Metabolic Analysis of the Regulatory Mechanism of Sugars on Secondary Flowering in Magnolia

Lingjuan Xuan
Zhejiang A&F University
Qianqian Wang
Zhejiang A&F University
Zhigao Liu
Zhejiang A&F University
Shaoyu Cheng
Zhejiang A&F University
Yingjia Zhang
Zhejiang A&F University
Danying Lu
Zhejiang A&F University
Bin Dong
Zhejiang A&F University
Dongmei Zhang
Shanghai Academy of Landscape Architecture Science and Planning
Lang Zhang
Shanghai Academy of Landscape Architecture Science and Planning
Jingjing Ma
Zhejiang A&F University
Yamei Shen (yameishen@zafu.edu.cn)
Zhejiang A&F University

Research Article

Keywords: Magnolia, flower bud differentiation, MlTPS genes, sucrose and trehalose spraying, metabolite analysis

Posted Date: September 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-846337/v1

Abstract

Background: Magnolia is a traditional and important ornamental plant in urban greening, which has been cultivated for about 2,000 years in China for its elegant flower shape and gorgeous flower color. Most varieties of *Magnolia* bloom once a year in the spring, whereas a few others, such as *Magnolia liliiflora* Desr. 'Hongyuanbao', also bloom for the second time in summer or a little late. Such a twice flowering trait is desirable because of its high ornamental values, but its underlying mechanism remains unclear.

Results: To explore the peculiar metabolism twice-flowering in 'Hongyuanbao', the chemical metabolites and the relevant genes encoding them in the flower buds during the entire flowering period were analyzed. It appears that there was a significant variation between the metabolic profiles of flower differentiation between the first and secondary flower buds. The sucrose and trehalose metabolic pathways were significantly upregulated during both flowering periods as shown by a Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis. This was further verified by quantitative real-time polymerase chain reaction analysis which showed that the expression levels of a number of trehalose-6-phosphate synthase (*TPS*) genes including *MlTPS1*, *MlTPS5*, *MlTPS6*, *MlTPS7*, and *MlTPS9* were substantial increased. To further investigate the potential functional role of sucrose and trehalose in flowering regulation, the leaves of 'Hongyuanbao' were sprayed with the sucrose or trehalose solution with a gradient of concentrations including 30 mM, 60 mM, and 90 mM. While both trehalose and sucrose of 60 mM in concentration were able to accelerate the process of flower bud differentiation, their molecular pathways appeared to be different. Sucrose treatment increased the transcription levels of *MlTPS5* and *MlTPS6*, whereas trehalose treatment increased *MlTPS1*. The expression of a number of flowering-related genes, such as *MlFT, MlLFY*, and *MlSPL* was also increased in response to the sprays of sucrose and trehalose.

Conclusions: Overall, the findings on the functional roles of sucrose/trehalose and their underlying molecular mechanism in flowering of 'Hongyuanbao' improves our current understanding on of the biochemical triggers for flowering in *Magnolia* and provides a useful tool in Magnolia cultivation and flowering management.

Background

Flowering is an prominent feature and essential part of the reproductive process in flowering plants which constitute the largest and most diverse group of the plant kingdom [1,2]. It is also an important indication that plants can adapt to their environment, and the choice of flowering time is crucial in the life cycle of a plant to ensure maximum reproductive success [3–5]. During the process of cultivation and breeding, ornamental plants bearing flowers with rich colors, changeable patterns and longer flowering period are generally favored [2]. The majority flowering plants bloom once a year at certain seasons and these plants are called once flowering plants [6]. While some can bloom again within a year as twice flowering plants, or continue to bloom under favorable conditions as continuous flowering (CF) plants. In

horticulture, multiple flowering is a popular trait which has been introduced in a wide range of cultivated varieties [7].

In recent years, there have been a number of in-depth studies on the molecular mechanisms of plant flowering, especially in model plant *Arabidopsis thaliana*, which is a once flowering plant. It has been found that six major pathways including photoperiod, vernalization, thermosensory, gibberellin, autonomous, and aging, respond to multiple signaling pathways to regulate flower development [8–10].

The investigation on the multiple flowering habit has so far been focusing on strawberry and rose. In CF roses, the absence of the floral repressor provokes continuous blooming because of an insertion of retrotransposon in the second intron of the *TERMINAL FLOWER1*(*TFL1*) homologue [11,12]. Similarly, in woodland strawberry (*Fragaria vesca*,), a 2-bp deletion in the coding region of the *TFL1* homologue introduces a frame shift and is responsible for its CF behavior [13]. However, the function loss of *TFL1* resulting in CF during the whole growth phase appears to be not applicable to all the CF species in rose and strawberry [14].

