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The involvement of *PsTCP* genes in hormone-mediated process of bud dormancy release in tree peony (*Paeonia suffruticosa*)

Qianqian Wang[1], Bole Li[1], Zefeng Qiu[1], Jiayi Ying[1], Xuyichen Jin[1], Zeyun Lu[1], Junli Zhang[2], Xia Chen[1*] and Xiangtao Zhu[1*]

Abstract

Background The complete dormancy release determines the quality of bud break, flowering and fruiting. While in tree peony (*Paeonia suffruticosa* Andr.), the insufficient accumulation of cold temperatures results in incomplete dormancy release and poor flowering quality.

Results In order to investigate if phytohormone can replace the chilling requirement in south China and other similar regions, the roles of fluridone (Flu), gibberellin (GA_3), and their combination in the bud dormancy release process were analyzed. It demonstrated that the application of exogenous GA_3 and the mixture significantly expedited the dormancy release of tree peony at 23℃. Furthermore, the endogenous hormone levels provided evidence for the substantial impact of exogenous GA_3 on dormancy release, highlighting its potential involvement in the chilling-independent pathway of dormancy release. Transcriptome sequencing and analysis of expression profiles were conducted to identify the crucial genes implicated in the dormancy release mechanism of tree peony. Among numerous genes from diverse gene families, the particularly interesting were *TEOSINTE BRANCHED 1*, *CYCLOIDEA*, *and PROLIFERATING CELL FACTORS-like* genes (*PsTCP3*, *PsTCP4*, and *PsTCP14*), which had significant expression levels during the dormancy release process under different treatments. They were divided into two distinct sub-families based on their different domains. Specifically, *PsTCP14* was classified under Class I, while *PsTCP3* and *PsTCP4* were classified under Class II. The analysis of expression patterns revealed a significant accumulation of the three *PsTCPs* in buds undergoing dormancy release, with clear upregulation observed in response to GA_3 and the mixture treatments. Additionally, the analysis of promoter activity demonstrated the sensitivity of *PsTCP4* and *PsTCP14* to GA_3 and Flu.

Conclusion The application of exogenous GA_3 has been shown to effectively expedite the release of dormancy in tree peony through a pathway that is not dependent on chilling. Further research found that *PsTCP* genes might play a crucial role in this process. These findings contribute to the understanding of the molecular mechanism of *PsTCPs* in the hormone-mediated and temperature-independent release of bud dormancy in tree peony.

*Correspondence:
Xia Chen
cx2912@zafu.edu.cn
Xiangtao Zhu
zhuxt@zafu.edu.cn

Full list of author information is available at the end of the article

Keywords Bud dormancy release, Hormone signalling, *Paeonia suffruticosa*, TCP transcriptional factor, Transcriptome sequencing

Background

Tree peony (*Paeonia suffruticosa* Andr.), a renowned flowering plant originated from China, possesses notable ornamental, nutritional, and medicinal properties [1, 2]. As a deciduous shrub, tree peony exhibits bud endodormancy, which is the deepest stage of dormancy and is not released until chilling requirements have been fulfilled under natural condition [3]. The primary approach for breaking dormancy in tree peony, similar to other perennial plants, entails providing an adequate chilling period during winter, followed by stimulating growth in the subsequent spring through a suitably warmer environment [4, 5]. In regions such as south China and other warm-winter areas, the elevated ambient temperature during winter results in inadequate accumulation of cold temperatures for tree peony plants [4]. Consequently, this insufficient cold accumulation leads to incomplete release of bud dormancy and subsequently poor flowering quality in the following spring [6, 7]. As a result, the expansion of tree peony cultivation in warm-winter regions is significantly constrained.

Bud dormancy, characterized by the temporary cessation of visible growth, represents a vital stage in the physiological cycle necessary for survival during cold winter conditions, which was regulated by a multitude of external and internal factors [8]. Among these factors, temperature plays prominent roles [9]. Research conducted on *Populus* [10] and *Picea mariana* [11] has demonstrated that the occurrence of bud dormancy release can be influenced by the interaction between winter chilling and spring warming. Additionally, phytohormones such as gibberellin (GA) and abscisic acid (ABA) have been identified as crucial factors in the release of bud dormancy [12, 13]. For instance, the application of GA exhibited a substantial enhancement in the release of dormancy and stimulation of flowering in Japanese apricot [14]. Similarly, when *Paeonia lactiflora* encountered insufficient chilling accumulation, the treatment with GA_3 effectively disrupted dormancy, expedited sprouting, and facilitated subsequent growth and flowering [15, 16]. ABA is widely acknowledged as a fundamental hormone involved in the maintenance and regulation of bud dormancy, exerting an antagonistic effect to GA [17, 18]. Furthermore, some transcription factors have been involved in dormancy release process, and *TEOSINTE BRANCHED 1, CYCLOIDEA, and PROLIFERATING CELL FACTORS-like* genes (*TCPs*) play an important role [9]. *TCPs* are plant-specific transcription factors that have been shown to act as modulators of hormone synthesis, transport and signal transduction [9]. In *Arabidopsis*, *AtTCP12* could regulate the accumulation of ABA and promote axillary bud dormancy [19]. *GhTCP19* from *Gladiolus hybridus* could modulate corm dormancy release by increasing cytokinin levels [20]. In *Prunus persica*, *PpTCP20* acted as a transcriptional promoter to regulate bud dormancy release [21].

Over the past two decades, numerous studies have been conducted to investigate the release of dormancy and elucidate its molecular mechanism in tree peony. Various measures, including the application of artificial chilling, exogenous GAs, garlic paste, and 5-azacytidine (5-azaC), have been employed to facilitate dormancy release [22–24]. Notably, the adequate application of low temperature and exogenous GAs treatment have proven to be effective and widely adopted methods for breaking bud dormancy in tree peony [22]. The application of chilling treatment resulted in notable changes in morphology, specifically an increase in the percentages of sprouting and blossoming [22]. It showed that After a duration of about 96 hours at 0–4℃, nearly 100% of the apical buds sprouted and subsequently blossomed [22, 25]. In the context of tree peony, the introduction of exogenous GAs proved effective in partially substituting the chilling treatment to expedite dormancy release. Among the various GAs tested, GA_3 exhibited the most significant impact [23]. Mornya et al. (2018) observed that the combination of exogenous GA_3 and chilling treatments contributed to the promotion of dormancy release in *P. suffruticosa* 'Luoyanghong' by reducing ABA content [25].

Although GAs and ABA have demonstrated significant efficacy in inducing dormancy release, further investigation is required to determine if they can replace the chilling requirement in south China and other similar regions. Fluridone (Flu), a synthetic inhibitor of ABA, effectively reduces the ABA content of buds, which promotes dormancy release in plants [26]. In this study, the effects of GA_3 and Flu on dormancy release in tree peony were examined. The observed morphological changes indicated that both exogenous GA_3 and a combination of GA_3 and Flu significantly expedited bud dormancy release under 23℃ and natural conditions. Notably, the combined treatment exhibited a superior effect compared to the individual GA_3 treatment. To gain insights into the impact of exogenous GA_3, Flu, and their combination on dormancy release, we conducted transcriptome sequencing and identified differentially expressed genes (DEGs) in tree peony subjected to various treatments. Among the DEGs, we focused our attention on *PsTCPs* and conducted an analysis of their expression pattern and promoter activity under various exogenous

hormone treatments. The findings of this study offered significant contributions to understanding the mechanism underlying dormancy release in tree peony, as well as its potential applications in cultivation and anti-season culturing production in warm-winter regions.

Materials and methods

Plant materials and treatment methods

The plant material used for this study was *P. suffruticosa* 'High Noon', which was cultivated at Jiyang College of Zhejiang A&F University (Zhejiang, China, 29°75′52′′N, 120°26′12′′E). All materials for transcriptome sequencing were obtained from 5-year-old trees that had reached the flowering stage. All the plants were thrived in greenhouse with native light conditions, and the temperature was recorded in Supplementary Figure S1A. These materials were subsequently stored at -80℃.

All plants were divided into two groups, with one group cultivated in greenhouse under a 14-hour light/10-hour dark cycle at 23℃, and the other group grown in an open farm with natural conditions (natural conditions). Each group had twelve plants used for four different treatments (Control, Flu, GA_3, and the mixture of Flu and GA_3). Plants underwent treatments beginning on 25 November 2022 (designated as day 0) prior to cooling (Supplementary Figure S1). For the exogenous hormone treatments, the dormant buds of tree peony were subjected to a spray application of an aqueous solution consisting of 0.1% (v/v) phosphoric acid, 0.025% (v/v) Triton X-100, and 300 mg/L of GA_3, Flu and 300 mg/L of each in the mixture (Dingguo Biotechnology Co., Ltd., Guangzhou, China). The control plants were sprayed with a solution containing only 0.1% (v/v) phosphoric acid and 0.025% (v/v) Triton X-10. The treatments were administered daily at 2 pm for a duration of five days. Phenotypic observations and samples were taken every three days. For tissue specificity, all samples were collected under natural conditions, and except for dormant buds, others were sampled after dormancy release. For period specificity, all samples were also collected under natural conditions at different stages. The resulting phenotypic alterations in the tree peony were subsequently examined using a stereo microscope (Carl Zeiss, Oberkochen, Germany).

