figs. 2 and 3). In *Populus*, calcein also reaches the vegetative bud apex after fully chilling requirement. But different to our results, calcein is not transported in insufficiently chilled plants resembling the situation in dormant leaf buds of hybrid aspen (Rinne et al., 2011), which might be due to different species or bud types, as the bud of tree peony is a typical mixed bud (Xin et al., 2019). Oppositely, GA feeding instantly induced the expressions of *PsBGs*, increased glucanases activities and declined glucan contents in the buds, accompanied by rapid sprouting and growth (Fig. 1). Remarkably, the transcripts of *PsBG9* were up-regulated by about 2530 and 2130 folds shortly after GA_3 and GA_4 applications (Fig. 6). Sufficient low temperature accumulation and GA treatment are effective measures to relieve dormancy (Looney, 1997; Saure, 1985). Therefore, it was concluded that low temperature and gibberellin treatments promoted the enhancement of glucanase activity, which led to the massive hydrolysis of callose, and opened up the material transport channel of flower buds, which promoted dormancy release of tree

peony. Meanwhile, the symplastic channels were gradually opened up by chilling accumulation, but rapidly by GAs feeding, in accordance with their morphological performance in tree peony. The reopening of the transport corridor ensured the efficient transportation of nutrients and signal substances for bud sprouting.

4.2. GAs promotes dormancy release in a species-dependent manner

It has been proved that hormones involve in endodormancy regulation, and among them GAs are particularly important and widely used in practice. GAs application can partially substitute for chilling in both vernalization and dormancy release (Saure, 1985). As known, GA_1 , GA_3 , GA_4 and GA_7 are the major bioactive GAs, of which GA_1 and GA_4 are the major endogenously active molecules in most plant species, and exogenous GA_3 is widely applied in dormancy breaking, such as in tree peony (Zhang et al., 2018), *Paeonia lactiflora* (Evans et al., 1990), azalea (*Rhododendron simsii* Planch) (Nell and Larson, 1974), tea (*Camellia sinensis*) (Thirugnanasambantham et al., 2020), apricot (*Prunus armeniaca* L.) (Kojori et al., 2018), etc. In fewer cases, GA_1 and GA_4 are also used (Rinne et al., 2011; Zhuang et al., 2015). Conversely, exogenous application of GA_3 delays the bud break in grape (Zheng et al., 2018). Thus, different GA species present distinct effects in bud dormancy release.

In hybrid aspen, GA_4 is the one functions in shoot elongation (Israélsson et al., 2004), and exogenous GA_4 induces canonical bud burst and development after 5 d treated, but GA_3 only induces callus-like tissue at the junction between bud scales and stem. Meanwhile, exogenous GA_4 promotes calcein transport to the apex of the non-chilled bud resembling the fully chilled buds, but GA_3 can't (Rinne et al., 2011). In our result, both GA_3 and GA_4 applications promoted bud burst and normal development, and GA_3 was superior to GA_4 for the more rapidly consequent growth (Fig. 1). Consistent with the morphological changes, GA_3 rapidly induced higher BGs activity and lower glucan content than GA_4 treatment in the bud. Consequently, the transport channels were gotten through by GA_3 feeding faster than GA_4 application (Fig. 2). Therefore, different species have diverse responses to GAs, despite of the same physiological phenomenon.

4.3. Members of *PsBGs* involved in transport channels regulation during dormancy release

Net callose deposition around the transport channels need to be hydrolyzed by β -1,3-glucanases prior to dormancy release. BGs belong to a big family subdividing to α , β , and γ clades. In *Arabidopsis*, more than 50 individuals of β -1,3-glucanases are found with different functions, and 10% of which have a cell wall-related function (Doxey et al., 2007). Meanwhile, over 100 putative BGs are identified in the *Populus trichocarpa* genome (Rinne et al., 2011). We explored 79 putative *PsBGs* unigenes in a full-length transcriptome of dormant tree peony bud, and all the *PsBGs* were clustered to α and β clades (Fig. 4), while no γ clade BG was found. We suspected that γ clade BGs might not express in the dormant bud, or γ clade BGs didn't exist in tree peony, an ancient species. In *Populus*, some LB-related γ clades BGs might be associated with PD reopening of dormant bud (Rinne et al., 2011). What is the characteristic and function of LB-related BGs in tree peony? should be further concerned.