Further, it is known that the development of flowers requires the strict regulation of ecological processes accompanied by changes in sugar metabolism [15,16]. Sugars were found to act as signaling molecules interacting with the flowering pathways, as exemplified by the transcription factor *ATIDD8* which regulates flower by regulating sucrose transport and metabolism in *A. thaliana* [17]. In addition, trehalose has been identified as a signaling molecule that regulates many important metabolic and developmental processes in plants [15, 18–20]. Trehalose-6-phosphate (T6P) is synthesized from UDP-glucose and glucose-6-phosphate by T6P synthase (*TPS*) and then converted to trehalose by T6P phosphatase [21,22]. Since T6P exists in plants in trace amount, it is thought to act as a signaling molecule for the regulation of sugars [23]. In many plants, the content of T6P is closely related to that of endogenous sucrose at both day and night. This significant correlation suggests that T6P may be a signal of sucrose availability and a negative feedback regulator of sucrose accumulation [18]. In studies of *A. thaliana*, it was found that the T6P pathway can directly regulate flowering at two sites: *TPS1* activity is required for the induction of the florigen *FT*, which provides a convenient way for the plant to integrate an environmental signal [24]; in addition, the T6P pathway affects the expression of important flowering-time and flower-patterning genes such as *SPLs* via the age pathway to regulate flowering [25]. Taken together, sugars may have been involved in the regulation of flowering processes in plants in through various flowering pathways. To our knowledge, the role of sugars in twice flowering process has not been studied, which prompted our current investigation on the interactions between sugars and continuous flowering in Magnolia.

Magnolia is a traditional and important ornamental plant in urban greening, which has been cultivated for about 2,000 years in China for its elegant flower shape and gorgeous flower color [26,27]. Most species of *Magnolia* bloom once a year in spring, while fewer were found to be able to flower again in summer or later [28]. The characteristic of twice flowering of *Magnolia* has greatly enhanced its ornamental and research values, and therefore, it is of significant interest to understand the molecular mechanism

controlling the secondary flowering in order to explore the commercial application of this valuable trait in *Magnolia*. *Magnolia liliiflora* 'Hongyuanbao' is one of the twice flowering Magnolia varieties that bloom in both spring and summer with regular flowering time [29]. In this study, we investigated the biochemical metabolism during the differentiation of flower buds in *M. lifiiflora* 'Hongyuanbao and identified a variety of metabolic compounds that are shown to influence the flowering process, which provides important clues to understanding the metabolic regulation of twice flowering and its underlying molecular mechanisms.

Results

Identification of two distinct periods of flower bud differentiations in *M. lifiiflora* 'Hongyuanbao'

M. lifiiflora 'Hongyuanbao' flowers in both spring and summer. The entire flower bud differentiation process was studied by microscopy observation of paraffin sections of flower buds sampled at regular intervals (Fig. 1A). In spring, 'Hongyuanbao' flowers from late March to the middle of April, which is relative later than the once flowering *Magnolia* plants. In summer, it started to flower from early June to middle August and continued to bloom for a longer period than the flowers in spring (Fig. 1B).

The flower bloomed in spring was borne primarily at the top of the raw branches from the previous year. Unlike other Magnolia, the flowering in 'Hongyuanbao' was rather synchronized. When the spring flowers had fallen, the axillary buds sprouted new shoots and formed flower buds at the top. The differentiation of buds occurred with simultaneous elongation of new shoots. In summer, these differentiated flower buds would bloom from early June to middle August, which lasted longer than the spring flowers. It appeared that some variations existed in the development and opening process of flower buds between spring and summer flowers, which could be attributed to the variations in plant nutritional status and environmental conditions.

Untargeted metabolomic analysis of various primary metabolites between the first and second flower bud differentiation

In order to explore the mechanism of twice flowering trait in 'Hongyuanbao', the primary metabolites of different flower buds periods during twice flowering were analyzed using gas chromatography-mass spectrometry (GC-MS).

In most metabolomic data analyses, compounding factors orthogonal to the variables of interest may obscure the intended class separation, and an orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used as a common analytical method. In the study, OPLS-DA

was used to filter out the orthogonal variables that are not related to the classification variables and analyze both the nonorthogonal and orthogonal variables in order to investigate the relationship between the metabolites and twice flower bud differentiation(Fig. 2). The coordinates value for to1 and t1 showed a clear separation of the first predicted component between the two groups (Fig. 2D-F), and the value of the Q2 is deemed to represent the prediction ability of OPLS-DA model. In this study, Fig. 2-A was compared with Fig. 2-D, which had an overall cross-validation coefficient, Q2(y), of 58%. In addition, when Fig. 2-B was compared with Fig. 2-E, it had an overall cross-validation coefficient, of 78% and the model had an overall cross-validation coefficient, of 77% when Fig. 2-C was compared with Fig. 2-F. Thus, the OPLS-DA model can be used to reliably identify the categories, and OPLS-DA is more suitable than principal components analysis to identify the source of 'Hongyuanbao' samples.