Transcriptome sequencing

RNA extraction was conducted on a total of 12 samples from four different treatments under 23℃, including Control, GA_3, Flu, and the mixture of GA_3 and Flu. Each treatment had three biological repeats. The RNA prep Pure Plant Kit (Vazyme, Nanjing, China) was utilized for the extraction, following the instructions provided by the manufacturer. Specifically, according to the phenotypic changes, the samples subjected to the mixture treatment were collected on the 12th day, while the samples treated with Control, Flu and GA_3 were collected on the 15th day. Each sample contained three buds from different plants, which were taken randomly. The integrity of the extracted RNA was assessed using the Bioanalyzer 2100 system (Agilent Technologies, USA). The Ribo-ZeroTM Magnetic Kit was utilized for the purpose of enriching prokaryotic mRNA through the removal of rRNA, and eukaryotic mRNA was enriched using Oligo (dT) beads (Epicentre). Subsequently, the enriched mRNA underwent reverse transcription into cDNA employing random primers and fragmentation buffer. The second-strand cDNA synthesis was accomplished using DNA polymerase I, RNase H, dNTP, and buffer. Following the end-repair of the cDNA fragments, poly(A) was introduced. The isolation and ligation of Illumina sequencing adapters were carried out using the QiaQuick PCR extraction kit. Illumina NovaSeq 6000 sequencing was performed by the Novogene Experimental Department located in Beijing, China. The ending reading of 150 bp pairing is generated. By deleting adapter-containing, N base-containing, and low-quality reads from the raw data, clean data were produced. Direct downloads of the reference genome and gene model annotation files were made from the genome website (https://db.cngb.org/search/project/CNP0000281/). Hisat2 (v2.0.5) was used to create an index of the reference genome and to match paired-end clean reads to the reference genome (v2.0.5). The length of each gene and the number of reads mapped to it were used to compute the FPKM of each gene.

Analysis of differentially expressed genes (DEGs) and enrichment

The DESeq2 R package version 1.20.0 was employed for the analysis of DEGs within samples or groups. This package utilized a model to offer statistical procedures for discerning differential expression in digital gene expression data. To adjust the obtained *P*-values, the Benjamini and Hochberg method was employed to reduce the false discovery rate. Genes were considered significant DEGs when they exhibited the absolute fold change on the log scale ≥ 2 and a *P*-value below 0.05 in a comparison.

In the case of GO terms, they were considered substantially enriched by DEGs if the adjusted *P*-value was below 0.05. The KEGG database is utilized for the interpretation of complex biological system functions and the extraction of valuable information from molecular-level data, specifically large-scale molecular datasets generated through genome sequencing and high-throughput experimental techniques (http://www.genome.jp/kegg/). To assess the statistical enrichment of differentially expressed genes (DEGs) within KEGG pathways, we employed the cluster Profiler R program.

RNA extraction for quantitative Real-Time PCR analysis

Total RNA was isolated using the RNAprep Pure Plant Kit (TianGen, Beijing, China), and its quality was evaluated using a nucleic acid analyzer (Implen Company in Germany). First-strand cDNA synthesis was performed using the PrimeScriptTM RT reagent Kit (TaKaRa, Dalian, China).

The related gene sequences were identified from the genomic data of tree peony downloaded from the genome website through the Blast X annotation [27]. Primer Premier 5 software was utilized for the design of qRT-PCR primers (Supplementary Table S1), with *PsACT* serving as the reference gene [27]. The expression levels of the relevant genes were assessed through quantitative real-time polymerase chain reaction (qRT-PCR) using the Light Cycler 480II Real Time PCR system (Roche, Basel, Switzerland). The reaction system consisted of 10 μL SYBR Premix Ex Taq, 2 μL cDNA, 0.8 μL each of upstream and downstream primers (10 μmol/L), and ddH_2O to a final volume of 20 μL. The reaction procedure was 95℃ for 30 s, 95℃ for 5 s, 60℃ for 30 s, a total of 40 cycles; 95℃ for 5 s, 60℃ for 1 min, 95℃ for 15 s. All qRT-PCR experiments were conducted with three biological replicates. The relative expressions were calculated by $2^{-\triangle\triangle}$CT method. Data analysis was performed by SPSS 19.0 statistical software and GraphPad Prism 8.

Sequence and phylogenetic analysis

Full-length open reading frames (ORFs) of PsTCPs were amplified using the first strand cDNA of buds from 'High Noon' with cloning primers (Supplementary Table S1). Sequences from *Paeonia lactiflora* (PlTCP3), *Camellia lanceoleosa* (ClTCP3), *Jatropha curcas* (JcTCP3), *Camellia sinensis* (CsTCP3), *Vitis vinifera* (VvTCP3), *Pistacia vera* (PvTCP4), *Populus alba* (PaTCP4), *Ricinus communis* (RcTCP4), *Hevea brasiliensis* (HbTCP4), *Mangifera indica* (MiTCP4), *Juglans regia* (JrTCP14), *Senna tora* (StTCP14), *Nicotiana tabacum* (NtTCP14), *Populus trichocarpa* (PtTCP14), *Cucumis melo* (CmTCP14) were downloaded from GenBank. Sequence alignment of *PsTCPs* sequences from 'High Noon' was performed using DNAMAN 7.0, and the maximum likelihood method PhyML was constructed using MEGA X.

Promoter cloning and prediction of the cis-elements in the promoter of*PsTCPs*.

DNA was extracted from the buds of the 'High Noon' using a DNA extraction kit (Vazyme, Nanjing, China), following the instructions provided by the manufacturer. The upstream sequences of the *PsTCPs*-coding sequences were obtained from the genome database of *P. ostii* 'Fengdan' [27]. The primers for the *PsTCPs* promoter were designed using Primer Premier 5 software, based on the transcriptome and genome of tree peony. The cis-acting regulatory elements in the PsTCPs promoter were analyzed using the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 23 November 2023).

Determination of endogenous hormone content

The sampling method and time of samples for endogenous hormone content were similar to transcriptome sequencing. The endogenous hormone content was measured by Ruiyuan Biotechnology Co. Ltd (Nanjing, China) through HPLC-MS/MS method. About 1 g plant sample was weighed and grounded in liquid nitrogen; Besides that, acetonitrile solution with 1:10 v/v (sample: acetonitrile solution) and 4 μL internal standard liquid were added (all the hormone standards were purchased from sigma company); Extraction was performed overnight at 4℃, and then centrifuged at 12,000 g for 5 min. Supernatant was absorbed and acetonitrile solution with 1:5 v/v was added to the supernatant and extracted twice. Added about 35 mg C_{18} was purify impurities, followed by centrifugation at 12,000 g for 5 min. After that, the supernatant was taken. Subsequently, the samples were blown dry with nitrogen and dissolved with 200 μL methanol. After that, they were passed through a 0.22 μm organic phase filter, and placed in a refrigerator at -20℃ for testing on the machine.

Luciferase assay

To investigate the regulatory effects of various plant hormones on *PsTCPs*, the Luciferase assay was conducted. The promoter region of *TCPs* was cloned into the pGreen0800II-LUC vector, and the vectors containing *PsTCPspro*::LUC and pGreen0800II-LUC were separately introduced into GV3101 (including pSoup-p19). Subsequently, *PsTCPspro*::LUC and pGreen0800II-LUC were transiently transformed into the leaves of *N. tabacum*. Following a 24-hour dark cultivation period at 23℃, different treatments were administered, including Control, Flu, GA_3, the mixture of Flu and GA_3 with 300 mg/L, and different ambient temperature. The activities of the luciferase enzyme were measured post-treatment.The activities of firefly luciferase (LUC) and renilla luciferase (REN) were measured through the GLOMAX® multifunctional instrument (Promega, USA).

Predicting protein interaction networks

To facilitate a deeper understanding of the potential interactions between *PsTCPs* and other proteins, we utilized the online software SRING v11.5 (https://cn.string-db.org/) to construct a protein interaction network map. The chosen approach involved utilizing the '*PsTCPs*' as the method and '*Arabidopsis*' as the organism. Default parameters were employed for all other aspects. Ultimately, the protein interaction network map was generated by selecting *A. thaliana* proteins with the highest

similarity of PsTCPs proteins based on the bit score and E-value.

Statistical analysis

The statistical analyses were performed using SPSS (16.0 version; SPSS Inc., Chicago, IL, USA). Data was expressed as the mean ± SD with three biological replicates for each. According to Duncan's multiple range test, differences were assessed by one-way analysis of variance (ANOVA) at $P < 0.05$.

Results

The dormancy release of tree peony under different treatments

To assess the effects of GA_3 and Flu on bud dormancy release of tree peony in warm-winter regions, *P. suffruticosa* 'High Noon' was treated with 300 mg/L GA_3, Flu and the mixture under both the 23℃ and natural condition. The growth status of buds was assessed and the results showed that under natural conditions, both the treatments of exogenous GA_3 and mixed solution of GA_3 and Flu could promote bud dormancy release (new leaves protruding from the tip of bud) (Fig. 1, Supplementary Table S2). It suggested that dormancy release was observed on the 25th day under the mixture treatment and the 30th day under GA_3 treatment. While in contrast to the control group, the independent application of exogenous Flu treatment did not yield any noticeable impact on the release of bud dormancy in tree peony, as the bud dormancy release was observed on the 80th day (Fig. 1A, Supplementary Figure S2).

