

# Metabonomic analysis reveals EMP pathway activation and flavonoid accumulation during dormancy transition in tree peony

**Tao Zhang**

Qingdao Agricultural University <https://orcid.org/0000-0003-0028-6080>

**Yanchao Yuan**

Qingdao Agricultural University

**Yu Zhan**

Qingdao Agricultural University

**Xinzhe Cao**

Qingdao Agricultural University

**Chunying Liu**

Qingdao Agricultural University

**Yuxi Zhang**

Qingdao Agricultural University

**Shupeng Gai** (✉ [spgai@qau.edu.cn](mailto:spgai@qau.edu.cn))

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## Research article

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# Abstract

## Background

Bud dormancy is a sophisticated strategy in which plants evolved to survive in tough environments. Chilling exposure is an effective method to promote dormancy release in perennial plants including tree peony, and endodormancy is a key obstacle for forcing culture of tree peony. However, the mechanism of dormancy release is still poorly understood, and there are few systematic studies on the metabolomics during chilling induced dormancy transition.

## Results

A total of 535 small molecules were obtained in tree peony buds treated with chilling, belonging to flavonoids, amino acid and its derivatives, lipids, organic acids and its derivatives, nucleotide and its derivatives, alkaloids, hydroxycinnamoyl derivatives, carbohydrates and alcohols, phytohormones, coumarins, and vitamins, respectively. 118 differential metabolites were detected, and they involved in several metabolic pathways related to dormancy. Sucrose was the most abundant carbohydrate in peony bud. Starch degradation and EMP activity were enhanced during the dormancy release, according to the variations of sugar contents, related enzyme activities and key genes expression. Flavonoids synthesis and accumulation were also promoted by prolonged chilling, which might attribute to the activated expression of *PsDFR* and *PsANS*. Moreover, the variations of phytohormones (SA, JA, ABA, and IAA) indicated they played different roles in dormancy transition.

## Conclusion

Our study suggested that starch degradation, EMP activation, and flavonoid accumulation were crucial and associated with bud dormancy transition in tree peony.

## Background

The bud dormancy of woody plants is a complex process that allows plants to survive harsh environments such as cold and drought. Bud dormancy is classified as paradormancy, endodormancy, and ecodormancy [1], of which endodormancy is regulated by internal factors [2]. External environmental factors, short-day and low temperature, play an essential role in endodormancy induction, and sufficient low temperature accumulation is a necessary prerequisite to bud break [3–5]. In *Paeonia lactiflora*, low temperature accumulation at 5°C for nine weeks was required to ensure the re-growth of buds [6]. The optimal temperatures for bud dormancy release in apple and sweet cherry were -2 °C to 5.5 °C and -2 °C to 7 °C, respectively, which was determined by temperature control experiments [7]. Also, the buds of tree peony need to undergo 21 d chilling at 0–4°C to ensure the following normal development at growth condition [8].

Furthermore, it is well known that external factors always work through internal factors during bud dormancy release, and hormones play an important role in dormancy regulation. With prolonged exposure to dormancy-inducing conditions (short-day or low temperature), the expression of growth-promoting signals gene (*FLOWERING LOCUS T*, *FT*) is inhibited, leading to reduce gibberellins (GAs) levels and increase abscisic acid (ABA) contents, and the ABA response could induce the close of plasmodesmata, thereby mediating the establishment of dormancy [9, 10]. GA and ABA not only involved in the establishment of growth cessation but also played a key role in dormancy release. During dormancy release, the reopening of plasmodesmata could restore the supply of growth-promoting signals with the increasing biosynthesis of GAs [11]. And, the degradation of ABA is necessary for bud dormancy release in grapes, while ABA is accumulated during dormancy establishment [12].

Recently, some conservative regulatory factors were identified during the bud dormancy transition. An important advancement for this field was to determine the relationship between Dormancy Associated MADS-box (*DAM*) and bud dormancy, which was up-regulated during induction into dormancy and down-regulated during dormancy release [13]. Since then, the *DAM* gene had been extensively studied in perennial woody plants, including Japanese pears [14], Japanese apricot [15], tea [16], kiwi fruit [17] et al. The recent results showed that *SHORT VEGETATIVE PHASE (SVP)-like (SVL)* with sequence homology to the *DAM* genes [18], played a vital role in the dormancy of poplar [19]. SVL is a critical component in the genetic regulatory network of bud dormancy in a recent model: low-temperature decreases ABA levels and reduces *SVL* expression, leading to the induction of *FT1* expression and GA biosynthesis, which promotes dormancy release. Without enough low-temperature accumulation, SVL directly binds to the promoters of *GA2 oxidase 8 (GA2ox8)* and *CALLOSE SYNTHASE 1 (CALST1)* and upregulates their expression, which have vital roles in growth cessation and blocking of plasmodesmata conduits, respectively. SVL also plays a critical role in the establishment of dormancy through a self-reinforcing loop along with ABA biosynthesis, activation of TCP/BRC1 (a member of TEOSINTE BRANCHED 1, CYCLOIDEA, PCF family) and repression of *FT1* [18, 19].

Carbohydrates play multiple roles in plant growth and development, of which the most important aspect is to provide energy. Before oxidative phosphorylation, Embden Meyerhof Parnas (EMP), Tricarboxylic Acid (TCA), and Pentose Phosphate Pathway (PPP) are the main respiration pathways in plants. EMP starts from glucose, which is an end product of starch degradation. Additionally, maltose and fructose also involved in the EMP pathway after conversion to glucose. The anabolic metabolism of sucrose is mainly carried out by two enzymes, sucrose synthase (SUS; EC 2.4.1.13) and invertase (INV; EC3.2.1.26) [20]. SUS reversibly catalyzes the formation of sucrose from UDP-glucose and fructose [21]. And INV, which irreversibly decompose sucroses into hexose, can be divided into three categories: cell-wall invertase (CWIN), vacuolar invertase (VIN), and cytoplasmic invertase (CIN) [22, 23].

It had been reported that EMP, TCA, and PPP were strictly related to dormancy release. For example, the TCA cycle was enhanced, while the PPP pathway slowly decreased during apple bud sprouting [24]. In grape, dormancy release induced by chemical and low temperature was found related to PPP, EMP, and

TCA cycles[25–27]. Furthermore, carbohydrates could also act as a sugar signaling molecule. Mason et al. found that sucrose could serve as signaling molecule involving in paradormancy release [28].

Flavonoids are widespread secondary metabolites in plants, which mainly contain six subclasses: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins [29]. The pathway of flavonoid biosynthesis is quite conservative and well researched in some model plants [29]. Some genes involved in the production of common precursors, such as *chalcone synthase (CHS)*, *chalcone isomerase (CHI)*, *flavanone 3-hydroxylase (F3H)*, and *flavonoid 3'-hydroxylase (F3'H)*, are called early biosynthetic genes (EBGs). Correspondingly, downstream genes for flavonoid biosynthesis are called late biosynthetic genes (LBGs). The pathway of flavonoid biosynthesis was affected by biotic and abiotic factors (e.g., pathogen infections, temperature, drought, plant hormones) [30]. Moreover, recent reports had shown that flavonoids were involved in plant stress response [31], pollen development [32], color formation [33], etc. However, it remained unknown whether flavonoids participate in the process of dormancy release.

Tree peony (*Paeonia suffruticosa* Andr.) is one of the most ancient ornamental and medicinal plants in the world. As a woody plant, it must undergo a period of low temperature to ensure the sprouting and flowering in the next year. Due to the short and concentrated florescence every year, its anti-season production becomes an essential content of the tree peony industry. Until now, the primary method of anti-season production is to provide sufficient low temperature exposure alone or combining with gibberellin application. Therefore, it was of great value to understand the mechanism of chilling induced dormancy release in tree peony. Our previous study characterized the relationship between chilling accumulation and dormancy status: the physiological status of tree peony 'Luhehong' after 14 d chilling treatment was regarded as the transition stage from endodormancy to endodormancy release, and that after 21 d chilling treatment was defined as dormancy release, and after 28 d chilling as a state of ecodormancy [34]. GA pathway plays a crucial role in endodormancy release induced by chilling [35]. And the activity of PPP pathway also increased during the process, suggesting that it played a role in dormancy release of tree peony [8]. As known, traits are more closely related to metabolites, which may provide a new perspective for the understanding of dormancy transition in tree peony.

Here, metabolic changes of tree peony buds during the chilling induced dormancy transition were analyzed. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that differential metabolites were involved in various metabolic pathways such as carbon metabolism, secondary metabolite synthesis, and hormone metabolism. It was revealed that starch degradation and EMP activity were enhanced during dormancy release. Interestingly, flavonoid anabolism was also activated by chilling accumulation, and its increasement might in return promote flower bud development. Furthermore, the variations of plant hormones (abscisic acid, jasmonic acid, and indole-3-acetic acid) during the dormancy transition were also evaluated in this research. Significantly, the roles of flavonoids were firstly discussed during the dormancy transition in perennial plants. All results would provide valuable information for the molecular mechanism of dormancy transition in tree peony.

## Results

# Metabonomic analysis during chilling induced dormancy transition

To study the metabolic changes during chilling-induced dormancy release in tree peony, flower buds were picked at five time points after 0–4 °C treatment (0, 7, 14, 21 and 28 days), and metabolic profiling was analyzed by ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) (Fig. 1a). The Principal Component Analysis (PCA) analysis was employed to evaluate the repeatability of the metabolite profiles (Fig. 1b). As shown, quality control (QC) samples were separated from tested samples, and the two principal components accounted for 28.8% and 14.5% of the total variance, respectively (Fig. 1b).