To investigate the impact of twice flowering on metabolism in flower buds, the metabolic profiles of both the first and the secondary flower buds, each with five replications, were used to conduct hierarchical cluster analysis (Fig. 2). A total of 41 differential metabolites were identified (Supplementary Table 1).

For the purpose of identifying the metabolites during various stages of flower bud differentiation, the responses of all the 41 metabolites were analyzed. Based on the Mass Bank and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolome Database analyses, the metabolites were divided into six groups: Carboxylic acids and derivatives (including 12 compounds), Organooxygen compounds (13), Fatty Acyls (5), Prenol lipids (3), Indoles and derivatives (2), and other compounds (6). The levels of most sugars tended to increase compared with the twice differentiation in flower buds,while the levels of glutamate and pyroglutamate were lower in the flower buds, and the level of organic acids, such as pyruvate, tended to significantly increase. The level of malate tended to decrease in the second flower buds. Furthermore, the levels of citrate and 2-oxoglutarate were lower in the flower buds. These data suggest that many metabolites, especially sugars, are necessary for the differentiation of secondary flower buds.

The correlation analysis of metabolite-metabolite interaction network in *M. lifiiflora* 'Hongyuanbao'

To study the correlation between the metabolites in different stages of flower bud differentiation in *M. lifiiflora* 'Hongyuanbao', Pearson correlation coefficients were used to calculate the data of differential metabolites between the first and secondary flower bud differentiations in three different developmental stages. MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) was used to map the correlation network among metabolites. There were 22 groups of metabolites in S1, 41 groups in S2, and 25 groups in S3 that were measured and the False Discovery Rate (FDR) was 0.05. The correlation network of S2 was found to be much closer than that of S1 and S3 (Fig. 3). These data suggest that various metabolic activities of S2 are active during flower bud differentiation and we would focus on the correlations between the metabolites in S2.

KEGG Enrichment Analysis of sugar metabolites and the expression of *MlTPS* genes

KEGG enrichment analysis showed that sucrose and trehalose in the sucrose and starch metabolic pathways were significantly upregulated. To reveal how the sugars affect continuous flowering, genes in relevant metabolic pathways were mined and five *MlTPS* genes, which were related to sugar pathways, were obtained from transcriptome data. The expression of *MlTPS* genes during the second differentiation process was significantly upregulated as revealed by qRT-PCR analysis. Further analysis found that *MlTPS1* and *MlTPS7* were the most prominent in expression, and *MlTPS5* was barely discernible during the second differentiation. The overall trend demonstrates that the level of expression of *MlTPS* genes increased significantly during the middle stage during the second flower bud differentiation (Fig. 4), suggestive of potential function role of *MlTPS* genes in the secondary flower bud differentiation.

Trehalose promoted early flowering

In order to confirm the effects of sucrose and trehalose on the twice flowering trait in *M. lifiiflora* 'Hongyuanbao', the plants grown in the nursery of Zhejiang A&F University, were sprayed with a solution of sucrose and trehalose (Fig. 5A). Once the plants stopped flowering, leaf spraying commenced with three concentrations (30 mM, 60 mM, 90 mM) of sucrose or trehalose. The leaves were sprayed once every 5 days and the samples were taken for microscopy observation on the 20th and 35th day following the first spraying.

Following 20 days spaying, the plants that had been sprayed with solutions of 90 mM and 30 mM of sucrose were still in the undifferentiated status, while the plants sprayed with a solution of 30 mM trehalose had reached the sepal differentiation status. Simultaneously, the flower buds of the plants sprayed with a solution of 60 mM trehalose had reached petal differentiation stage. Among the samples taken after 35 days spraying, the control (CK- plants without any treatment) was still in the stage of pistil development, but the plants that had been sprayed with solutions of 60 mM and 90 mM of trehalose had started to show flower bud differentiation. However, the plants treated with a 30 mM solution of sucrose were in the petal developmental stage, while those treated with a 60 mM solution of sucrose were in the pistil developmental stage. The plants treated with a 90 mM solution of sucrose were in the stamen developmental stage. Taken together, these results demonstrate that spraying of 'Hongyuanbao' leaves with different concentrations of sugars, either sucrose or trehalose, have variable effects on flower bud differentiation, with trehalose showing prominent promotion effects on the process of flower bud differentiation (Fig. 5B).