Moreover, similar results were observed at a temperature of 23℃. The phenotypic changes observed indicated that dormancy breaking occurred on the 12th day under the mixture treatment, while dormancy release occurred on the 15th day under exogenous GA_3 treatment. Additionally, all buds treated with exogenous Flu remained dormant during the treatment phase and their phenotype did not significantly differ from the control (Fig. 1B, Supplementary Figure S2, Supplementary Table S2). These findings suggest that in south China, exogenous GA_3 and the mixture of Flu and GA_3 treatment has a significant impact on bud dormancy release under both natural and 23℃ conditions. Additionally, the exogenous Flu treatment had limited effect, resulting in outstanding performance of the mixture treatment than separate GA_3 treatment, but exogenous Flu treatment had no obviously function on dormancy release.

Transcriptome sequencing of buds under different treatments in tree peony

To screen the vital genes involved in dormancy release of tree peony, we collected samples from Control, Flu, GA_3 and the mixture treatments for RNA-seq analysis. A total of 85.1 Gb of RNA-seq clean reads were generated from the 12 samples with a range from 6.5 to 7.6 Gb in each sample (Supplementary Table S3). In those libraries, the Q20 was > 96.64%, and the Q30 was > 91.18%. The GC content was 43.9% on average in the 12 libraries (Supplementary Table S3). We filtered 95,021 differentially expressed unigenes (DEGs) under three comparisons, including Control vs. Flu (C1), Control vs. GA_3 (C2), and Control vs. the mixture (GA_3 + Flu, C3), which were annotated using NR [28], NT [28], KO [29], KOG [30], PFAM [30], GO [31] and SWISSPROT [32] databases. The covering annotation data based on the above seven databases were 39.69%, 25.04%, 12.37%, 26.29%, 27.78%, 27.35% and 6.82%, respectively (Supplementary Table S3).

In addition, we analysed the DEGs in the three comparisons, including C1, C2, and C3 (Fig. 2). It was found that 1266 DEGs were upregulated and 1216 DEGs were downregulated in the C1 comparison. Meanwhile, 3493 and 4970 DEGs in the C2 comparison were up- or downregulated, respectively. In the C3, 4360 DEGs were upregulated, and 5718 DEGs were downregulated (Fig. 2B). From the Venn diagram, 639 DEGs were upregulated in all three comparisons, and 195 DEGs were upregulated in both the C1 and C2 comparisons. A total of 237 and 1374 DEGs were upregulated in the C1 and C3 comparisons, respectively (Fig. 2C). Simultaneously, 783 DEGs were downregulated in the three comparisons, and only 77 DEGs were downregulated in both the C1 and C2 comparisons. A total of 173 and 2775 DEGs in the C1 and C3 comparisons were downregulated, respectively (Fig. 2D).

GO enrichment and KEGG pathway analysis of DEGs

In all three comparisons, 'cellular process' and 'metabolic process' were the most significantly enriched GO terms in the biological process (Fig. 3A). In the cellular component, 'cellular anatomical entity', 'intracellular' and 'protein-containing complex' were highly enriched, whereas 'binding' and 'catalytic activity' from molecular function were obviously enriched among the three comparisons (Fig. 3A). Among the KOG functional classifications, 6488 DEGs were categorized into 26 groups. The most abundant annotations were enriched in 'posttranslational modification/ protein turnover/chaperones' (O, 946; 14.58%), 'general function prediction' (R, 812;12.52%) and 'Translation, ribosomal structure and biogenesis' (J, 781; 12.04%). The least abundant annotations were enriched in 'Cell motility' (N, 2; 0.03%) and 'Extracellular structures' (W, 5; 0.08%; Fig. 3B). For KEGG pathway enrichment, the DEGs were annotated into 323, 373 and 369 pathways in the three comparisons respectively. In the three comparisons, Metabolic pathways was the most highly enriched pathway, followed by Biosynthesis of secondary metabolites (Fig. 3C-E, Supplementary Table S4).

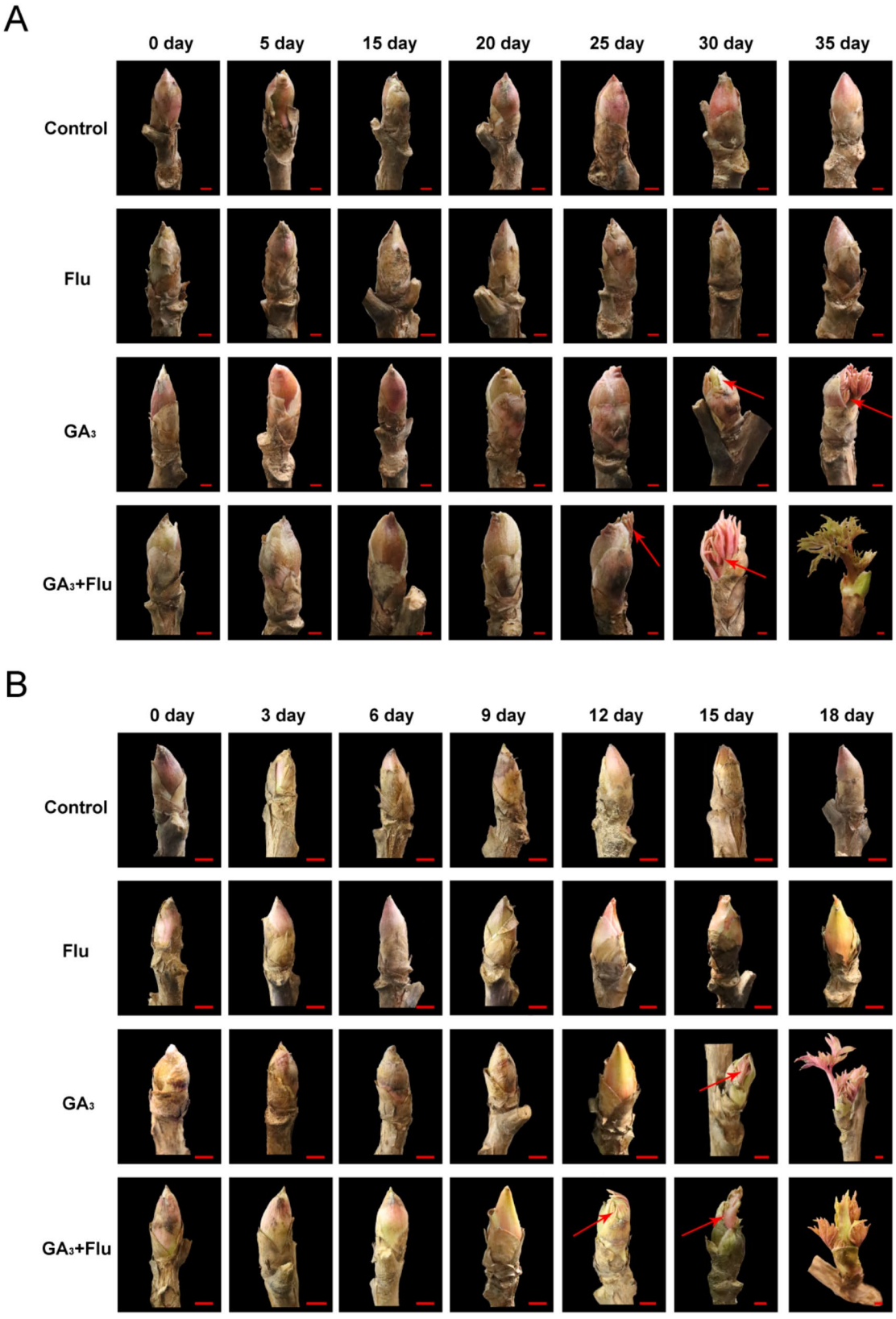

Fig. 1 The effect of exogenous hormone in bud dormancy release of tree peony. (**A**) The phenotype changes of buds under different treatments at natural condition. (**B**) The phenotype changes of buds under different treatments at 23℃. Control: the control without exogenous hormone treatment. GA: exogenous gibberellin treatment. Flu: exogenous fluridone treatment. Red arrow: the dormancy release buds with new leaves protruding from the tip. Red bars: 0.5 cm