LB-related BGs and individuals with GPI and/or X8 domain might be recruited to PD and function in callose breakdown during dormancy release processes (Doxey et al., 2007; Levy et al., 2007; Rinne et al., 2011). The members with GPI and/or X8 responding to chilling and GA_4 might function in the extracellular site of PD, while LB-related BGs function intracellularly in both chilling- and GA_3 -induced dormancy (Doxey et al., 2007; Rinne et al., 2011; Simpson et al., 2009). In tree peony, 92.4% *PsBGs* contained CBM43/X8 domain and 40.5% with GPI anchor. As mentioned above, chilling accumulation and GAs applications activated the expressions of β -1,3-glucanases genes and improved

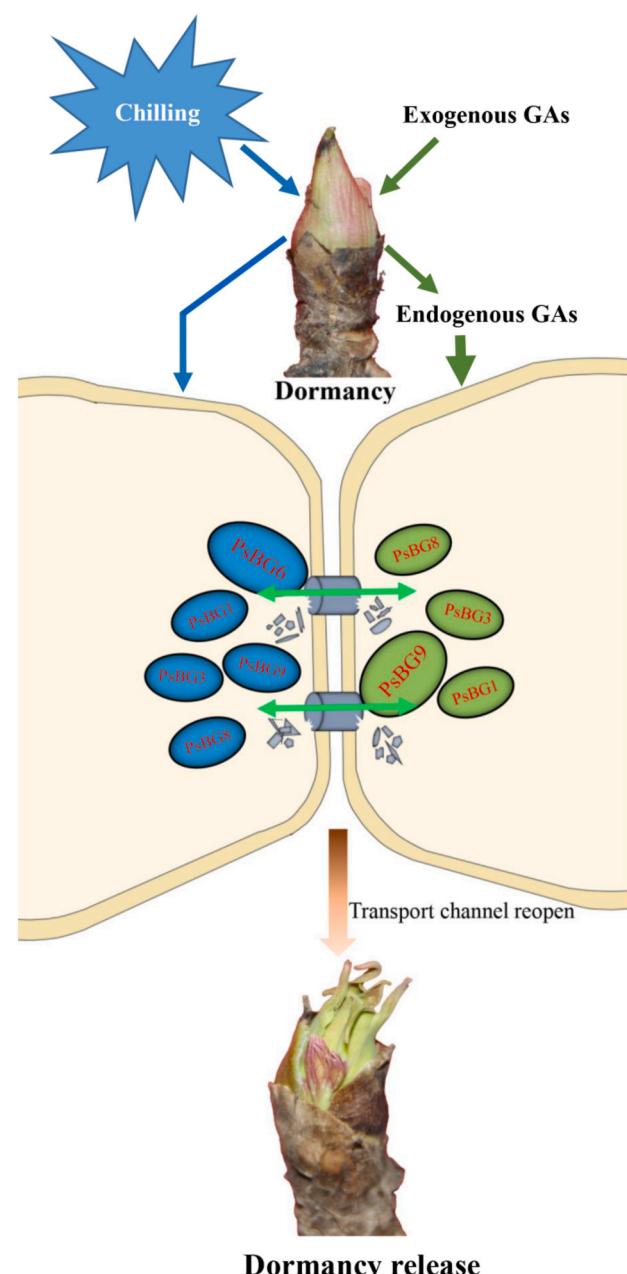
their activities benefiting to dormancy release. Thus, we attempted to screen the candidate BGs involving in the process. Differentially expressed *PsBGs* were screened and identified through a microarray database (Gai et al., 2013) and qPCR. Three criteria were set to determine the right BGs, i. e. presenting an inducibility to chilling accumulation and/or GAs, localization to PD and processing glucanases activity to hydrolyze glucan.

The results indicated that *PsBG1*, *PsBG3*, *PsBG4*, *PsBG7*, *PsBG8* and *PsBG9* were activated by low temperature with similar expression tendency, increasing only at the end of dormancy, while *PsBG6* was up-regulated at the early of chilling accumulation. The responses of *PsBGs* to both GA₃ and GA₄ were similar, but in different degrees. *PsBG1*, *PsBG3*, *PsBG4*, *PsBG8* and *PsBG9* genes were induced by GAs (Fig. 6). *PsBG6* and *PsBG9*, representing members with both X8 and GPI domain and sole X8 domain respectively, were localized to intracellular and extracellular site of plasma membrane, respectively (Fig. 7). The candidate *PsBGs* were prokaryotically expressed, and the purified products were tested for their ability to hydrolyze glucan. We found that the recombinational *PsBG1*, *PsBG3*, *PsBG5*, *PsBG6*, *PsBG8* and *PsBG9* had abilities to hydrolyze laminarin. The functions of *PsBG4* and *PsBG7* need to be tested further. Therefore, *PsBG1*, *PsBG3*, *PsBG6*, *PsBG8* and *PsBG9* involved in chilling-induced transport channels reopening, and *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* functioned in GAs-induced job.