A total of 535 small molecules were detected in the metabonomic analysis, and 511 of them were annotated with MassBank, KNAPSACK, HMDB [36], MoTo DB and METLIN [37](Table S1). The metabolites were divided into eleven groups, including flavonoids, amino acid and its derivatives, lipids, organic acids and its derivatives, nucleotide and its derivatives, alkaloids, hydroxycinnamoyl derivatives, carbohydrates and alcohols, phytohormones, coumarins, and vitamins (Table 1). The metabolic pathway of these metabolites was analyzed using KEGG databases. The results showed that the metabolites were mapped to 14 KEGG pathways during dormancy release (Fig. 1c). Of them, most metabolites were assigned to the pathway of global and overview, followed by amino acid metabolism, biosynthesis of other secondary metabolites, metabolism of cofactors and vitamins, nucleotide metabolism, carbohydrate metabolism, and so on (Fig. 1c).

Table 1  
Overview of annotated metabolites

Type	Number	Percentage (%)
Flavonoids	132	25.83
Amino acid and its derivatives	79	15.46
Lipids	64	12.52
Organic acids and its derivatives	62	12.13
Nucleotide and its derivatives	51	9.98
Alkaloids	36	7.05
Hydroxycinnamoyl derivatives	27	5.28
Carbohydrates and alcohols	21	4.11
Phytohormones	14	2.74
Coumarins	9	1.76
Vitamins	9	1.76

## Differential metabolites analysis

The Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA, VIP  $\geq 1$ ) and the Student's t test ( $P < 0.05$ ) were applied to detect the differential metabolites (DMs) among different groups (Fig. S1). The results indicated that the amounts of up-regulated metabolites increased along with the accumulation of low temperature when compared with 0 d (Fig. 2a). In detail, there were 4, 18, and 29 up-regulated metabolites at 14, 21 and 28 d vs 0 d, respectively, indicating that some metabolism pathways were activated by prolonged chilling exposure. Interestingly, the number of down-regulated metabolites was dominant in all DMs when compared with that of 0 d. To better understand the substance changes caused by different chilling durations, the venn diagram of 118 DMs showed that four metabolites were shared among the four comparisons, i.e. 7 d vs 0 d, 14 d vs 0 d, 21 d vs 0 d, and 28 d vs 0 d. And of the four common metabolites, one was up-regulated, and three were down-regulated (Fig. 2b; Fig. S2). Besides, 30 metabolites were unique in 28 d vs 0 d, implying that this group had more variation than other groups. The KEGG pathway annotation of 118 DMs was performed and the DMs were involved in amino acid metabolism, nucleotide metabolism, and biosynthesis of other secondary metabolism, and so on (Fig. 2c). Additionally, KEGG enrichments between each two treatments were listed in Fig. S3. Carbohydrate metabolisms, such as pyruvate metabolism, starch and sucrose metabolism, PPP pathway and so on, were frequently presented in the different enrichments analysis. Plant hormone signaling transduction was enriched in 7 comparable groups. Besides above, several amino acid metabolisms, pyrimidine and purine metabolism, nitrogen metabolism and others were also frequently enriched in the comparisons.

# The metabolic processes related to dormancy release in tree peony

To study the crucial metabolic processes related to dormancy release in tree peony, totally, 50 DMs of 14 d vs 7d and 21 d vs 7 d were identified and presented in a clustering heatmap, which reflected the metabolites change between the endodormancy and the endodormancy release transition (Fig. 3a). The heatmap showed that 21 DMs were significantly up-regulated, 18 DMs were down-regulated after prolonged chilling endurance, and the other 11 DMs fluctuated with a peak at 14 d. The KEGG analysis of the 50 DMs showed that 25 components participate in the metabolic pathway, which accounted for 83.33% of all the 30 annotated metabolites (Fig. 3b, Table S2). The 25 DMs which identified by KEGG enrichment analysis involved in the pathways of glucose metabolism (glucose 6-phosphate), amino acid metabolism (aspartate) and hormone metabolism (abscisic acid) (Table S3), suggesting that these metabolic pathways might play a critical role during dormancy release in tree peony.

## Carbon metabolism during dormancy transition in tree peony

A metabolic network containing EMP pathway, TCA cycle, shikimate pathway, and amino acid metabolism was presented to visualize the carbon flow during dormancy release of tree peony (Fig. 4). The levels of glucose at 0, 7, 14, 21 d were lower than that at 28 d. However, glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) had the higher levels at 7 and 14 d (Fig. 4), indicating that the EMP was activated during chilling induced endodormancy release in tree peony. In the TCA cycle, the levels of citrate declined until 14 d and then climbed. The succinic acid amount showed an significant upward at 14 d. In terms of amino acid metabolism, some amino acids increased with the release of endodormancy, such as leucine, proline, etc. In contrast, the others decreased, such as valine, aspartic acid, glutamic acid, etc. (Fig. 4). In the shikimic acid pathway, the level of shikimic acid and phenylalanine were increased after chilling exposure (Fig. 4).

## The variation of carbohydrates during dormancy transition

Sugars play critical roles in energy metabolism and substance metabolism, whose variation might reflect the status of bud dormancy after chilling exposure. The levels of 15 sugars in metabolic profiles were analyzed throughout the same process (Fig. 5a). Some monosaccharide (G6P, glucosamine, and trehalose 6-phosphate) and polysaccharides (maltotetraose, melezitose, and melezitose *O*-rhamnoside) were up-regulated after 7 d chilling exposure, then their contents reduced (Fig. 5a). The monosaccharides (fucose, glucose, and arabinose) had the maximum level at the stage of endodormancy (28 d chilling treated, Fig. 5a), indicating a well preparatory status for the following regrowth. A Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS) measurement was then employed to further analyze the change of sugars during the chilling process in tree peony, and the results were similar to the metabolomics data. Fructose, glucose, and inositol were the most abundant three kinds of

monosaccharides. The content of maltose significantly decreased after 7 d chilling treatment. The content of sucrose was the highest of all the 13 tested sugars, which significantly increased and reached a maximum of 92.9 mg/g at chilled 14 d, and then declined rapidly (Fig. 5b), suggesting that it might play a vital role in the whole process. To further investigate the role of sucrose in dormancy release of buds, the expression patterns of *sucrose synthase* (*PsSUS1* and *PsSUS2*) and *sucrose invertase* genes were analyzed (Fig. 5c). The expression of *PsSUS1* continued to decline after 7 d chilling treatment, but *PsSUS2* was up-regulated at 7 and 14 d chilling. The expressions of *cytoplasmic invertase* (*PsCIN*), *vacuolar invertase* (*PsVIN*), and *cell-wall invertase* (*PsCWIN*) were significantly increased at different chilling periods (Fig. 5c). Taken together, it was presumed that sucrose catabolism was dominant during chilling duration process, to provide sufficient sugars for respiratory metabolism and energy metabolism. Starch, the main storage carbohydrate in higher plants, was measured during dormancy transition in tree peony. The results indicated that starch content decreased after chilling exposure and reached its minimum at 14 d, which might correspond to the activity of amylases (AMY) during the same process (Fig. 5d).

## The changes of flavonoids during dormancy transition

Flavonoids are secondary metabolites that are widely present in plants. Moreover, the biosynthesis of secondary metabolites accounted for a large part of KEGG enriched terms (Fig. 3b). Thus a flavonoids metabolic pathway analysis was performed to help understand its changes during dormancy transition in buds. Most flavonoids (naringenin, apigenin, luteolin, etc.), flavonols (kaempferol, quercetin, etc.), and anthocyanins (cyanidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, etc.) presented similar change tendency, and most of them were down-regulated at the beginning of dormancy (0–14 d) and up-regulated at 28 d. Thus, their highest contents were usually detected at 28 d, such as cyanidin-base anthocyanins, cyanidin 3-*O*-glucoside etc (Fig. 6a). According to the results of LC-ESI-MS/MS, we analyzed the relative contents of anthocyanins at 28 d. The results indicated that five anthocyanins, including cyanidin 3-*O*-glucoside (Cy3Glu), cyanidin 3-*O*-rutinoside (CyRut), cyanidin *O*-syringic acid (CySyr), cyanidin 3,5-di-*O*-glucoside (Cy3Glu5Glu), and pelargonidin 3-*O*-glucoside (Pg3Glu), were dominant in flower buds of tree peony, and the others were relatively rare (Fig. 6a and b). In detail, cyanidin-base anthocyanins (Cy3Glu5Glu) accounted for the majority, followed by Cy3Glu (Fig. 6a and b). Two EBGs (*PsCHS* and *PsF3H*) and two LBGs (*PsANS* and *Ps3GT*) showed the similar patterns. Their transcripts were relatively abundant at the beginning (0 d), and dramatically declined at 7 d chilling, but prolonged chilling promoted their expression compared to 7 d treatment. The exception was *PsDFR*, which remained very low expression level till dormancy release period, but increased by ten folds at 28 d (Fig. 6c). The expression patterns of the related genes were accordance with their content variations during chilling duration period.