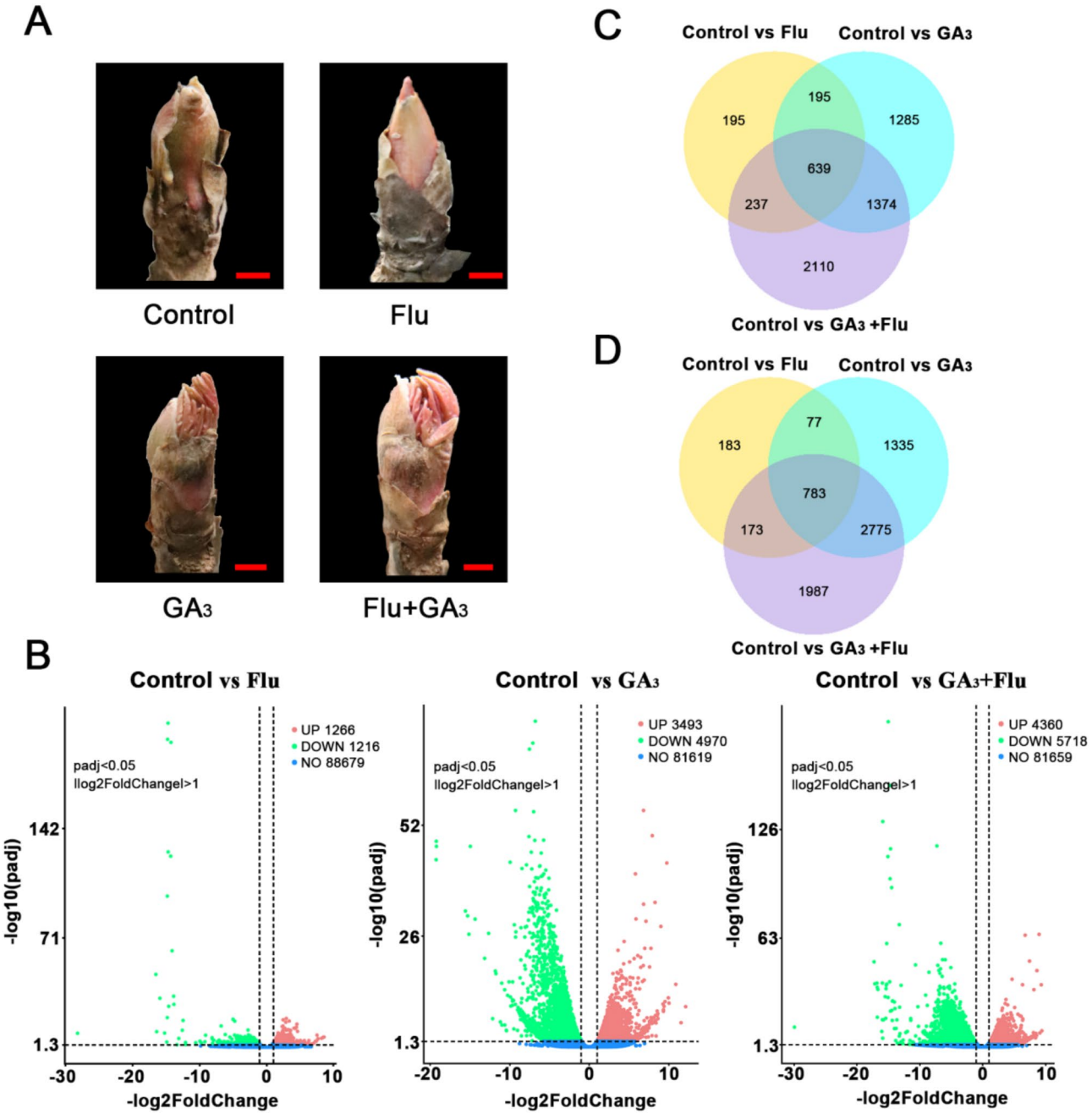

Fig. 2 Comparative analysis of DEGs among different treatments. (**A**) Samples for transcriptome sequencing analysis. Control: the control without exogenous hormone treatment. GA: exogenous gibberellin treatment. Flu: exogenous fluridone treatment. Red bars: 0.5 cm. (**B**) The expression trends of DEGs in the three comparisons. Green circles: down-regulated genes; red circles: up-regulated genes; blue circles: no differential expressed genes. (**C**) Venn diagram of the number of up-regulated genes among the three comparisons. (**D**) Venn diagram of the number of down-regulated genes among the three comparisons

DEGs under different treatments in tree peony

In order to ascertain the potential genes implicated in the release of dormancy under various phytohormone treatments, an analysis was conducted on the genes exhibiting significant differential expression in the three comparisons (Fig. 4). The findings revealed differential expression of numerous factors belonging to diverse families associated with dormancy release, such as ERF/AP2, IAA, MYB, TCP, WRKY, bHLH, bZIP, NAC, and HSF.

In the ERF/AP2 family, the expression levels of *PsERF14*, *PsERF109*, *PsERF10*, *PsERF21*, *PsRAP2-12*, *PsCRF2*, and *PsERF1* exhibited a clear decrease in all three treatments. Conversely, seven genes belonging to the ERF/AP2 family (*PsERF54*, *PsERF20*, *PsERF113*,

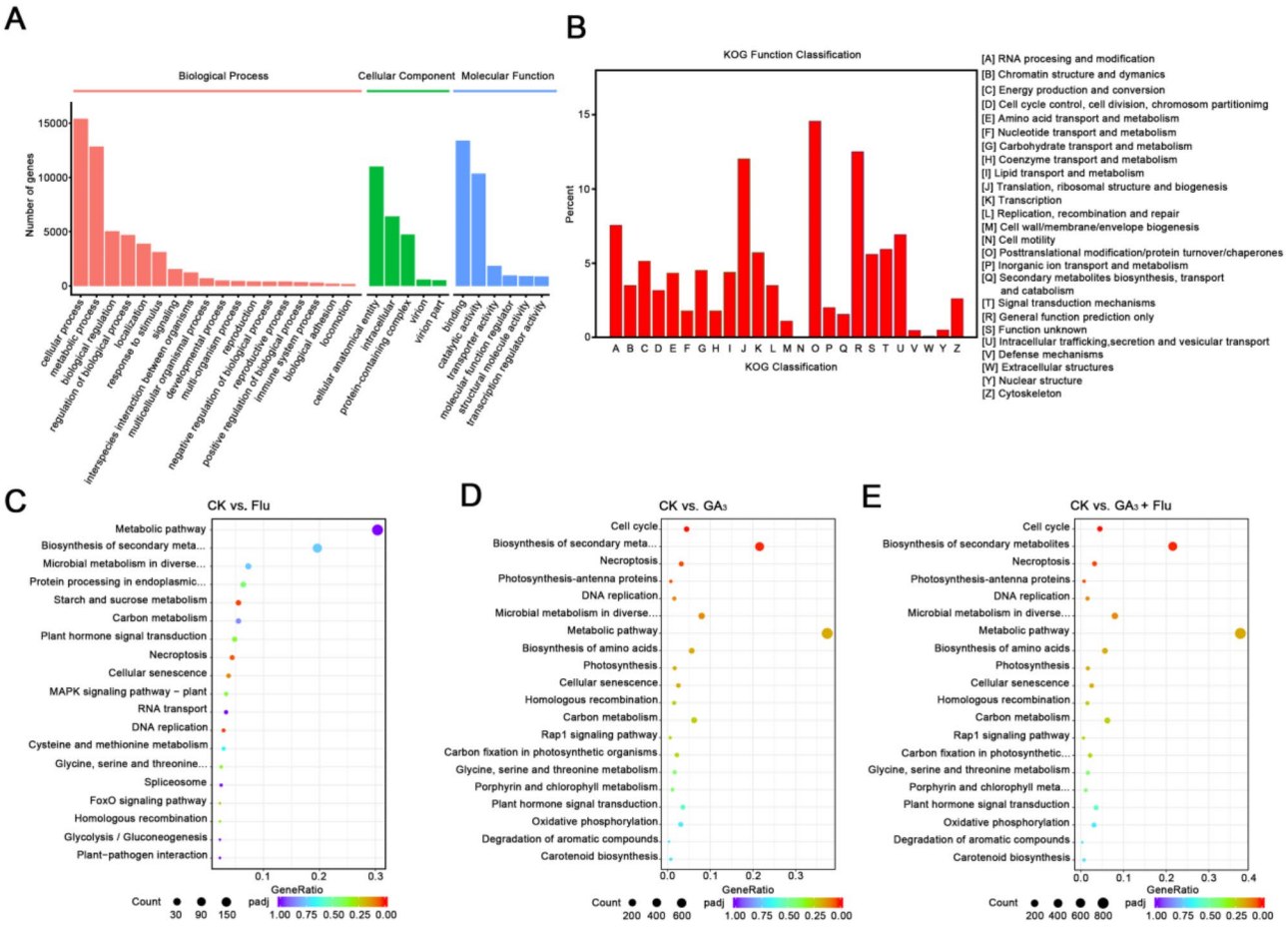

Fig. 3 Enrichment analysis of differentially expressed genes among different treatments. (**A**) GO function classification of DEGs among different treatments. (**B**) KOG function classification of DEGs among different treatments. (**C**) The top 20 of KEGG pathway enrichments of DEGs in Control vs. Flu comparison. (**D**) The top 20 of KEGG pathway enrichments of DEGs in Control vs. GA_3 comparison. (**E**) The top 20 of KEGG pathway enrichments of DEGs in Control vs. GA_3 + Flu comparison