Expression of *TPS* genes and flowering genes under sucrose treatment

To investigate the effect of trehalose and sucrose on the levels of expression of the *MlTPS* genes in 'Hongyuanbao', the expression patterns of the *MlTPS* genes in 'Hongyuanbao' leaves that had been treated with 60 mM of trehalose or sucrose were examined (Fig. 6). It was apparent that the *MlTPS* genes were widely expressed throughout the flower bud differentiation period. In the treatment with a solution of 60 mM trehalose, relative to CK, the expression of *MlTPS1* increased during the middle and later stage, the expression of *MlTPS5* continuously increased, the expression of *MlTPS6* decreased following an

initial increase, while the expression of *MlTPS7* and *MlTPS9* remained unchanged. Under the treatment with 60 mM sucrose, the expression of *MlTPS1* was barely detectable, the expression of *MlTPS5* decreased following an initial increase, the expressions of *MlTPS6* and *MlTPS7* was always higher than that of the CK, and the *MlTPS9* expression remained unchanged.

Moreover, the flowering integrators also responded to sugar treatment (Fig. 6). *MlFT* might have influenced the differentiation of flower buds in the beginning, particularly when the plants were sprayed with a solution of trehalose. The level of expression of *MlLFY* was higher in the beginning of flower differentiation compared to the later stages, suggestive of its functional role in the development of floral meristem. The expression of *MlCO* was raised moderately by the sugar treatments, while *MlAP1* was not responsive. These results directly demonstrated that *Ml*TPS genes could be responsive to sugar signal to regulate floral differentiation and the acceleration in flowering promotion may depend on the enhanced expression of *MlFT* and *MlLFY*.

Analysis of the expression of transcription factor *SPL* following sugar treatment

SPL genes have been shown to be regulated by diverse flowering signals and to form the molecular output of a pathway that regulates flowering as a function of a plant's age [30]. In the current studies, the age pathway gene *SPL3* have been suggested as a participant in the T6P pathway and affect the process of flowering. Two *MlSPL3* genes (*MlSPL3-1* and *MlSPL3-2*) were identified from the 'Hongyuanbao' transcriptome. *MlSPL3-1* showed significantly higher expression relative to CK under both sugar treatments, with the sucrose treatment showing relatively higher expression than the trehalose treatment. While the expression level of *MlSPL3-2* did not differ significantly between the spraying treatments with trehalose and sucrose, the overall expression of *MlSPL3-2* was higher following spraying with trehalose (Fig. 7). These results suggest that the T6P pathway can directly affect the expression of important flowering-time and flower-patterning genes *MlSPL* via the age pathway to promote the floral differentiation process.

Discussion

A different multiple flowering mechanism exist in *M. lifiiflora* 'Hongyuanbao'

As the timing of flowering is critical for successful sexual reproduction, crop productivity and yield [30,31], numerous models have been established in model plants such as Arabidopsis and some crop plants. These models have also been used to study the molecular mechanisms that underlie the control of flower bud differentiation in ornamental plants. Nevertheless, the specific requirements for multiple flowering in ornamental plants have only recently become a popular research topic due to the increasing commercial interest. Flower bud differentiation is a physiological and morphological sign of the transformation from vegetative to reproductive growth [32], and the whole process consists of the induction stage before

flower bud differentiation and the specific processes of inflorescence and flower differentiation [33]. ‘Hongyuanbao’ is a *M. lifiiflora* variety that was introduced to China in 2001 [5], and its ornamental features have been relatively stable for many years, especially the twice flowering phenotype. The phenomenon of twice flowering has been described in one previous study [5], but the in-depth understanding on its flowering mechanism is still lacking. In this study, we made observations on the flowering time and the differentiation of flower buds throughout the entire flowering period and it was revealed that ‘Hongyuanbao’ had two distinct flower bud differentiations, the first and secondary differentiations in spring and summer respectively, not the whole growth phase, which is different from the continuous flowering species in rose and strawberry.

In previous reports, the functional loss of *TFL1*, and *KSN* caused continuous flowering in rose and strawberry [34–36], and multigenic control of continuous flowering was proposed in cultivated strawberry [37,38]. However, the unaltered expression pattern of *TFL1* gene in the transcriptome of ‘Hongyuanbao’, prompt us to consider for an alternative mechanism which regulates the formation of flowers twice a year in ‘Hongyuanbao’.