## The variation of phytohormones during dormancy transition

Phytohormones play vital roles in plant growth and development, flowering, stress response, and so on. With metabolomics analysis, 14 phytohormones or analogues, including salicylic acid (SA), jasmonic

acid (JA), gibberellin (GA), and abscisic acid (ABA), were detected in dormant buds of tree peony, and their contents during chilling duration were shown in a heatmap (Fig. 7a). Nine metabolites presented the highest contents at chilled 0 d (nonchilled period), and the other five hormones peaked at chilled 28 d (Fig. 7a). Cluster analysis divided them into three subgroups. The first subgroup showed a up-regulated tendency, including SA, GA15, JA, methyl jasmonate (MeJA) and jasmonic acid-isoleucine (JA-Ile), all with a peak at 28 d. Dihydrozeatin, salicylic acid *O*-glucoside, trans-zeatin *N*-glucoside and ABA were categorized as the second subgroup with an obviously down-regulated tendency. The others fluctuated during chilling exposure process with a peak at non-chilling point.

The contents of JA, SA, indole-3-acetic acid (IAA) and ABA were evaluated by LC-MS/MS analysis, which was similar to the metabolomic data. JA level was relatively low from 0 to 21 d chilling, and dramatically peaked to 299.167 ng/g with a ten fold increase at chilled 28 d (Fig. 7b). The MYC2 (myelocytomatosis protein 2) transcription factor plays a central role in JA signal transduction [38]. Consistent with the variation of JA content, the transcript of *PsMYC2* increased sharply at chilled 28 d (Fig. 7c). The contents of SA was down-regulated from 0 d to 21 d, and then recovered the initial content of 0 d.

The content of IAA fluctuated during the chilling duration process, reached the highest level of 44.57 ng/g at 14 d, and its content of 28 d was also higher than 0 and 21 d (Fig. 7b). The expression pattern of two auxin receptors genes (*TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN2, TIR1/AFB2*) were analyzed during the chilling duration. *PsAFB2* was down-regulated in the whole process, and *PsTIR1* was up-regulated at 7 d, and then down-regulated from 7 to 28 d (Fig. 7c). The content of ABA persistently decreased from 1140.37 ng/g at chilled 0 d to 803.83 ng/g at chilled 28 d (Fig. 7b), indicating that ABA was an inhibitor of dormancy release in tree peony buds, and chilling treatment could gradually reduce the content of ABA.

## Discussion

The dormancy transition of woody plants is a complicated process, which was synergetically regulated by photoperiod and low temperature [39]. Chilling treatment as an effective method to promote dormancy release had been verified in many species including tree peony [3–5]. Transcriptomics, proteomics and microRNAs analysis had been performed to investigated the concealed mechanism of chilling induced dormancy release in tree peony, which revealed the roles of GA and carbohydrate metabolism [8, 35, 40]. Here, substance changes were detected by metabolomics analysis during the chilling duration process.

## Starch degradation and EMP pathway were enhanced during dormancy release

Carbohydrate is the basic energy substance of primary metabolism and secondary metabolism. Sugars metabolism and signal transduction involving in bud dormancy process had been revealed by RNA-seq [41]. Sugar metabolism genes (*beta-amylase 5, alpha-amylase-like 1, sucrose synthase 3, and trehalose-phosphatase/synthase 7*), sugar transporter genes (*GT-2 like 1, sucrose-proton symporter 2, protein O-*

*mannosyltransferase 5*, and *senescence-associated gene 29*) and sugar signal transduction genes (*glucose insensitive 2* and *beta-fruct 4*) had been proven to involve in dormancy release in poplar, grape and *P. mume*. [41–43]. In this study, the content of carbohydrates was continuously measured during the chilling induced dormancy transition of tree peony. Starch and maltose showed a downward trend during dormancy release, which were consistent with the increasing of amylase activity and sugar catabolism (Fig. 5b, d). Our recent results also revealed that the transcripts of *PsAMY* and *PsBMY* were up-regulated [8, 41]. At the same time, the enzyme activity of AMY gradually increased. Together, these results indicated that chilling treatment promoted the degradation of starch during dormancy release of tree peony. After endodormancy release (21–28 d), the buds maintained active respiratory metabolism and amylase activity, but the starch and maltose contents did not decrease significantly (Fig. 5b, d). It was speculated that a considerable amount of carbohydrates were transported from other parts of the plant to the flower buds, such as the root system (tree peony has a developed succulent root system), to meet the consumption of flower buds. The reopening of the material transport channel also enables the long-distance transport of carbohydrates [11].

The content of sucrose was the most abundant among all the measured sugars, which implied that sucrose might also be used to transport of assimilates in tree peony rather than sorbitol as in apple because sucrose was about 3 200 times higher than sorbitol. Sucrose was up-regulated till to 14 d and decreased afterward according to GC-MS/MS results (Fig. 5b). The starch was rapidly degraded at the beginning of the chilling duration due to high amylase activity, and sucrose is a kind of its intermediate product. Taken together, we speculated that sucrose mainly came from starch degradation. It was also found that sucrose accumulated, and sucrose synthase genes were up-regulated at the early stage of dormancy process in poplar and *P. mume* [41]. Therefore, sucrose might be used as an energy center to ensure the supply of glucose in dormancy transition.

In addition, extensive degradation of starch should lead to glucose accumulation, but glucose and fructose were at a low level or down-regulated until to ecodormancy stage (Fig. 5a, b). Meanwhile, the content of F6P and G6P increased significantly after chilling exposure, but decreased thereafter (Figs. 4 and 5a). Also, Our previous study showed that the transcripts and enzyme activities of Hexokinase (HK) and glucose 6-phosphate isomerase (G6PI) were also significantly up-regulated [8, 41]. These results indicated that the EMP pathway was activated during dormancy release of tree peony. Metabolome results showed that dormancy release was an energy-consuming process. A large number of carbohydrates were broken down to produce enough substances and energy to promote dormancy release while also providing a carbon chain for secondary metabolism.

## **Flavonoids accumulation at ecodormancy stage**

As secondary metabolites, flavonoids play important roles in many processes of plant growth and development, such as color formation, stress resistance, etc. [29]. However, the changes and functions of flavonoids during dormancy transition were still poorly understood. Variations of flavonoids were observed during chilling duration process in our work. Flavonoids (e.g. quercetin, kaempferol, and apigenin) synthesized in the early step of the flavonoid biosynthesis pathway were down-regulated in the

whole endodormancy stage, i. e. the treatments before 21 d, due to the decreasing expression level of *PsCHI* (Fig. 6). Subsequently, their levels were sharply upregulated at 28 d, an ecodormancy stage (Fig. 6a). The primitive high level of flavonoids might be related to cold acclimation for winter survival. It was considered that the initial products of the flavonoid biosynthesis pathway, such as quercetin, kaempferol, apigenin, etc., inhibited the transport of polar auxin and regulate plant development [44]. Also, the correlation between pollen fertility and flavonoids had been found in maize and peanuts [29], and the silencing of *chalcone synthase* gene resulted in parthenocarpy in tomato [45]. Flavonols (in particular quercetin) is essential for pollen germination [32]. In our results, flavonols showed higher levels at ecodormancy stage (Fig. 6a), when the flower buds are well-differentiated, and stamens are clearly visible [46]. Therefore, flavonols might be involved in flower bud development at ecodormancy stage in tree peony.

In a recent study, Gu et al. suggested that anthocyanin accumulation occurred 10 d before anthesis in tree peony 'Qing Hai Hu Yin Bo'[47]. Here, we found anthocyanins were up-regulated after endodormancy release, accumulating in large amounts at ecodormancy stage in tree peony 'Luhehong' (Fig. 6). The results implied that the floral pigments might begin their synthesis before the bud entering into endodormancy with a early peak at ecodormancy period. Cyanidin-based glycosides such as Cy3Glu and Cy3Glu5Glu were the most abundant anthocyanins in the petal blotches of 35 cultivars [48], and they were also the most abundant anthocyanins in the buds of 'Luhehong' (Fig. 6b). We hypothesized that dormancy release induced by chilling duration synchronously activated anthocyanin synthesis and accumulation.

Taken together, Flavonoid accumulation during chilling induced dormancy release might accelerate flower bud development by promoting flower organ development (petal and stamens). To our knowledge, it was the first report to describe the changes and role of flavonoids during dormancy transition in perennials.

## The roles of phytohormone during dormancy transition

In perennial woody plants, ABA and GA have been widely proven to regulate bud dormancy. Recent researches revealed the antagonism between ABA and GA in bud dormancy. A MADS-box (DAM) family gene, *SHORT VEGETATIVE PHASE-like (SVL)* had been shown to play a key role in ABA-mediated bud dormancy in poplar. In *SVL* RNAi strain, the expression of *FT1* was significantly up-regulated, and GA biosynthesis gene *GA20ox* was up-regulated to promote bud rupture [18]. Further research found that *SVL* could directly bind to the promoters of *GA20ox8* and *CALS1* to induce their expression, reduce the level of active GAs and control the closure of plasmodesmata, thereby maintain dormancy status [49]. In our work, ABA was down-regulated along with the chilling duration (Fig. 7). Gibberellin 15, the precursor of bioactive gibberellins, was up-regulated gradually (Fig. 7a). Meanwhile, the expression of *PsGA20ox*, a key enzyme in GA biosynthesis, was up-regulated, and the content of GAs increased with chilling accumulation [50, 51]. Therefore, there might be similar mechanisms between ABA-regulated dormancy in poplar and chilling-induced dormancy in tree peony. Chilling accumulation reduces the level of ABA,

which in turn suppresses the expression of *SVL* and promotes the biosynthesis of GAs, and finally break bud dormancy [35].