PsERF3, *PsERF61*, *PsAP2-L1*, and *PsERF62*) displayed an increase in expression across all three treatments. Within the IAA family, only two members (*PsIAA14* and *PsIAA17*) showed downregulation under all three treatments, and the remaining genes exhibited significant upregulation in the GA_3 treatment. In the TCP family, it was observed that three genes (*PsTCP3*, *PsTCP4*, and *PsTCP14*) were notably upregulated in all three treatments. Three genes belonging to the bZIP family, namely *PsTGA2*, *PsTGA9*, and *PsTGA1*, exhibited significant downregulation across all three treatments. Conversely, five genes from the same family, namely *PsbZIP53*, *PsbZIP18*, *PsbZIP11*, *PsbZIP34*, and *PsbZIP61*, were upregulated. Within the WRKY family, the majority of genes displayed downregulation in the GA_3 treatment, with only *PsWRKY70* and *PsWRKY40* being concurrently increased in both the GA_3 and mixed treatments. In the NAC family, the expression levels of seven genes (*PsNAC71*, *PsNAC98*, *PsNAC21*, *PsNAC55*, *PsNAC47*, *PsNAC92*, and *PsNAC100*) were observed to decrease, while *PsNAC8*, *PsNAC35*, and *PsNAC75* exhibited increased expression across all three treatments. In one of the three comparisons, 16 genes belonging to the bHLH family exhibited notable differential expression, with four genes being downregulated and 12 genes being upregulated. Conversely, within the MYB family, the expression levels of *PsMYB1*, *PsMYB44*, and *PsMYB105* were found to be decreased in all three treatments. On the other hand, *PsMYB5*, *PsMYB3R-5*, *PsMYB113*, and *PsMYB3R-1* displayed an opposing trend, with *PsMYB3R-5* and *PsMYB3R-1* showing increased expression in all three treatments. Additionally, four genes (*PsHSFB-2a*, *PsHSFA-6b*, *PsHSF24*, and *PsHSF30*) within the HSF family exhibited significant decreased expression in all three treatments (Fig. 4).

Endogenous hormone content and related gene expression analysis

To assess the levels of endogenous GAs and ABA in response to different treatments, the separate buds had

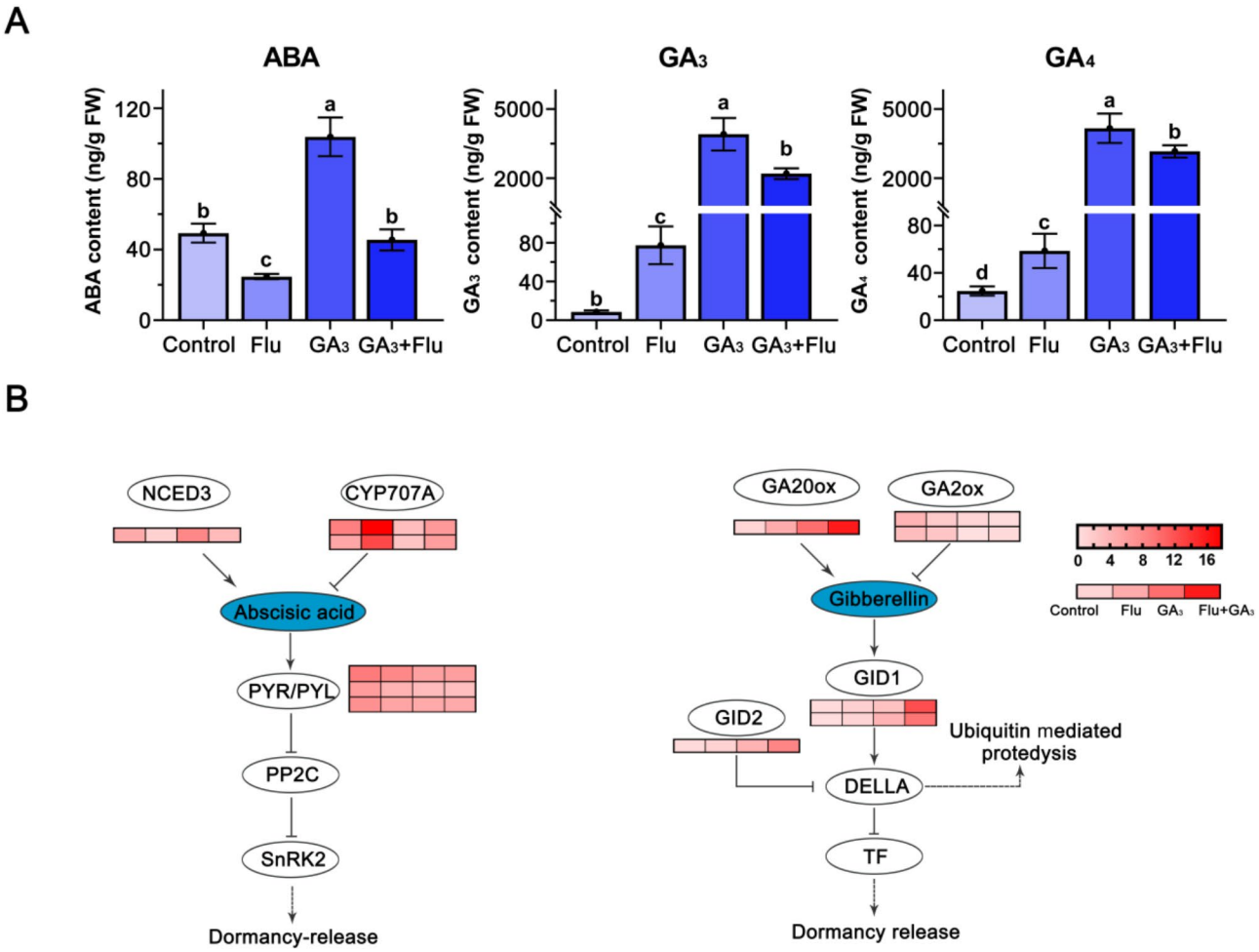

Fig. 4 The determination of endogenous hormone content under different treatments. (**A**) The determination of endogenous hormone content. Different letters indicated significant differences, Student's t test, $P < 0.05$, $n = 3$. (**B**) The heatmap of plant hormone signal transduction-related DEGs under different treatments. The data used in the heatmap sections was the multiple of FPKM difference from the transcriptome data. Means ± SD, $n = 3$, $P < 0.05$

relative development stage of that to the transcriptome sequencing were collected for measure. This experiment indicated that the content of GA_3 and GA_4 were higher under the three treatments than Control, suggesting that GA displayed an important role in dormancy release process (Fig. 5A). While the content of GA_1 and GA_7 was too low, which could not be measured. In addition, the content of ABA was increased under the GA_3 treatment and decreased under the Flu treatment. The results suggested that in tree peony, exogenous GA_3 treatment could promote ABA biosynthesis, while exogenous Flu treatment could increase GA content.

Gene expression analysis showed that in the ABA signaling pathway, the expression level of *PsCYP707A* was significantly decreased after GA treatment, and significantly increased after Flu and the mixed treatment, which was consistent with the endogenous ABA content in plants (Fig. 5B). The expression level of *PsNECD3* gene was significantly increased after GA treatment. In addition, the expression levels of the ABA receptor *PsPYL/PsPYLR* were significantly decreased, which were consistent with the contents of endogenous ABA (Fig. 5B). While in the GA signaling pathway, the GA synthesis promoter *PsGA20ox* was upregulated in the Flu and GA_3 treatments (Fig. 5B). On the contrary, the expression level of *PsGA2ox*, which could promote GA degradation, was decreased under the three treatments. These results were consistent with the determination of endogenous GA_3 and GA_4, suggesting the exogenous application of GAs induced their endogenous synthesis. In addition, it was found that the GA receptor *GIBBERELLIN INSENSITIVE DWARF1* (*PsGID1*) was upregulated under the three treatments, especially the exogenous GA_3 and the mixture applications. PsGID2 is a known F-box protein and encode a subunit of ubiquitin E3 ligases, which was increased after exogenous GA_3 and the mixture treatments [15].