In recent years, increasing amounts of attention have been paid to plant metabolism, and, sugars have been deemed as signaling molecules that regulate a variety of genes involved in various aspects of plant developments. In order to further uncover the potential mechanism underlying the twice flower bud differentiation in ‘Hongyuanbao’, the whole flower bud differentiation process was categorized into six stages. A metabonomic analysis of the differential flower buds of these six stage suggested that the flower bud differentiation mainly occurred in the middle stage at which the content of sugars, such as sucrose and trehalose, and the content of isozymes had increased significantly. It indicated that sucrose and trehalose may play important roles during the process of flower bud differentiations, especially the secondary flower bud differentiation.

Sucrose and trehalose play important roles during twice flower bud differentiation and blossom

Sugars, as the basic molecules of carbon metabolism, can be used as energy substances or signaling molecules playing an important role in the entire life cycle of plants. A number of plant systems, such as Chrysanthemum [39], *A. thaliana* [15,40], and apple [41,42] have been well established for studying sugar metabolism. Recent studies found that sugars could influence the phase transition from vegetative to reproductive growth in plants, by acting as a signaling molecule which could interact with other inorganic regulatory networks [43,44]. Consistent with previous researches, metabolomics analysis of flower buds in ‘Hongyuanbao’ verifies the importance of sugars during flower bud differentiation process. In order to investigate the functional role that sugars may play during the twice flower bud differentiation and bloom in *M. lifiiflora* ‘Hongyuanbao’, solutions of sucrose or trehalose were sprayed on plant leaves, revealing that both sucrose and trehalose can promote flower formation, with trehalose showing more prominent effect (Fig. 5).

As illustrated in Fig. 8, T6P acts as a signaling molecule that relays information about carbohydrate availability to other signaling pathways, and plays an important role in sugar metabolism pathway [15]. T6P pathway responses to sugar signal and regulate flowering based on two different aspects. In the plant leaves, *TPS1* is activated by the elongation in day length in spring, which produces T6P and induces the expression of florigen *FT*, integrating the environmental and physiological signals for flowering [15,45]. The signals ensure the expression of *FT* when the conditions are optimal to support the energy demanding processes of flowering. Besides, the T6P pathway affects the expression of *SPL3* via the age pathway directly at the SAM independently of the photoperiod pathway (Fig. 8).

Therefore, we screened and characterized five *MlTPS* and two *MlSPL3* genes derived from *M. lifiiflora* 'Hongyuanbao'. The *MlTPS* genes that were expressed in various stages was simultaneously verified using qRT-PCR and the expression levels of *MlTPS1*、*MlTPS6*、*MlTPS7* increased significantly under sugars treatment, indicating the important function of *MlTPS* during the flowering pathways that are regulated by sugars. Previous studies [45] in Arabidopsis have also found that *TPS1* can promote flowering, while knocking out *TPS1* gene will result in delay in flowering. But the correlation of *TPS6* and *TPS7* on the mechanism of flowering had not been studied further. *TPS9*, however, was more inclined to be involved in stress resistance [46]. It is attempting to assume that the longer photoperiod leads to the accumulation of polysaccharides, which promote the expression of *MlTPS1* and the secondary flowering (Fig. 8). The T6P pathway may also function by the pathway of aging to promote flowering through the expression of *MlSPL3* [15, 47], that the activation of *MlSPL3* expression by sugars as revealed in this study could promote the expression of floral integrator (Fig. 8).

In conclusion, our study demonstrates that *M. liliiflora* 'Hongyuanbao' could go through two cycles of flower bud differentiations in spring and summer, distinct from the continuous flowering in roses and strawberry. Sucrose and trehalose are important players during the secondary flower bud differentiation and bloom by activating the expression of *TPS* and *SPL* genes.

Materials And Methods

Plant materials

The experiment was conducted with flower buds from 10-year-old *M. liliiflora* 'Hongyuanbao', that were collected from the entire flower bud differentiation process on April 22 and August 21, 2018, from the Magnolia trees grown in the Zhejiang Agricultural and Forestry University, Zhejiang, China. The flower buds were stored at -80°C until extraction.

Gas chromatography-mass spectrometry analysis

A total of 50 mg of each sample was placed in a 2 mL polyethylene tube, mixed with 400 μL cold methanol, and 20 μL of methanol as the internal standard. A steel ball was added to the tube to assist the grinding with a 40 Hz grinder (JXFSTPRP-24, Shanghai Jingxin Experimental Technology, Shanghai, China) Following grinding for 4 min, the samples were treated with sonication in an ice bath for 5 min.