Auxins and CKs play antagonistic roles in meristems of many plants [52]. Previous researches had shown that CKs play a positive role in hydrogen cyanamide-induced bud dormancy release in grape [53], but that of IAA is still ambiguous until now. However, in our study, the level of CKs (dihydrozeatin, *trans*-zeatin *N*-glucoside and kinetin 9-riboside) decreased after chilling exposure, and IAA was up-regulated by chilling, confirmed by metabolomics and LC-MS analysis (Fig. 7). TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) family are known as auxin receptors [54]. The transcript of *PsTIR1* was significantly induced and consistent with IAA variation (Fig. 7c), which implied a positive role of IAA during dormancy release in tree peony. These results were also different from the upregulation of CKs in *P. kingianum* and *P. mume* during dormancy release [55, 56]. Therefore, the regulations of CKs and IAA in dormancy release were not a common mechanism in different perennial plants, and they might not be the key factors in dormancy regulation.

Usually, SA and JA are regarded as stress response hormones, rather than function on dormancy regulation [57, 58]. Recently, Ionescu et al. proposed that the upregulated JA-Ile induced the expression of *MYB21* and *MYB108* to participate in the flower development process during HC-induced dormancy release in sweet cherry [56]. In our results, jasmonates were dramatically upregulated when entering endodormancy period, along with a transcriptional climb of *PsMYC2*, a key transcription factor for JA signal transduction (Fig. 7). The results indicated that JA signal transduction was activated after sufficient chilling duration. In recent studies, the mechanism of promoting the anthocyanins accumulation by JA was revealed in *Arabidopsis*. When the JA content increased, the inhibitory effect of JAZs protein on MYB-bHLH-WD40 complex was released, which promoted the expression of *DFR* and *ANS*, and the accumulation of anthocyanins [59].

Interestingly, *PsDFR* and *PsANS* also significantly increased, and anthocyanins presented higher levels at 28 d chilling treatment in our study (Fig. 7). It was hypothesized that jasmonate was additionally involved in anthocyanin accumulation after endodormancy release in tree peony. Additionally, SA involve in the response to low-temperature stress, for SA and glucosyl SA accumulating after low-temperature exposure [57]. The use of exogenous SA improves the cold tolerance of corn, cucumber, and rice [58]. The accumulations of SA and Salicylic acid *O*-glucoside were also observed during chilling induced dormancy release in tree peony (Fig. 7), it might be the response of buds to low-temperature stress.

## Conclusions

In summary, we systematically revealed the metabolomic changes during the chilling induced dormancy transition of tree peony, and a total of 511 substances and 118 DMs were identified. Chilling accumulation promoted the degradation of starch and enhanced the activity of EMP, providing adequate energy and substances for secondary metabolism required by dormancy release and bud burst. Flavonoid was accumulated by sufficient chilling duration along with endodormancy release.

Furthermore, we also reported phytohormone changes in during the dormancy transition in tree peony. Prolonged chilling exposure declined ABA content, but promoted JA and GA accumulation at the end of dormancy. Taken together, we proposed a work model of dormancy transition induced by chilling according to the metabonomics analysis (Fig. 8). Our results might help people better understand the dormancy transition of perennial plants.

## Materials And Methods

### Plant materials

Four-year-old tree peony plants (*Paeonia suffruticosa* 'Luhehong') were treated with continuous artificial chilling (0–4 °C) from Nov 12, 2018 as described previously [8]. At 0, 7, 14, 21 and 28 d after refrigerating treatment, buds were picked in each time point, frozen in liquid nitrogen, and stored at -80°C until further analysis. The samples from each three plants were harvested and mixed in each treatment. Three replicates (3 plants/replicate) per group were set.

### Sample preparation and extraction

The freeze-dried samples were crushed at 30 Hz for 1.5 min using a mixing mill with zirconia beads (MM 400, Retsch). Then 100 mg powder was mixed with 1.0 mL 70% methanol solution (containing 0.1 mg/L lidocaine as an internal standard) at 4°C overnight. After centrifugation at 10,000 × g for 10 min, the supernatant was filtered (scaa-104, aperture 0.22 µm; ANPEL, Shanghai, China, [www.anpel.com.cn/](http://www.anpel.com.cn/)) and analyzed by LC-MS/MS. Quality control (QC) samples were mixed with all samples to test the repeatability of the whole experiment [60].

### Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS)

The extracted compounds were analyzed using an LC-ESI-MS/MS system (UPLC, Shim-pack UFLC SHIMADZU CBM20A, <http://www.shimadzu.com.cn/>; MS/MS, Applied Biosystems 4500 QTRAP, <http://www.appliedbiosystems.com.cn/>) [60]. Firstly, 5 µL samples were injected into a Waters ACQUITY UPLC HSS T3 C18 chromatographic column (2.1 mm × 100 mm, 1.8 µm) with a flow rate of 0.4 mL/min at 40 °C. The water and acetonitrile after acidification with 0.04% acetic acid were used as the mobile phases of stages A and B, respectively. The following gradient was used to separate the compounds: the ratio of phase A and B was 95:5 at 0 min, 5:95 at 11.0 minute, 5:95 at 12.0 minute, 95:5 at 12.1 min, and 95:5 at 15.0 minute. The effluent was connected to the ESI- triple quadrupole linear ion TRAP (Q TRAP) - MS.

LIT and triple quadrupole (QQQ) scans were performed on a triple quadrupole linear ion TRAP mass spectrometer (QTRAP). The AB Sciex QTRAP4500 system was equipped with an ESI-Turbo Ion-Spray interface, ran in positive ion mode, and was operated by the Analyst 1.6.1 software (AB Sciex). Operating parameters were as follows: ESI source temperature was 550 °C; Collision activation dissociation (CAD)

was set to the highest; Ion-spray voltage (IS) was 5500 V; The m/z range was set to 50 to 1000. The QQQ scan was obtained as a multiple reaction monitoring (MRM) experiment, with the optimal solution cluster potential (DP) and collision energy (CE) for each MRM transformation.

## Qualitative and quantitative determination of metabolites

The qualitative analysis of metabolites was performed based on the public metabolite database (e.g. MassBank and KNApSAcK) and the METLIN database (MWDB) [36, 37]. The repetitive signal of  $K^+$ ,  $Na^+$ ,  $NH_4^+$ , and other large molecular weight species were removed during the analysis. The metabolites were quantified by MRM of triple quadrupole mass spectrometry. The total ion chromatogram (TIC) and extracted ion chromatogram (EIC or Xic) of QC samples were derived to summarize the metabolite spectra of all samples and calculate the area of each peak. The MultiaQuant software (v 3.0.3) was used to integrate and calibrate the peaks.

## Principal component analysis (PCA) and Orthogonal projection to latent structures-discriminant analysis (OPLS-DA)

To initially visualize the differences between the groups, the R package “ropls” was employed for principal component analysis (PCA) (<http://bioconductor.org/packages/release/bioc/html/ropls.html>).

Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) is an extension of PLS-DA, which incorporates an Orthogonal Signal Correction (OSC) filter into a PLS model. The OPLS-DA model was used to analyze all comparison groups. Subsequent model tests and differential metabolite screening were analyzed using OPLS-DA results.

## Differential metabolites analysis and KEGG analysis

The most distinguishable metabolites between every two groups were ranked by the variable importance of the projection (VIP) score using the OPLS model. The threshold for VIP was set to 1. Besides, Student's t test was used as a univariate analysis to screen different metabolites. Those with  $P < 0.05$  and  $VIP \geq 1$  were considered as differential metabolites between two groups.

The KEGG Orthology software (<http://kobas.cbi.pku.edu.cn/>) was used for KEGG pathway analysis.

## RNA Extraction and Real time Quantitative PCR analysis

Total RNA was extracted from homogenized buds of tree peony using RNA isolation kit according to the manufacturer's protocol (TaKaRa, Dalian, China). Genomic DNA was removed from total RNA by DNase I (TaKaRa, Dalian, China). The first-stand cDNA was synthesized using HiScript III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme Biotech Co., Ltd, Nanjing, China). qRT-PCR was carried out using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China) following the manufacturer's protocol. The *P. suffruticosa Actin* was used as an internal control to normalize the transcriptional levels. The specific primers used in qRT-PCR were designed by Primer Premier 6.0 according to the full-length

cDNA sequences, and were listed in Table S4. The relative expressions of these genes were performed using  $2^{-\Delta\Delta Ct}$  method as described by Livak and Schmittgen [62].

## Measurements of sugar contents

Three flower buds of every repeat at five chilled phase were crushed by a crusher (MM 400, Retsch) containing zirconia beads at 30 Hz. A total of 20 mg powder was added into 500  $\mu$ L of methanol: isopropanol: water (3: 3: 2 V/V/V) solution. After vortexed for 3 min and sonicated for 30 min, 50  $\mu$ L of the supernatant were taken and evaporated in nitrogen, with adding internal standard. After evaporated in a nitrogen stream and freeze-drying, the residue was further derivated as follows: Firstly, the small molecule carbohydrate was mixed with 100  $\mu$ L methoxine hydrochloride solution in the 1.5 mL Eppendorf tubes. Secondly, 100  $\mu$ L of bistrifluoroacetamide was added to the solution after 37 °C for 2 h. After vortexed, the mixture was incubated at 37 °C for another 30 min. N-Hexane was used as dilution fusion. The mixture was detected by MetWare (<http://www.metware.cn/>) based on the Agilent7890B-7000D GC-MS/MS platform [63].