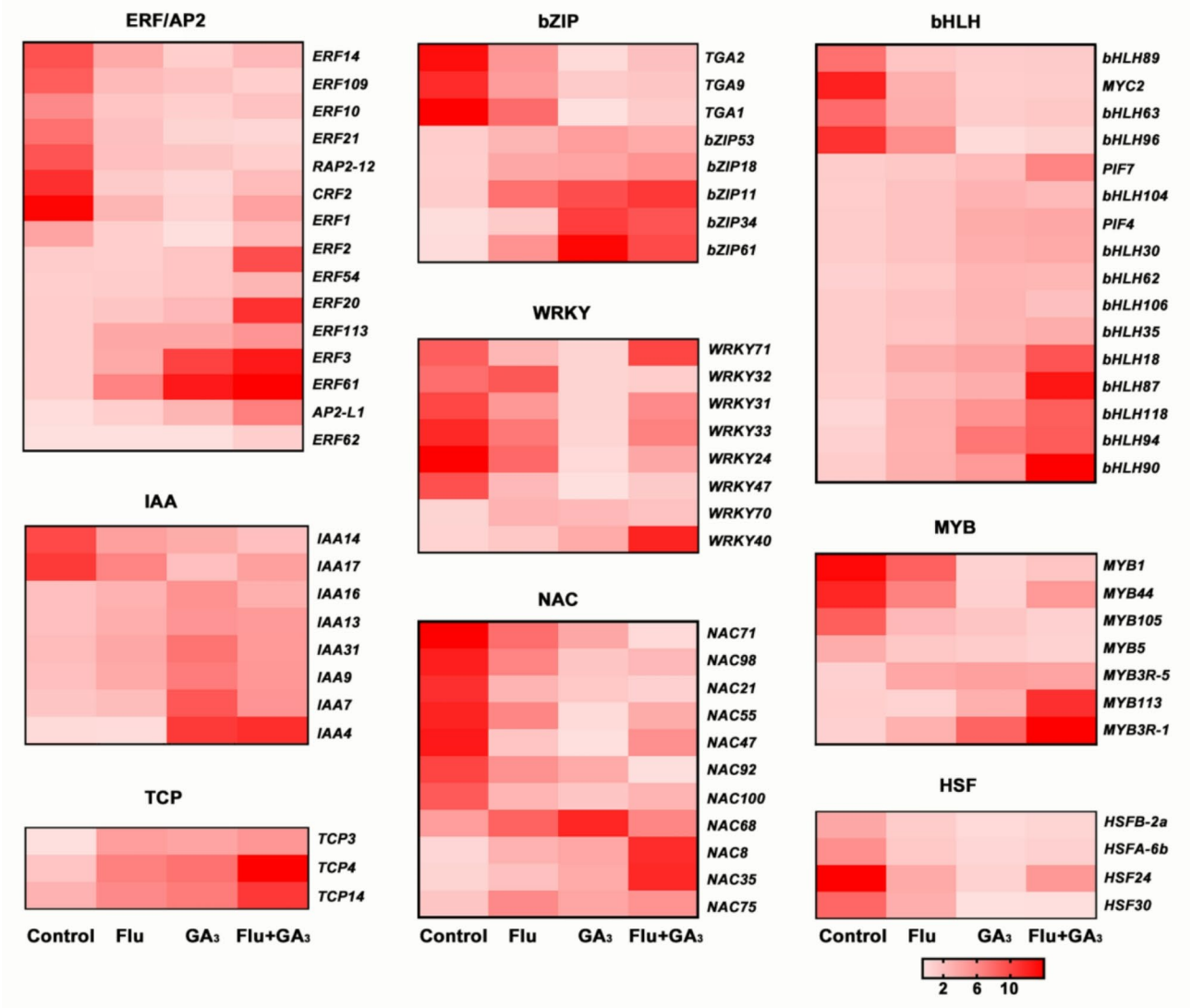

Fig. 5 Comparative analysis of DEGs among different exogenous hormone treatments. The data used in the heatmap sections was the multiple of FPKM difference from the transcriptome data. Means ± SD, $n = 3$, $P < 0.05$

Promoter analysis of the *PsTCP* Genes

According to previous studies and the results of transcriptome sequencing analysis, the TCP family might play a vital role in bud dormancy release process [9]. Here, we focused on *PsTCP3*, *PsTCP4* and *PsTCP14*, which were significantly expressed in the three comparisons. The promoters of *PsTCPs* were obtained from the genome database and identified by PCR. According to the genome database, *PsTCP3* was located at the very front of the chromosome and only 821 bp was identified as the promoter. The cis-regulatory elements (CREs) in the promoter of *PsTCPs* were identified and analyzed using PlantCARE web tool. It showed that a large number of the CREs were related to light signaling, plant growth/development, hormones, stress, and that they contained different transcription factor binding sites (Fig. 6).

In order to illustrate the responsiveness of *PsTCPs* to external hormone signals, an analysis was conducted on their expression pattern and promoter activity during the treatments. The results indicated the expression of the *PsTCPs* was notably upregulated under the GA_3 and mixture treatments, while experiencing a slight increase under the Flu treatment (Fig. 7A). Furthermore, the analysis of promoter activity revealed that the promoter of *PsTCP4* exhibited sensitivity to exogenous hormones, as evidenced by a significant increase in luciferase signal intensity under all three treatments (Fig. 7B). The promoter activity of *PsTCP14* was found to be significantly enhanced by treatments with GA_3 and the mixture. However, there was no significant change in promoter activity under the Flu treatment, suggesting that the expression of *PsTCP14* might be directly influenced by exogenous

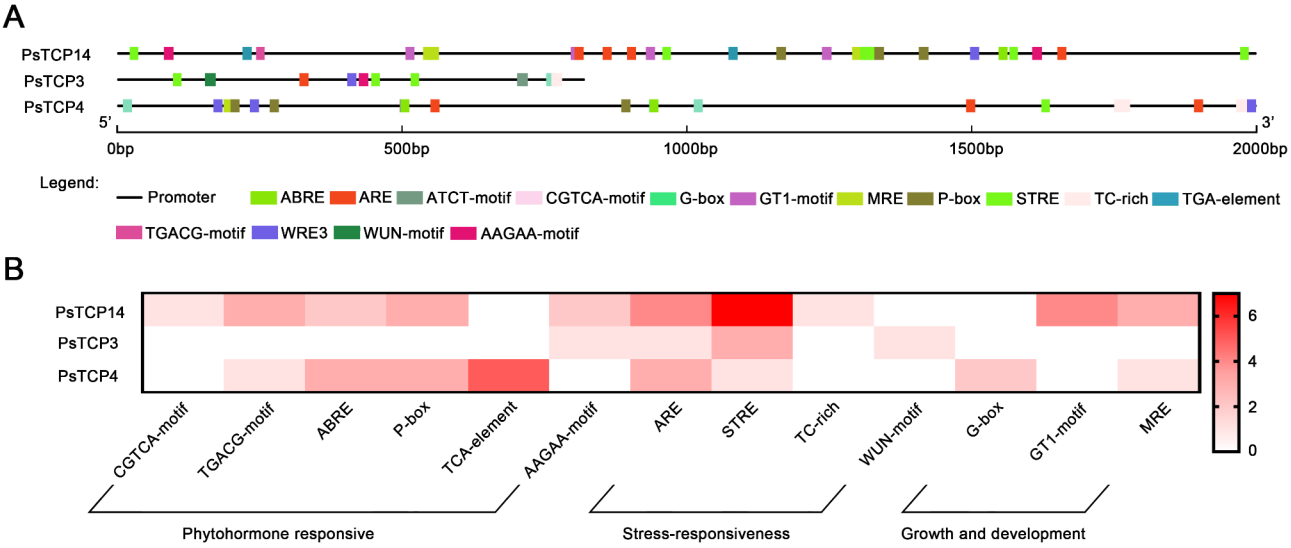

Fig. 6 The Cis-regulatory element (CRE) analysis of *PsTCPs* gene promoters. **(A)** The location of CREs in the *PsTCP* gene promoters. The black line represented the promoter region and the different color boxes correspond with the different kinds of CREs. The horizontal value represented the promoter length. **(B)** The heatmap of CREs in the *PsTCP* gene promoters. The different boxes indicated the number of CREs in *PsTCP* gene promoters, and the white boxes represented none corresponding CRE

GA_3 signaling. On the other hand, the promoter activity of *PsTCP3* did not show any noticeable variation in response to exogenous hormone treatments compared to the control, indicating that its promoter is not sensitive to hormone signaling. Under different ambient temperature treatments, in showed that compared to 23℃, the promoter activity of three *PsTCPs* exhibited sensitivity to 10℃ (Supplementary Figure S3). These findings support the hypothesis that *PsTCPs* are involved in dormancy release via distinct hormone-regulated pathways.

Identification and expression analysis of *PsTCP* genes

To gain insights into *PsTCPs*, they were identified by PCR sequencing. Amino acid sequence alignment showed that the three *PsTCPs* were divided into two subfamilies based on their different domains. It was found that *PsTCP14* was divided in Class I and *PsTCP3* and *PsTCP4* were divided in Class II (Fig. 8C). Phylogenetic analysis showed that the three PsTCPs were clustered into in different clades (Supplementary Figure S4).

qRT-PCR was used to examine *PsTCP* expression in growth and development process and various tissues, including roots, leaves, stems, flower buds, and leaf buds. It showed that *PsTCPs* exhibited significantly lower expression levels in both root and leaf tissue (Fig. 8A). Notably, distinct expression patterns were observed between dormant buds and buds released from dormancy, and the three genes had higher expression levels in dormancy release bud compared to dormant bud. During the growth and development processes of tree peony, *PsTCPs* expression levels from S1 to S5 were analyzed (Fig. 8B). It showed that the three genes had similar expression patterns and were significantly upregulated from S1 to S2 and S4 to S5 stage, suggesting that those genes may have important role in dormancy release and flower opening process. These results indicated the vital roles of *PsTCPs* in growth and development processes of tree peony.

The interaction network prediction of *PsTCPs*

To investigate the regulatory pathways involving PsTCPs and other proteins, a network map was constructed using the STRING website (Supplementary Figure S5). The three PsTCPs proteins exhibited a structural similarity to the *A. thaliana* protein AtTCP3, AtTCP4 and AtTCP14 respectively. PsTCPs exhibited interactions with a range of other proteins, including SPL, NAC, and additional members of the TCP family, all of which were implicated in the dormancy release process. These findings implied that PsTCPs not only form heterodimers to exert their function in tree peony but also participate in interactions with other proteins to modulate the dormancy release processes.