The samples were then mixed with 200 µL of chloroform and 400 µL of water and centrifuged at 13,500x g for 15 min at 4°C. Finally, 200 µL of supernatant was collected and evaporated by vacuum drying in a glass sampling vial. The samples were analyzed using gas chromatograph-mass spectrometry (GC-MS) Agilent 7890 GC-TOFMS which was equipped with an Agilent DB-5 ms capillary column (30 M × 250 µm × 0.25 µm, J & W Scientific, Folsom, CA, USA) [53].

Data processing and analysis

The peak extraction, baseline correction, deconvolution, peak integration, and peak alignment of the mass spectrometry data were carried out using chromat of software (v. 4.3x, LECO Corporation, St. Joseph, MI, USA). SIMCA software (v.14) was used for multivariate pattern recognition and data normalization. Principal component analysis and the OPLS-DA method were used to discriminate the metabolic changes in the experimental group. The corresponding metabolic pathways were mapped in the KEGG database, and P values were calculated with the level of statistical significance set at $p < 0.05$. Pathway enrichment analysis was conducted using metaboanalyst3.0 and the heat map was generated by OriginPro2015 (OriginLab Corporation, Northampton, MA, USA).

Screening and identification of metabolic differences

A total of 569 non-targeted metabolites were obtained based on the naming conditions of ion peak matching. All the differentially expressed compounds in the treated group were selected by comparing the compounds in the treated group with the control using the multivariate statistical method. For biologically duplicated metabolites, the combination of P-value and Variables with variable importance in the projection (VIP) value of the OPLS-DA (29 Thévenot E A, 2015) model was used to screen differential metabolites. The screening criteria were $P < 0.05$. $VIP > 1.0$ was considered relevant for group discrimination.

The induction of flowering by sucrose and trehalose treatments

Eighteen red and golden plantlets of similar size and growth were selected to grow in the Pingshan base experimental station. The leaves were sprayed from top to bottom with different sucrose or trehalose solutions with concentrations of 30 mM, 60 mM, and 90 mM. There were seven equal groups of three plants each. The stem tips were collected for cytological observation when the shoot apical meristems begin to expand which is used as the indicator of floral bud initiation.

The qRT-PCR analysis of *MlTPS* genes in response to sucrose treatment

The sequences of *MlTPS* genes were identified from *M. liliiflora* ‘Hongyuanbao’ and all qRT-PCR experiments were conducted in triplicates with three biological replicates. The statistical analyses were performed using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA), and the relative expression was calculated using the 2-ppCt method [22].

Abbreviations

KEGG	Kyoto Encyclopedia of Genes and Genomes
TPS	trehalose-6-phosphate synthase
T6P	trehalose-6-phosphate
OPLS-DA	orthogonal projection to latent structures discriminant analysis
CK	plants without any treatment
FT	FLOWERING LOCUS T
LFY	LEAFY
AP1	APETALA1

Declarations

Acknowledgments:

We are thankful to the editor and two anonymous reviewers for their comments on this paper. We thank Dong Meng (Beijing Forestry University) for carefully providing advice for this article, Dr. Chao Zhang for sharing knowledge about the experiments, and Rohul Amin (Beijing Forestry University) for editing the language.

Author Contributions:

Conceptualization, Y.S., J.M. and B.D.; methodology, Y.S. and L.X.; software, L.X.; validation, Y.S. and B.D; formal analysis, L.X., Q.W. and Z.L.; investigation, S.C., Y.Z. and D.L.; resources, D.Z. and L.Z.; data curation, Y.S. and L.X.; writing—original draft preparation, L.X. and Q.W.; writing—review and editing, L.X., Q.W. and Z.L.; visualization, L.X., Q.W. and Z.L.; supervision, Y.S., B.D. and J.M.; project administration, Y.S.; funding acquisition, Y.S. All authors have read, reviewed, and agreed to the published version of the manuscript.

Funding:

This study was funded by “Zhejiang Provincial Key Laboratory of Germplasm Innovation and Utilization for Garden Plants, Zhejiang Agriculture & Forestry University, Hangzhou 311300” and funding came from “Project Supported by Zhejiang Provincial Key Laboratory of Germplasm Innovation and Utilization for Garden Plants”

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Any additional data generated during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of Interest:

Authors declare no competing interests.