Considering the channels were gradually opened up, there must exist some members to function at the early stage of dormancy. Among all the chilling induced members, *PsBG6* was the only candidate at the early dormancy stage (7 d), and with a 98-folds increase at 21 d when the channels were thoroughly reopened, while the others were only induced at the end of dormancy. The channels have been already gotten through at 21 d (Fig. 2), and there is no necessary to maintain high glucanases activities at 28 d unless other requirement. BGs function in many processes, such as pathogenesis, phloem transport and sputum movement, cell division, microtubule tissue transport regulation, plant aging and other life processes (Zavaliev et al., 2011). We concluded that *PsBG6* played a vital role in chilling-induced transport conduits reopening during dormancy breaking in tree peony.

Intriguingly, some members such as *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* were overlapped in chilling and GAs treatments. GA production is a downstream effect of chilling (Hazeboek et al., 1993; Yamauchi et al., 2004), and Mornya and Cheng (2013) believed that the accumulated GA is the key regulator of dormancy release in tree peony. Several key GA biosynthesis genes such as *GA3ox* and *GA20ox* are up-regulated by chilling accumulation and the bioactive GA content increases at the end of dormancy (Gai et al., 2013; Zhang et al., 2014, 2020). Similar results were reported in tea and *Populus* (Yue et al., 2018; Rinne et al., 2011). Meanwhile, up-regulation of GA deactivator *GA2ox1* at the end of chilling indicates the increase of GA biosynthesis during chilling accumulation (Thomas et al., 1999; Rinne et al., 2011), and still other evidence comes from the GA signaling pathway in Japanese apricot (Lin et al., 2018). Recently, we also found that exogenous GAs feedings enhance the endogenous GAs levels and induced faster bud burst, higher shoot and more flowers (Zhang et al., 2021). Thus, the increases of *PsBG1*, *PsBG3*, *PsBG8* and *PsBG9* transcripts by chilling might partially be due to the activation of GA biosynthesis.

In most cases, GA₄ induced higher *PsBGs* expression than GA₃ almost at the whole processes, but *PsBG9* was an exception. *PsBG9* transcript was quickly up-regulated just 0.5 d after GA₃ feeding with 2528 times increase, but required 2 d to reach the similar level after GA₄ feeding. Histologically, the transport conduits were opened up in depth after GA₃ application for 1 d, and more time required for GA₄ application. The results were also in accordance with morphological variation between the two GAs treatments. Taken together, we believed that *PsBG9* was the most essential member in GAs-induced transport conduits reopening, and the superiority of GA₃ to bud burst in tree peony might partially attribute to its higher expression at the early stage of GA₃ application.



Dormancy release

Fig. 9. Model of the *PsBGs* roles during chilling- and exogenous GAs- induced dormancy release in tree peony. Chilling accumulation accelerated the synthesis of endogenous GAs and gradually enhanced the expression of β -1,3-glucanases genes (shown as ellipses) and their activities, and finally reopened the PD channels by hydrolysis of callose along with dormancy release; The exogenous GAs improved the levels of endogenous GAs, and enhanced the expression of β -1,3-glucanases genes and their activities to reopen the PD channels, but functioned quickly. Cylinders indicated plasmodesmata (PD) connecting two cells. *PsBG6* played a central role during chilling-induced dormancy release (blue), and *PsBG9* was central in GAs-induced dormancy release (green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

5. Conclusion

The transport channel apertures are blocked by callose when tree peony buds enter into endodormancy, and must be reopened by β -1,3-glucanase before dormancy release. Chilling accumulation gradually enhanced the expression of β -1,3-glucanases genes and their activities, and finally got through the channels along with dormancy release.

PsBG6 played a central role during chilling-induced dormancy release. Both GA₃ and GA₄ applications rapidly activated β -1,3-glucanases and unchoked the channels, and accelerated bud burst and subsequent growth, but GA₃ was preferable. GAs might function by stimulating the expression of *PsBG9* together with *PsBG1*, *PsBG3* and *PsBG8*. This paper assumed a potential mechanism how the dormancy breaking factors, chilling and GAs, work in reopening the transport channels, and also identified the key *PsBGs* involving in the process (Fig. 9). The results will benefit to understand the functions of *PsBGs* and the dormancy regulation mechanism, and also the improvement of the forcing culture protocol.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2021.09.002>.

Contributions

GSP and ZYX conceived and designed the experimental plan. GXK, YYC, LZQ, XH and LCY conducted the experiments. GXK, ZYX and YYC analyzed the data. ZYX and GSP prepared and revised the manuscript. All authors read and approved the final manuscript.

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