Discussion

The release of bud dormancy is a crucial factor in the growth and development of flowering plants, and is tightly regulated by intricate signalling networks [33]. While in south China and some other warm-winter regions, the incomplete release of bud dormancy always results in poor flowering quality in next spring [8, 9]. This limitation significantly hampers the distribution of important flowering plants, particularly the tree peony, in warm-winter regions [4]. Consequently, it is imperative

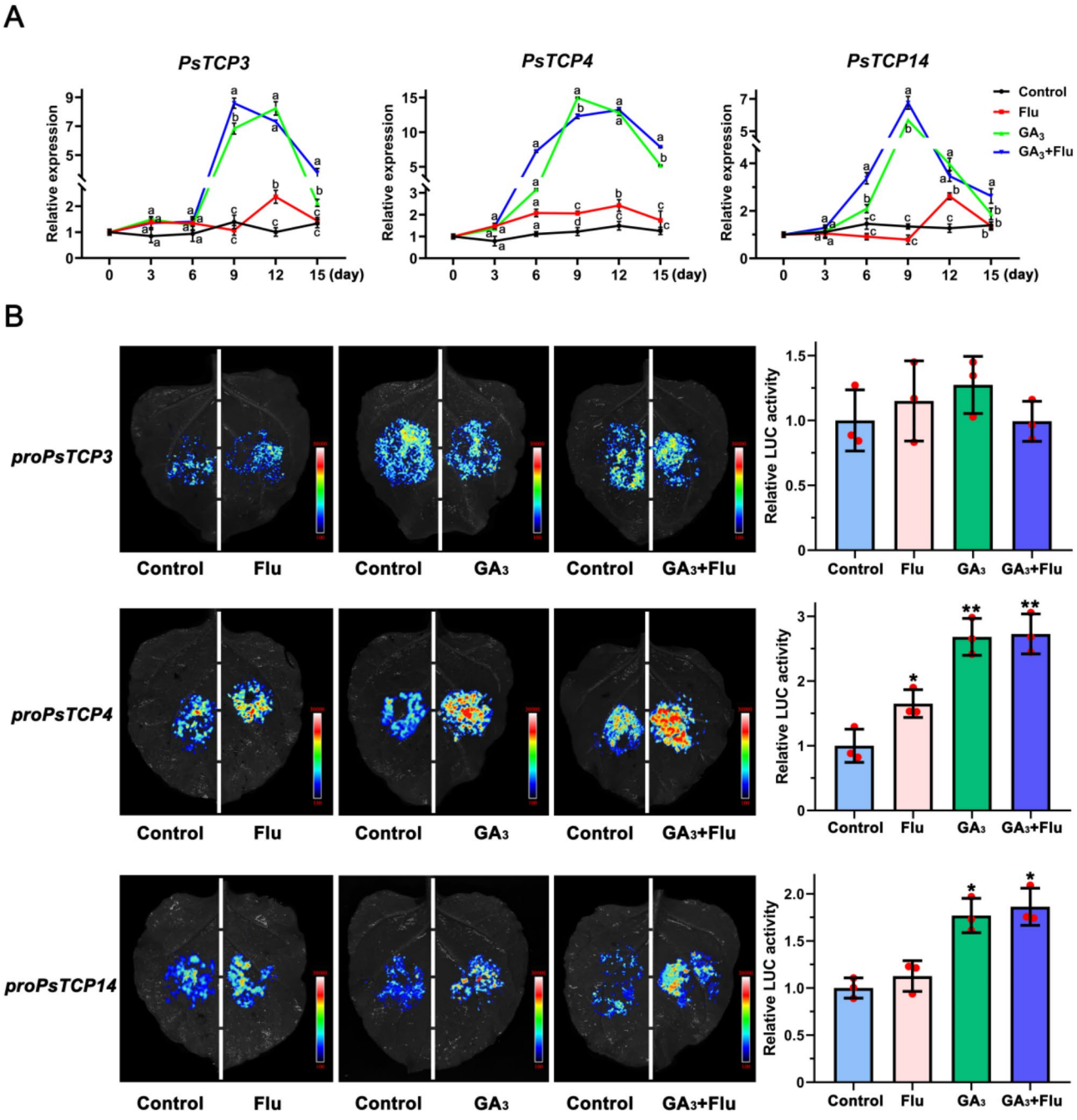

Fig. 7 The sensitivity of *PsTCPs* under different exogenous hormone applications. (**A**) The expression analysis of *PsTCPs* under different exogenous hormone applications. Different letters indicated significant differences, Student's t test, $P < 0.05$, $n = 3$. (**B**) The promoter activity of *PsTCPs* under different exogenous hormone treatments. The red dots represent the distribution of the data. Mean ± SD, $n = 3$, $^{*}P < 0.05$ and $^{**}P < 0.05$

to investigate a cultural practice to substitute for chilling in regions where it is usually inadequate, as this knowledge holds significant implications for production and cultivation purposes.

To investigate the potential cultural practice, we conducted exogenous hormone treatments. The findings demonstrated that, akin to natural conditions, treatments involving GA_3 and the combination of GA_3 and Flu significantly expedited the dormancy release of tree peony at 23℃ (Fig. 1). These results substantiate the significant impact of GA on the dormancy release of tree peony and shed light on its role in the chilling-independent dormancy release pathway. GAs are essential phytohormones that play a significant role in the processes of bud dormancy and sprouting transition in perennial plants, such as *Rhododendron simsii* [34], *Camellia*

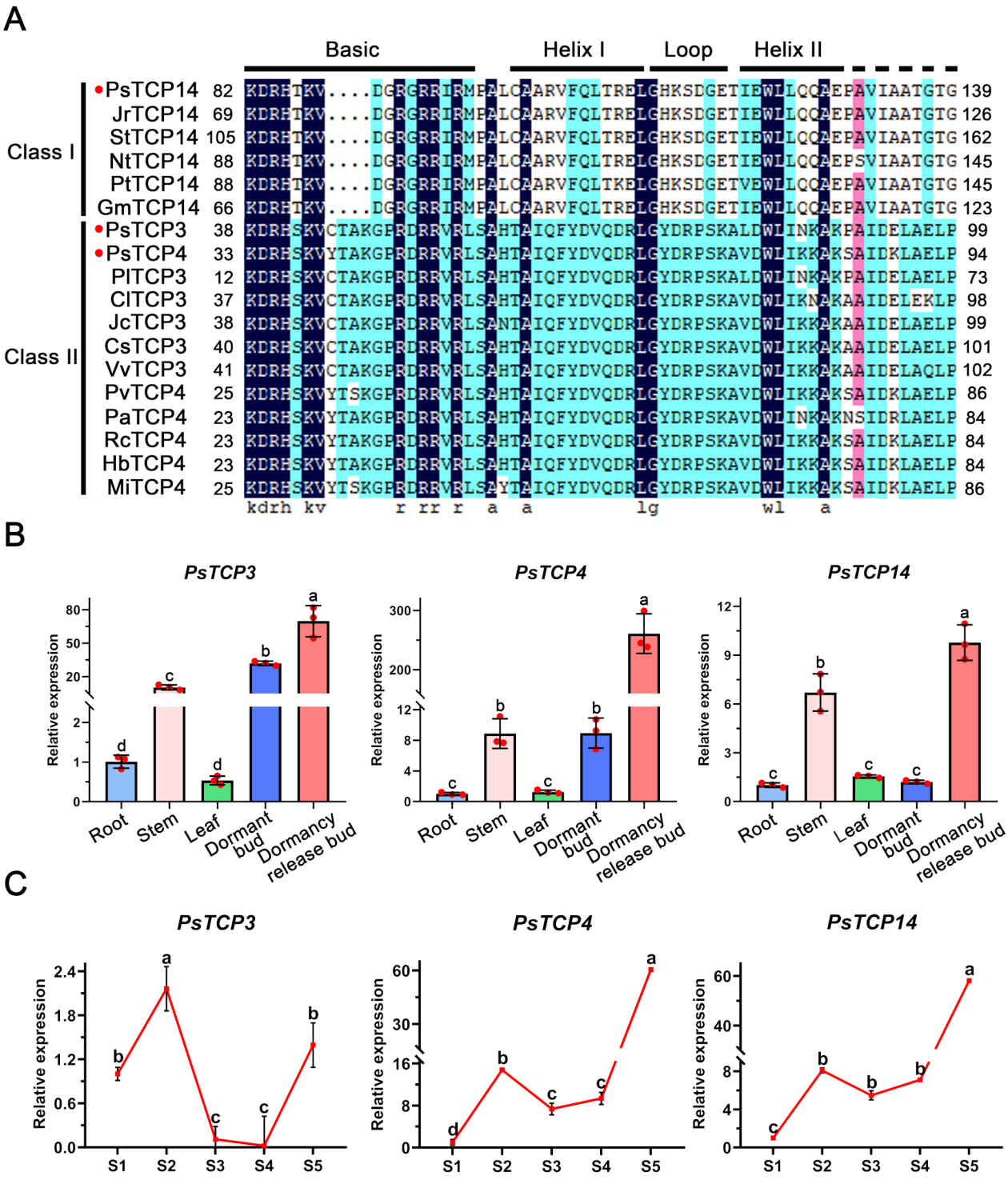

Fig. 8 Isolation and expression analysis of *PsTCPs* in tree peony. (**A**) Expression pattern of *PsTCPs* in different tissues. Different letters indicated significant differences. The red dots represent the distribution of the data. Means ± SD, $n = 3$, $P < 0.05$. (**B**) Expression pattern of *PsTCPs* during growth and development process of tree peony. Different letters indicated significant differences. Means ± SD, $n = 3$, $P < 0.05$. S1-S5 represented different development process. S1: bud dormancy stage; S2: bud dormancy release stage; S3: blooming bud stage; S4: wind bell stage; S5: full flowering stage. (**C**) Multiple amino acid sequences alignment of TCP proteins in different species