Statement of plant material

All experimental research and field studies on plants (either cultivated or wild) in this article, including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Author details

[1] School of Landscape and Architecture, Zhejiang A&F University, Hangzhou, Zhejiang 311300, China; 1075855127@qq.com(L.X); 1527103473@qq.com(Q.W); Vzhigao@zafu.edu.cn(Z.L); 1398383437@qq.com (S.C); 909621959@qq.com (Y.Z); 1760255397@qq.com (D.L); Dongbin@zafu.edu.cn(B.D); Jingjingma@zafu.edu.cn (J.M); yameishen@zafu.edu.cn(Y.S); [2] Shanghai Academy of Landscape Architecture Science and Planning, Shanghai 200232, China; 418517920@qq.com (D.Z); 1132467518@qq.com (L.Z).

References

1. Barry C; Rosa C; Yongbiao X; Zsuzsanna, S. S.; D Brendan. Tracing the Evolution of the Floral Homeotic B- and C-Function Genes through Genome Synteny. *Molecular Biology & Evolution* 2010, 27(11):2651–2664.

2. Chanderbali A S; Berger B A; Howarth D G;vSoltis, D. E.; Soltis, P. S. Evolution of floral diversity: genomics, genes and gamma. *Philos Trans R Soc Lond B Biol* 2017, 372(1713): 509–520.
3. Cho, L.H.; Yoon, J.; An, G. The control of flowering time by environmental factors. *Plant Journal for Cell & Molecular Biology* 2016, 90, 708–719.
4. Srikanth A., Schmid M. Regulation of flowering time: all roads lead to Rome. *Cellular & Molecular Life Sciences* 2011, 68, 2013–2037.
5. Miki, F.; Takanari, T.; Kaoru, U.; Saya, K.; Kazuo, S. RIPPS: A Plant Phenotyping System for Quantitative Evaluation of Growth Under Controlled Environmental Stress Conditions. *Plant Cell Physiol*. 2018, 59, 2030–2038.
6. Jinlei Shi; Aiwu Dong; Wenhui Shen. Epigenetic regulation of rice flowering and reproduction. *FRONT PLANT SCI* 2015, 5:803–816.
7. Sharma B; Batz T A; Kaundal R; EM Kramer; RB Larson. Developmental and molecular changes underlying the vernalization-induced transition to flowering in *Aquilegia coerulea* (James). *Genes* 2019, 10(10):734–751.
8. Craigon D J; Nick J; John O; H Janet; J Joan; M Sean. NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. *Nucleic Acids Research* 2004, 32(1):575–577.
9. Ream T S; Woods D P; Amasino R M. The Molecular Basis of Vernalization in Different Plant Groups. *Cold Spring Harbor Symposia on Quantitative Biology* 2011, 77:105–134.
10. Yano M. Natural Variation in *Hd17*, a Homolog of Arabidopsis *ELF3* That is Involved in Rice Photoperiodic Flowering. *Plant and Cell Physiology* 2012, 53(4):709–716.
11. Hikaru Iwata; Ame`lia Gaston; Arnaud Remay; Tatiana Thouroude; Julien Jeauffre; Koji Kawamura; Laurence Hibrand-Saint Oyant; Takashi Araki; Be´atrice Denoyes; Fabrice Foucher. The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. *The Plant Journal* 2012, 69:116–125.
12. Li-Na Wang; Yun-Feng Liu; Yu-Man Zhang; Rong-Xiang Fang; Qing-Lin Liu. The expression level of Rosa *Terminal Flower 1* (*RTFL1*) is related with recurrent flowering in roses. *Mol Biol Rep*. 2012, 39:3737–3746.
13. Elli A. Koskela; Timo Hytönen. Control of Flowering in Strawberries. *The Genomes of Rosaceous Berries and Their Wild Relatives* 2018, 35–48.
14. Timo Hytönen; Takeshi Kurokura. Control of Flowering and Runnering in Strawberry. *The Horticulture Journal* 2020, 89(2): 96–107.
15. Wahl, V.; Ponnu, J. Schlereth, A.; Arrivault, S.; Langenecker, T.; Franke, A.; Feil, R.; Lunn, J.E.; Stitt, M.; Schmid, M. Regulation of Flowering by Trehalose-6-Phosphate Signaling in *Arabidopsis thaliana*. *Science* 2013, 339, 704–707.
16. Eveland, A.L.; Jackson, D.P. Sugars, signalling, and plant development. *Journal of Experimental Botany* 2011, 63, 3367–3377.