## Measurements of starch content and AMY enzyme activity

The contents of starch was determined according to the previous method with little change [64]. The crushed tree peony buds (0.1 g) were extracted in 7.2 mL of 80% ethanol at 80 °C for 30 min. The extract was centrifuged for 30 min, and the precipitate was gelatinized in a boiling water bath for 15 min. Concentrated sulfuric acid was added to the precipitate to dehydrate monosaccharides into aldehyde compounds. The anthrone reagent was used to react with the test solution. The absorbance at 640 nm was recorded with a spectrophotometer (HITACHI, Japan).

The AMY enzyme activity was determined according to the method of Huggins and Russell with minor modifications [65]. About 0.2 g flower buds from tree peony was ground into a homogenate, and centrifuged to collect the supernatant. The supernatant was incubated at 70 °C for 30 min to inactivate  $\beta$ -amylase. The samples were reacted with 3,5-dinitrosalicylic acid. The absorbance at 525 nm was recorded with a spectrophotometer (HITACHI, Japan).

## Measurements of hormone contents

Approximately 2 g fresh weight buds per repeat were taken from different chilling treatments. The contents of phytohormones were determined by the Wuhan Greensword Creation Technology Company (<http://www.greenswordcreation.com>) based on LC-MS/MS analysis according to a previously reported method with minor modification [66].

## Statistical analysis

Means and standard errors were calculated using Graphpad Prism 7 (San Diego, USA). Analysis of variance (ANOVA) was used to compare statistical differences and levels of gene expression between treatments and control.

## Abbreviations

QC: quality control, OPLS-DA:Orthogonal projection to latent structures-discriminant analysis, DMs:differential metabolites, SD:standard deviation, VIP:variable importance in the project, GC-MS/MS:Gas chromatography-mass spectrometry, LC-MS/MS:Liquid chromatography electrospray ionisation tandem mass spectrometry.

## Declarations

## Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. The sequences of the genes used in this study are available at GenBank, and the accession numbers are as follows: PsCHS, JN105300.1; PsCHI, ADK55061.1; PsF3H, HQ283447.1; PsDFR, HQ283448.1; PsANS, KJ466969.1; Ps3GT, MT702582; PsAFB2, MT702583; PsTIR1, MT702589; PsMYC2, MT702586; PsCIN, MT702584; PsCWIN, MT702585; PsVIN, MT702590; PsSUS1, MT702587; PsSUS2, MT702588.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

The authors declare that they have no competing interests.

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## Authors' contributions

GSP and ZYX conceived and designed the experimental plan. ZT, CXZ, ZY and LCY conducted the experiments. ZT and YYC wrote the manuscript. GSP and ZYX revised the manuscript. Both authors have read and approved the manuscript.

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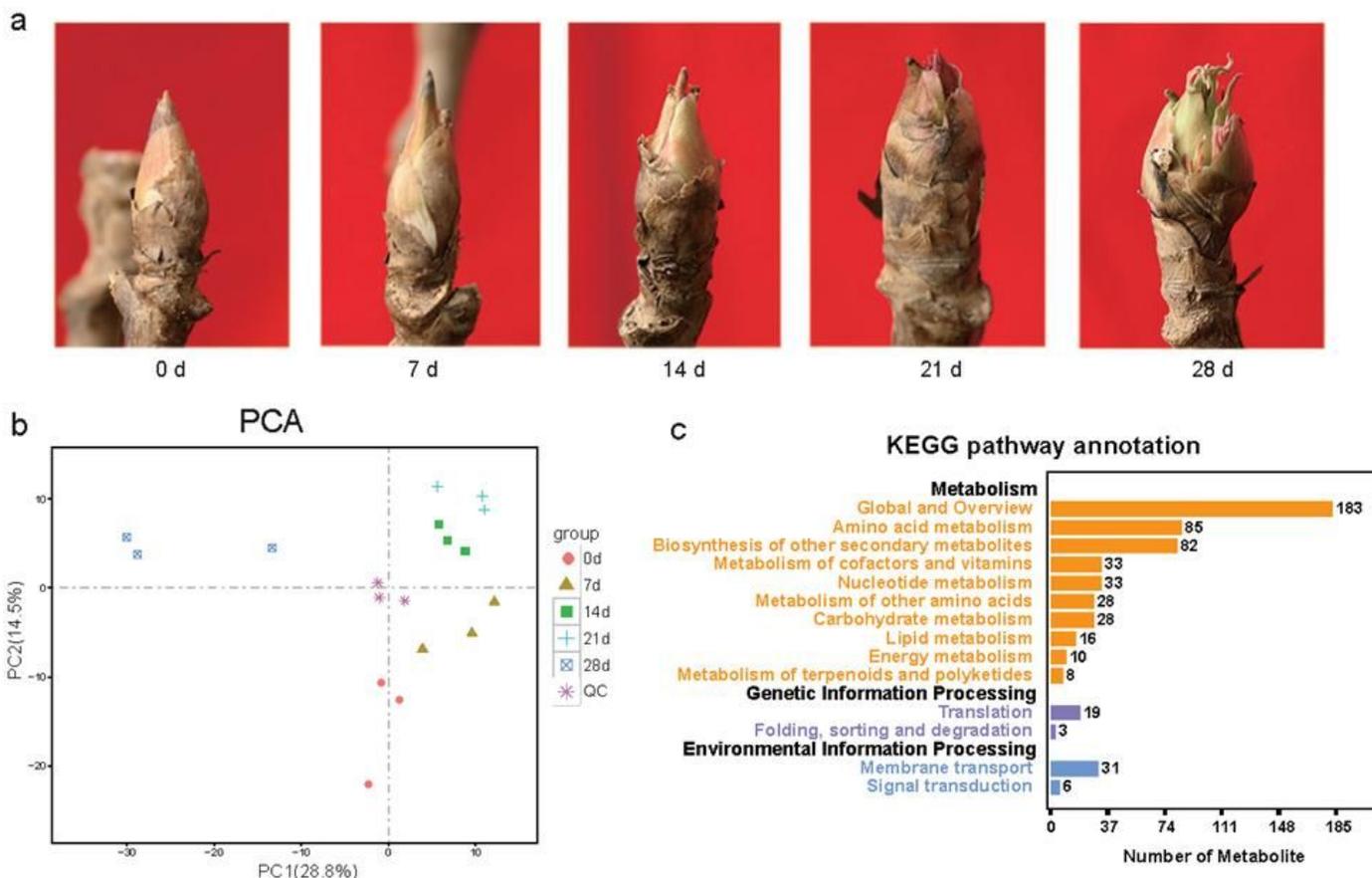
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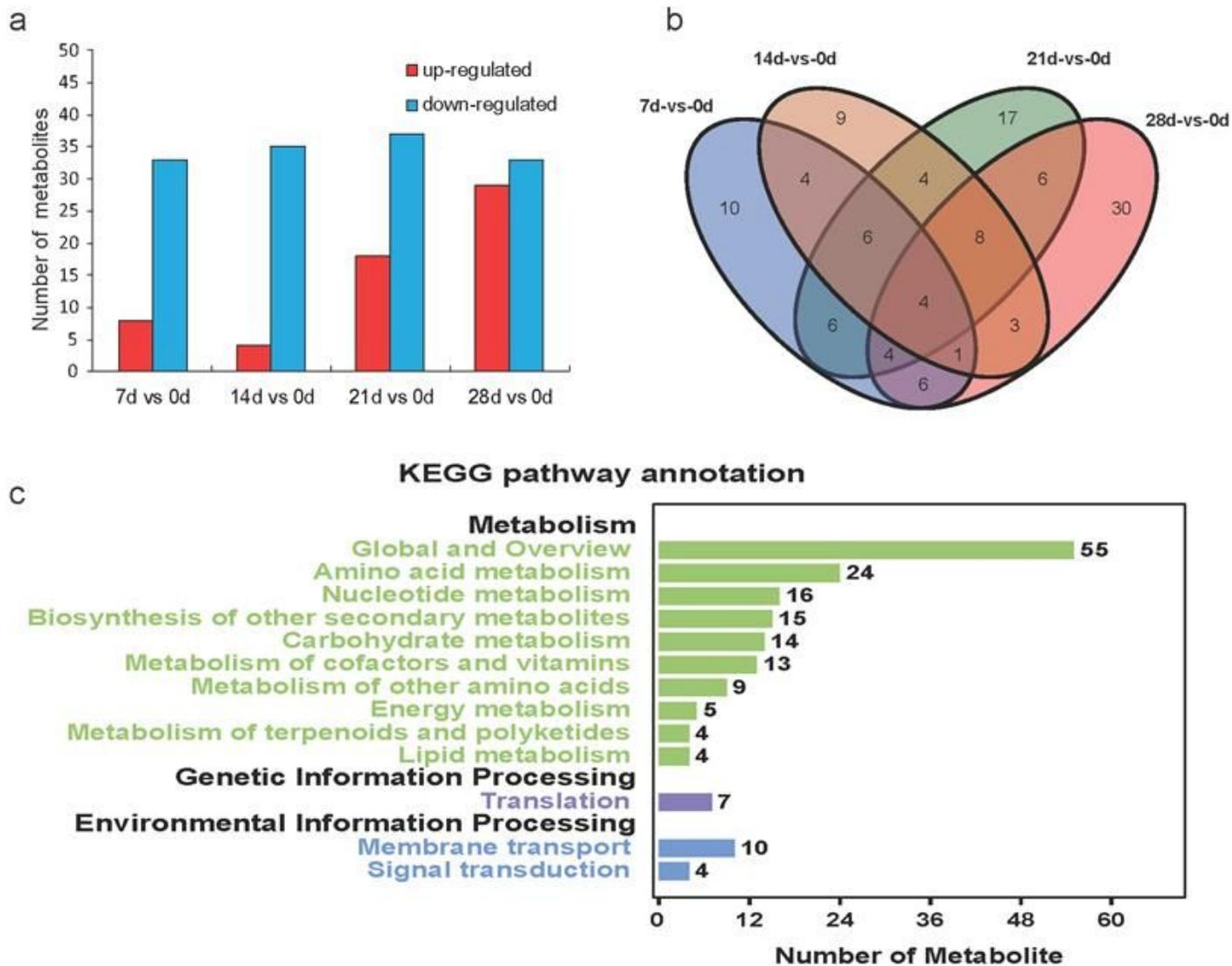
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## Figures