sinensis [35], and *P. armeniaca* [36]. Previous studies on tree peony have identified GA as a crucial regulator that effectively induces dormancy release [37]. Additionally, the release of bud dormancy is also associated with a decrease in ABA levels. In the woody model plant poplar, some phytohormone including ABA, GA, ethylene (ET), auxin and cytokinins are involved in bud dormancy, of which the decrease of ABA content was an important guarantee [9]. The similar results have been verified in some other plants like pear, *G. hybridus* [38] and grapevine [39]. We found that separate Flu treatment could decrease the ABA content in buds, while it had no obvious effect on dormancy release in tree peony (Fig. 5). The phenomenon might be due to the fact that Flu could inhibit the biosynthesis of new ABA through suppressing the synthesis of its precursor, but could not affect the ABA already produced. It indicated the differences in regulation mechanisms of dormancy release among different species, and in tree peony, GA played an essential role. Additionally, ABA is known to suppress the dormancy release, while after treatment with GA_3 at 23℃, there is an increase in ABA levels. According to the previous study, it showed that chilling treatment resulted in substantial drop in ABA levels [26]. Those results suggested that there may be differences in the mechanism of GA_3 and chilling treatments in dormancy release of tree peony, and the regulatory relationship between GA and ABA hormone needs further study.

In relation to the phenomenon of bud dormancy in flowering plants, a sophisticated genetic regulatory network exists. Within the metabolism of GA, the regulation of both "activating enzymes" such as *GA20-oxidase* (*GA20ox*) and *GA3ox*, and "deactivating enzymes" like *GA2-oxidase* (*GA2ox*) is tightly controlled [40]. Through transcriptome sequencing and endogenous hormone analysis in tree peony, this study suggested that the application of exogenous GA_3 could enhance GA levels by upregulating the expression of *GA20ox* and downregulating the accumulation of *GA2ox* (Fig. 5B). Moreover, it was discovered that the GA-GID1-DELLA complex facilitates the degradation of DELLA via the 26 S proteasome, resulting in the alleviation of repression and activation of GA responsive genes during the process of dormancy release [41]. This finding aligns with a previous investigation conducted on tree peony [15]. Additionally, numerous genes are implicated in the molecular mechanisms underlying dormancy release. Analysis of transcriptome data revealed that certain gene families, such as AP2/ERF, IAA, MYB, TCP, WRKY, bHLH, bZIP, NAC, and HSF, were associated with dormancy release in tree peony (Fig. 4). The AP2/ERF family is one of the largest transcription factor families in plants, and in *Prunus dulcis*, 13 members had significant expression under different low-temperature freezing stress treatments during the dormancy release process [42]. IAA is considered as an important regulator in plant growth and development. In various species, such as *Oryza sativa* [43] and tree peony [27], there was a notable increase in the levels of indole-3-acetic acid (IAA) and the upregulation of associated genes during the period of dormancy release. MYB-like proteins play a significant role in numerous physiological and biochemical processes [44]. In the case of tree peony, a specific R2R3-MYB protein called PsMYB1 was found to be involved in the budbreak process, exhibiting a more rapid response to temperature fluctuations in flower buds undergoing complete dormancy release [40]. The members of the WRKY family play crucial roles in regulating plant dormancy release process. In *P. persica*, the expression profiles of the *PpWRKYs* in bud dormancy were analysed, and six *PpWRKY* genes were thought to play important roles in dormancy [45]. In *Vitis vinifera*, the SA signaling pathway took part in bud dormancy release process through *VvWRKY70* mediated regulatory pathways [46]. The bHLH family have been found to display important roles in controlling seed dormancy and dormancy release process [47]. And here, we found that the members from these families had significant expression in bud dormancy release of tree peony. In addition, we also found that the HSF, bZIP and NAC family might response to exogenous hormone treatments to regulate the dormancy release process in tree peony.

The TCP genes, belonging to a plant-specific transcription factor family, are known to have significant impacts on plant growth and development by influencing cell proliferation and differentiation [48]. This study focused on the three *PsTCP* genes which were categorized into Class I and Class II (Fig. 8). In *Arabidopsis*, TCP genes were also classified into the two subfamilies based on their distinct domains [49]. The most notable disparity between the classes lied in the presence of four additional amino acids in the TCP domain of Class II. The investigation of various plant species has demonstrated that genes belonging to both classes exhibit diverse roles in controlling leaf type, developing axillary bud meristems, promoting plant height growth, influencing the asymmetry of floral organs, facilitating hormone signal transduction, and responding to both biotic and abiotic stresses [48, 49]. Specifically, *AtTCP14* and *AtTCP15* have been found to regulate embryonic growth through the GA signaling pathway during seed germination [50]. In poplar, *TCP18* has been identified as a negative regulator of bud dormancy release, which is itself influenced by low temperature [9]. The similar result was identified in blueberry, and *VcTCP18* negatively regulated the release of flower bud dormancy [51]. In this study, *PsTCPs* might have a positive role in bud dormancy release process, according to the expression profiles and promoter activity analysis,

while the specific gene function still needs to be further verified (Fig. 7).

Furthermore, the interaction network prediction revealed that *PsTCPs* not only formed heterodimers but also engaged in interactions with other proteins to influence the growth and development processes of tree peony (Supplementary Figure S5). Recent studies reported that *SPL* might form homodimers and interact with TCP transcription factor via the SPL dimerization and TCP interaction domain [52]. In eight hulless barley, WGBS, ChIP-seq and RNA-seq technology were used and the TCP transcription factor was predicted to target to NAC, bHLH and bZIP transcription factor genes [53]. In this study, it was observed that certain members from these families exhibited a tendency to co-express with the TCP gene. However, the specific regulatory mechanisms underlying this co-expression still require further investigation.

Conclusion

The present research aimed to analyze the roles of GA_3 and Flu in the temperature-independent process of bud dormancy release in tree peony. Results showed that both the separate application of exogenous GA_3 and mixed application of GA_3 and Flu can significantly accelerated the dormancy release of tree peony at 23℃. While only applying Flu could obviously promoted the dormancy release of. The determination of endogenous hormone content also proved the prominent effect of GA on dormancy release in chilling-independent pathway. Transcriptome sequencing and analysis of expression profiles revealed the involvement of numerous genes in the process of dormancy release in tree peony, particularly *PsTCP3*, *PsTCP4*, and *PsTCP14*. Additionally, the analysis of promoter activity demonstrated the sensitivity of *PsTCP4* and *PsTCP14* to GA_3 and Flu treatments. These findings establish a fundamental basis for comprehending the molecular mechanism of *PsTCPs* in the hormone-mediated and temperature-independent process of bud dormancy release in tree peony.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11439-7.

Supplementary Material 1: Table S1. The primer sequences used in the study. This table listed all the primer sequences used in the related experiments described in the text.

Supplementary Material 2: Table S2. The dormancy release ratio of buds under different treatments. The growth status of buds under natural conditions, both the treatments of exogenous GA_3 and mixed solution of GA_3 and Flu were assessed and recorded in the table.

Supplementary Material 3: Table S3. The transcriptome data of the 12 samples. The transcriptome data, including size, reads, the GC content and the covering annotation data were listed in the table.

Supplementary Material 4: Table S4. KEGG pathways annotation of the DEGs in the three comparisons. KEGG pathways annotation of the DEGs in the CK vs. Flu, CK vs. GA_3 and CK vs. Flu + GA_3 comparisons.

Supplementary Material 5: Figure S1. The changes of ambient temperature in 2022.

Supplementary Material 6: Figure S2. The phenotypic shifts of tree peony under different treatments in the later observation. Red arrow: the dormancy release buds with new leaves protruding from the tip. Red bars: 0.5 cm.

Supplementary Material 7: Figure S3. The sensitivity of ***PsTCPs*** under different ambient temperature treatments. The red dots represent the distribution of the data. Mean ± SD, $n = 3$, $^{*}P < 0.05$ and $^{**}P < 0.05$.

Supplementary Material 8: Figure S4. The Phylogenetic relationship of PsTCPs.

Supplementary Material 9: Figure S5. The prediction of TCPs protein interactions network. Each node represented the corresponding protein, and the different colored lines indicated the type of evidence of protein interactions.

Supplementary Material 10

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Author contributions
X. Z. and X. C. designed the research; Q. W., B. L., Z. Q. and Z. L. performed the experiments; Q. W., J. Y., X. J. and J. Z. analyzed the data; Q. W. and B. L. wrote the manuscript; X. Z. revised the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]College of Jiyang, Zhejiang A&F University, Zhuji 311800, China
[2]Weifang Vocational College, Weifang 262737, China

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