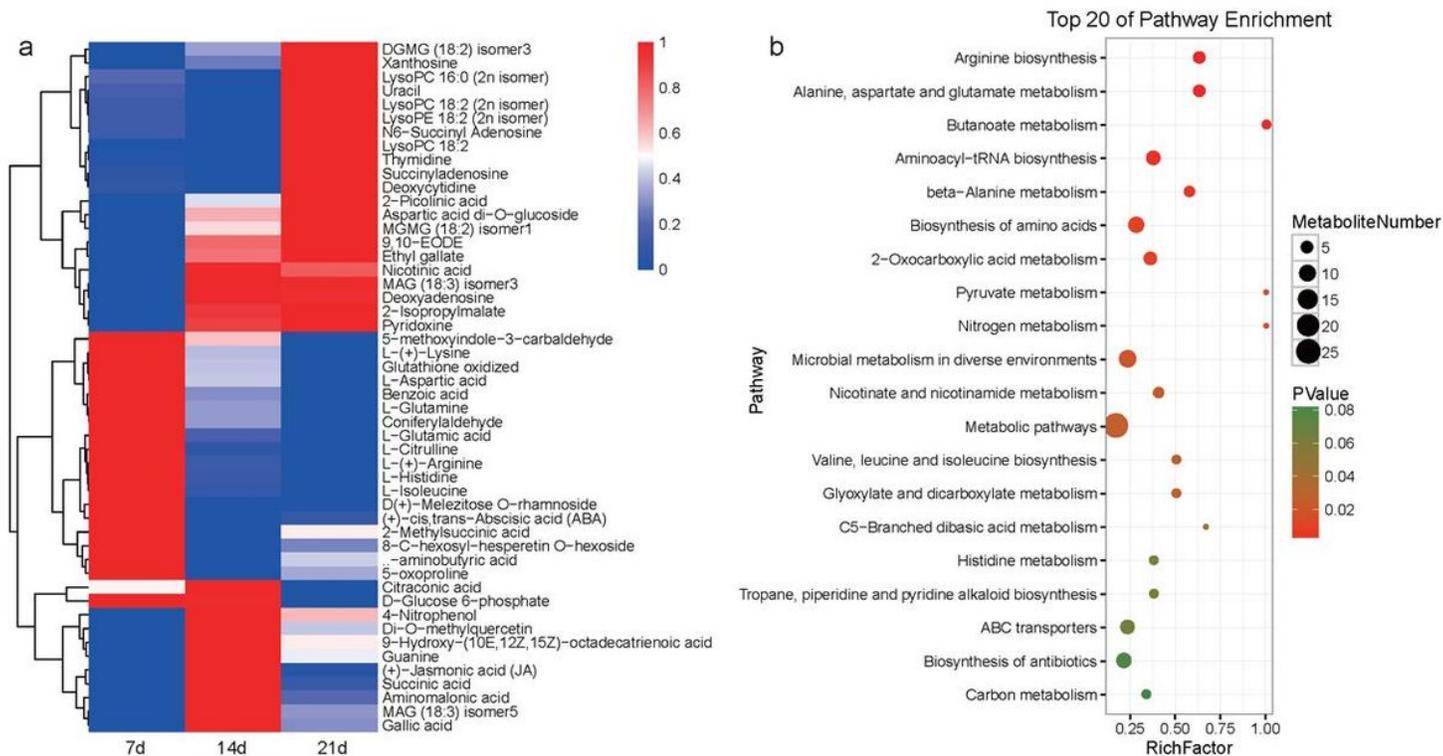
**Figure 1**

Metabonomics analysis of tree peony buds. (a) Bud morphology of tree peony buds during the chilling duration. (b) PCA plot of the tree peony buds at five stages. (c) The KEGG pathway annotation of metabolites.



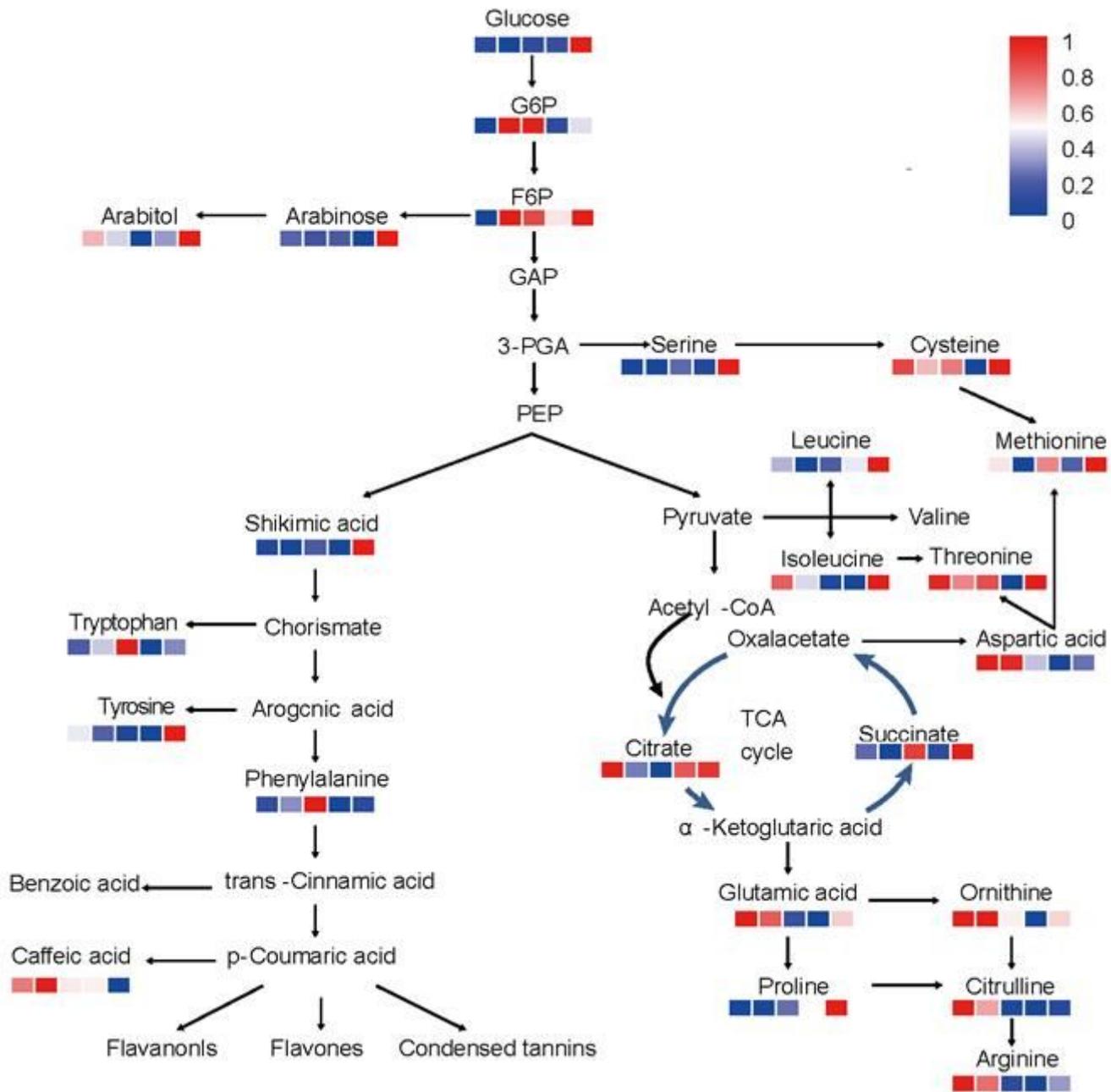
**Figure 2**

Expression dynamics and comparative analysis of metabolites in tree peony buds chilled at different stages. (a) Statistical map of intergroup metabolites ( $P < 0.05$ ,  $VIP \geq 1$ ). Red and blue represented up- and down-regulated metabolites, respectively. (b) Venn diagram of metabolites among four comparison groups. (c) The KEGG pathway annotation of all DMs.



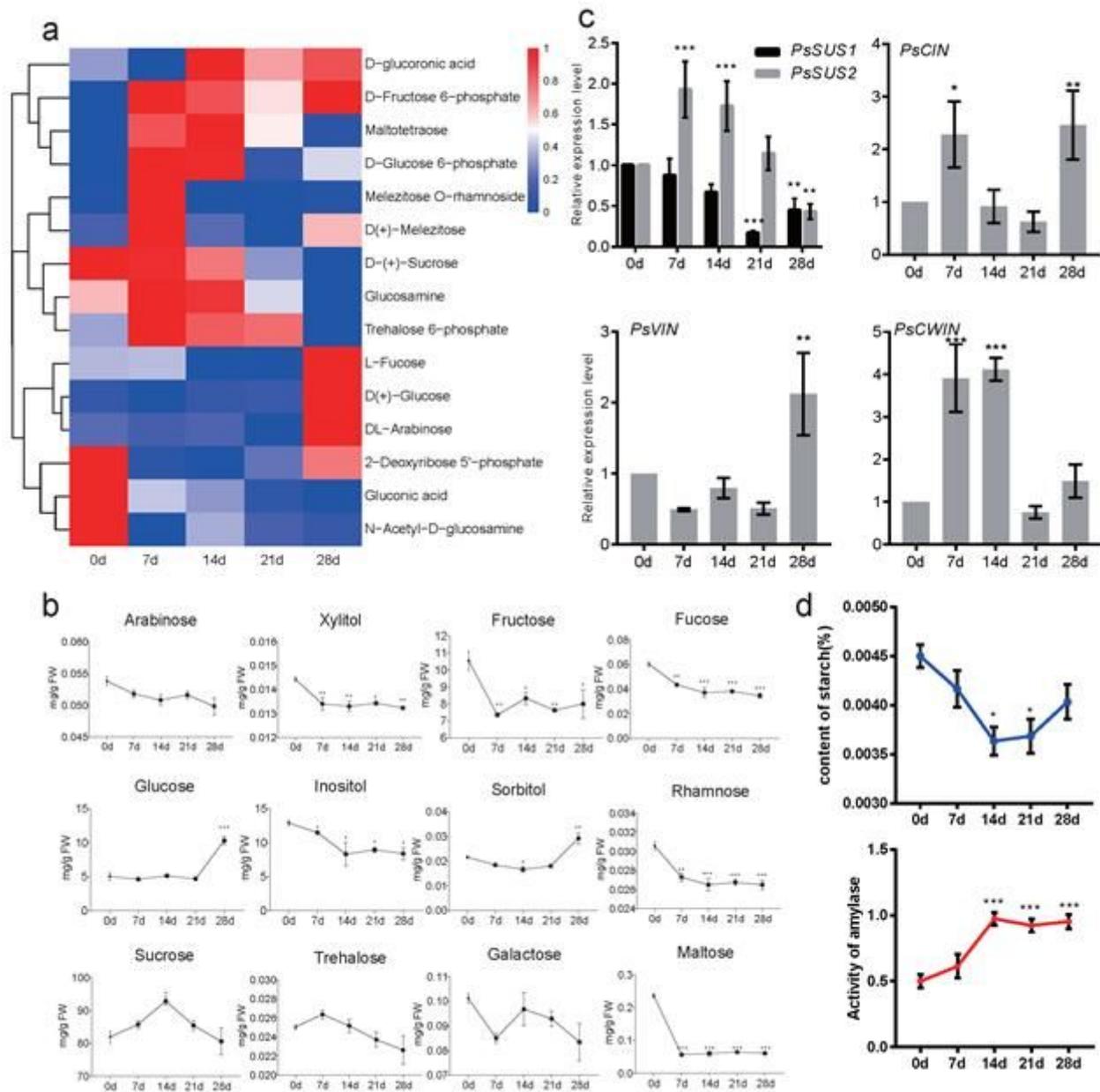
**Figure 3**

Changes of 50 DMs related to dormancy release in tree peony. (a) The clustering heatmap of 50 DMs at 14 d vs 7 d and 21 d vs 7 d. (b) The top 20 of KEGG enrichment pathways for DMs among 14 d vs 7 d and 21 d vs 7 d.



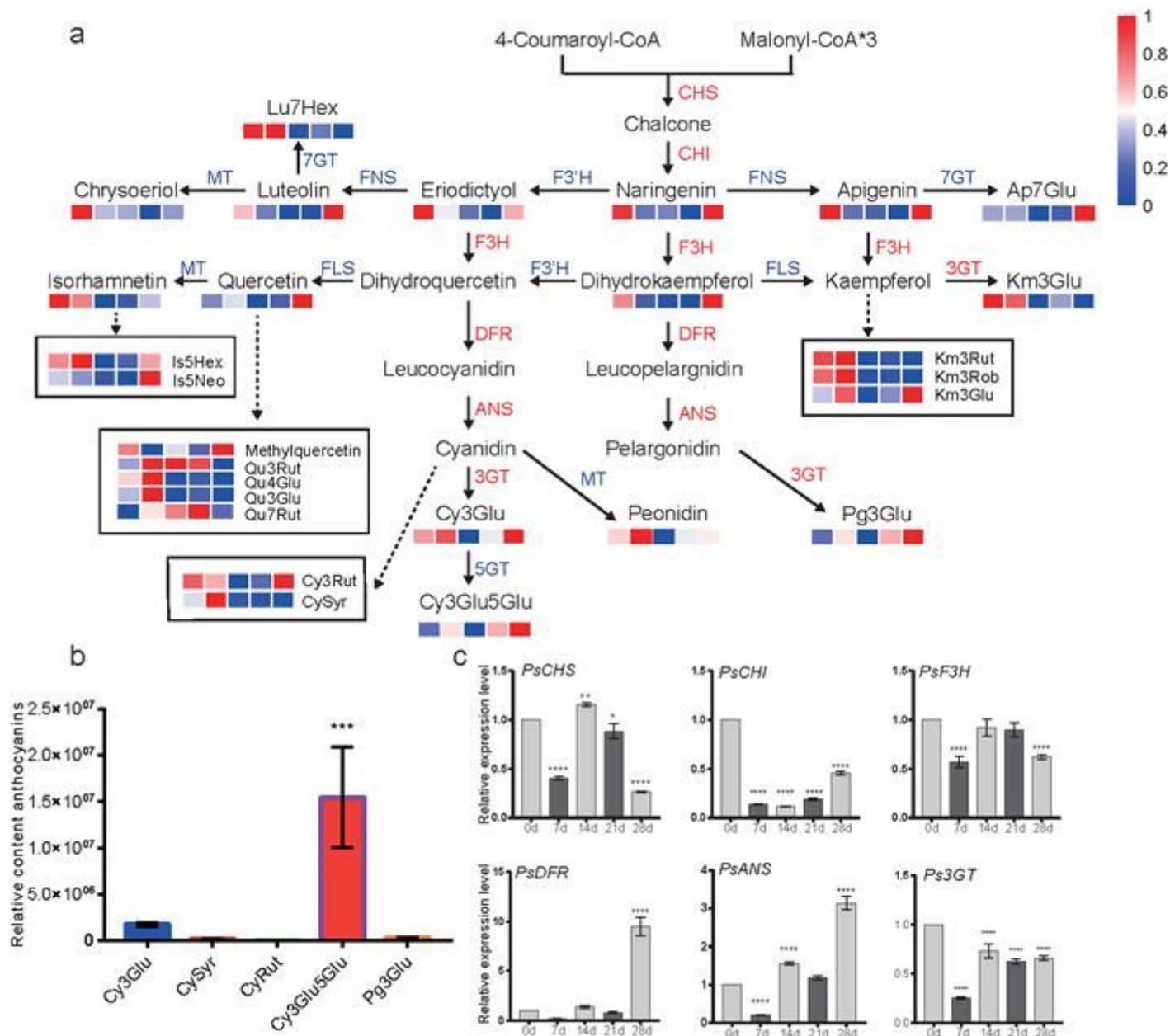
**Figure 4**

Dynamics of carbon metabolic pathways throughout the chilling duration process. The metabolite amounts were shown in heatmaps as 0, 7, 14, 21, and 28 d from left to right, respectively. G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), GAP (3-phosphoglyceraldehyde), PEP (phosphoenolpyruvate).



**Figure 5**

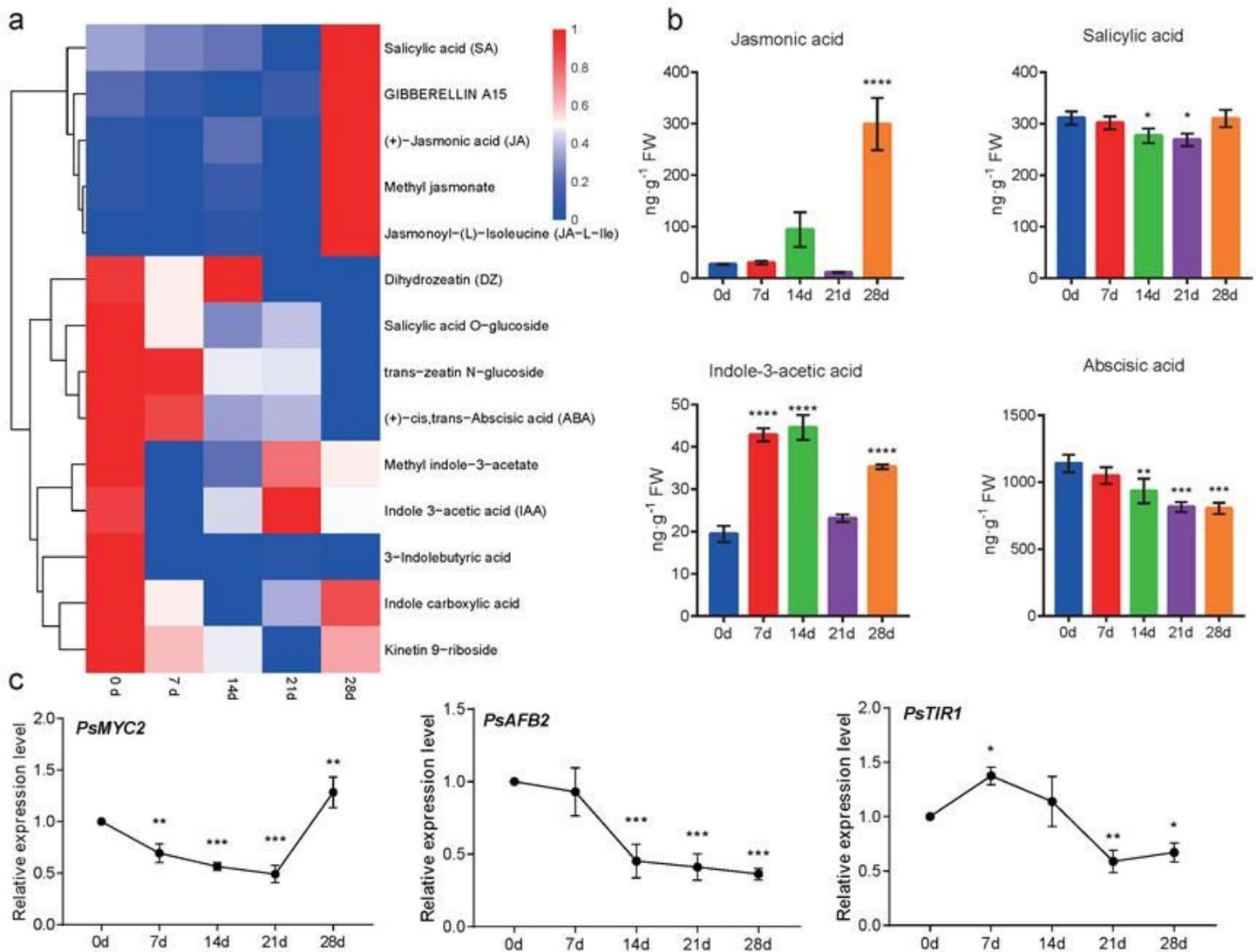
Changes of carbohydrates during dormancy transition induced by the chilling in tree peony. (a) The cluster heatmap of carbohydrates. (b) The variations of several sugars content. Data were represented as mean of three different determinations  $\pm$  SD. Asterisks indicate statistically significant differences (one-way ANOVA, \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ). (c) The relative expression levels of sucrose synthase and sucrose invertase genes. The mean  $\pm$  SD in three biological replicates was shown. \*, \*\* and \*\*\* indicated significant differences of one-way ANOVA at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively. (d) The variations of starch and amylases activity. The mean  $\pm$  SD in three biological replicates was shown. \*, \*\* and \*\*\* indicated significant differences of one-way ANOVA at  $P < 0.05$  and  $P < 0.001$ , respectively.



**Figure 6**

The changes of flavonoids content and the related genes expression during the chilling duration. (a) The flavonoids and anthocyanin pathway. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanin synthase; 3GT, anthocyanidin 3-O-glycosyltransferase; 5GT, anthocyanidin 5-O-glycosyltransferase; MT, anthocyanidin methyltransferase; 7GT, anthocyanidin 7-O-glycosyltransferase; FLS, flavonol synthase; FNS, flavone synthase; Lu, luteolin; Ap, apigenin; Is, isorhamnetin; Qu, quercetin; Km, kaempferol; Cy, cyanidin; Pg, pelargonidin; Glu, glucoside; Hex, hexoside; Neo, neohesperidoside; Rut, rutinoside; Rob, robinobioside; Syr, syringic acid. (b) The relative content of anthocyanins in tree peony flower buds after 28 d chilling duration based on the results of LC-ESI-MS/MS. (c) The relative expression level of key genes involving in anthocyanin

biosynthesis. Data were represented as mean of three different determinations  $\pm$  SD. Asterisks indicate statistically significant differences (one-way ANOVA, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ).



**Figure 7**

The changes of phytohormone content and the expression patterns of related genes during chilling duration. (a) The cluster heatmap of phytohormone during the chilling duration. (b) Phytohormone contents in tree peony flower buds during the chilling duration by LC-MS/MS. (c) The qRT-PCR results of anthocyanin synthesis gene *PsMYC2*, and two auxin receptors genes *PsAFBs*, and *PsTIR1*. The mean  $\pm$  SD ( $n = 3$ ) were shown. Asterisks indicated statistically significant differences (one-way ANOVA, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ).

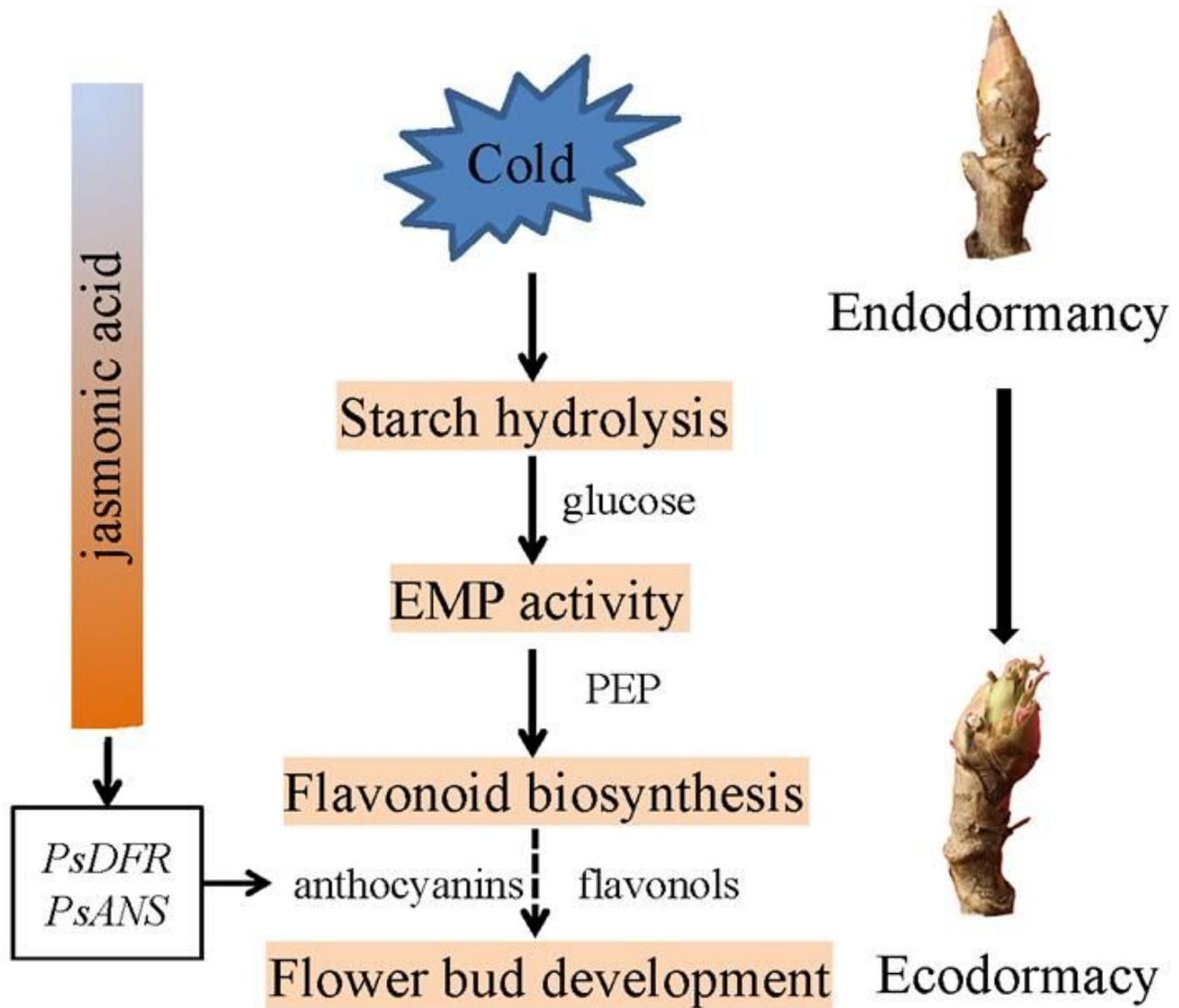


Figure 8

Model of metabolite role during dormancy transition induced by chilling in tree peony. Starch degradation and EMP activation provide energy and material basis for flavonoid accumulation during endodormancy release. Flavonoid and anthocyanin accumulation might promote flower bud development at ecodormancy stage. Meanwhile, the accumulation of anthocyanin may be regulated by jasmonic acid.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS4.xlsx](#)
- [TableS3.xlsx](#)
- [TableS2.xlsx](#)

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- [Fig.S2.pdf](#)
- [Fig.S1.pdf](#)