17. Seo, P.J.; Ryu, J.; Kang, S.K.; Park, C.M. Modulation of sugar metabolism by an *INDETERMINATE DOMAIN* transcription factor contributes to photoperiodic flowering in *Arabidopsis*. *The Plant Journal* 2011, 65, 418–429.
18. Figueroa, C.M.F.R.; Ishihara, H.; Watanabe, M.; Kolling, K.; Krause, U.; Hohne, M.; Encke, B.; Plaxton, W.C.; Zeeman, S.C.; Li, Z.; Schulze, W.X.; Hoefgen, R; Stitt, M.; Lunn, J.E. Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. *The Plant Journal* 2016, 85, 410–423.
19. Figueroa, C.M.; Lunn, J.E. A tale of two sugars - trehalose 6-phosphate and sucrose. *Plant Physiology* 2016, 172, 7–27.
20. Lunn, J.E.; Delorge, I.; Figueroa, C.M.; Dijck, P.V.; Stitt, M. Trehalose metabolism in plants. *The Plant Journal* 2014, 79, 544–567.
21. Paul, M.J.; Primavesi, L.F.; Jhurreea, D.; Zhang, Y. Trehalose Metabolism and Signaling. *Annu Rev Plant Biol.* 2008, 59, 417–441.
22. Leyman, B., Dijck, P.V.; Thevelein, J.M. An unexpected plethora of trehalose biosynthesis genes in *Arabidopsis thaliana*. *Trends in Plant Science* 2001, 6, 510–513.
23. Lunn, J.E.; Feil, R.; Hendriks, J.H.M.; Gibon, Y.; Morcuende, R.; Osuna, D.; Scheible, W.R.; Carillo, P.; Hajirezaei, M.R.; Stitt, M. Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochemical Journal* 2006, 397, 139–148.
24. Ohto M A; Onai K; Furukawa Y; E Aoki; Takashi Araki; Kenzo Nakamura. Effects of Sugar on Vegetative Development and Floral Transition in *Arabidopsis*. *Plant Physiology*, 2001, 127(1):252–261.
25. Wahl V; Ponnu J;, Schlereth A; S Arrivault; T Langenecker; A Franke; R Feil; JE Lunn; M Stitt; M Schmid. Regulation of Flowering by Trehalose-6-Phosphate Signaling in *Arabidopsis thaliana*. *Science*, 2013, 339(6120):704–707.
26. Chang Ha Park; Soo-Yun Park;, Sook Young Lee; Jae Kwang Kim; Sang Un Park. Analysis of Metabolites in White Flowers of Magnolia Denudata Desr. and Violet Flowers of Magnolia Liliiflora Desr. *Molecules* 2018, 23(7): 1558–1563.
27. Nimali I S, Alan J. L., Jian-Kui Liu, Saisamorn Lumyong; Saisamorn Lumyong. Phylogeny and morphology of *Lasiodiplodia* species associated with *Magnolia* forest plants. *Scientific Reports* 2019, 9: 14355–14365.
28. Fan, L.J.; Chen, M.Q.; Dong, B.; Wang, N.H.; Yu, Q.; Wang, X.L.; Xuan, L.J.; Wang, Y.L.; Zhang, S.Z.; Shen, Y.M. Transcriptomic Analysis of Flower Bud Differentiation in *Magnolia sinostellata*. *Genes* 2018, *9*, 212–226
29. Liu, Y.H.; Xia, N.H.; Yang, H.Q. The origin, evolution and phytogeography of magnoliaceae. *Journal of tropical and subtropical botanye* 1995, *4*, 1–12.
30. Yeoh, C.C.; Balcerowicz, M.; Laurie, R.; Macknight, R.; Putterill, J. Developing a method for customized induction of flowering. *Bmc Biotechnology* 2011. 11, 36–46.

31. Jung, C.; Muller, A.E. Flowering time control and applications in plant breeding. *Trends in Plant Science* 2009, 14, 563–573.
32. Simpson, G. G. Arabidopsis, the Rosetta Stone of Flowering time. *Sci*. 2002, 296, 285–297.
33. Ryan, P.T.; Omaoileidigh, D.S.; Drost, H.G.; Kwasniewska, K.; Gabel, A.; Grosse, I.; Graciet, E.; Quint, M.; Wellmer, F. Patterns of gene expression during *Arabidopsis* flower development from the time of initiation to maturation. *Bmc Genomics*. 2015, 16, 488–499.
34. Hikaru, I.; Amelia, G.; Arnaud, R.; Tatiana, T.; Julien, J.; Koji, K.; Laurence, H.S.O.; Takashi, A.; Beatrice, D.; Fabrice, F. The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. *Plant Journal for Cell & Molecular Biology* 2012, 69, 116–125.
35. Marie, R.J.J.; Tatiana, T. Gibberellins regulate the transcription of the continuous flowering regulator, *RoKSN*, a rose *TFL1* homologue. *Journal of Experimental Botany*. 2012, 63, 6543–6554.
36. Shannon, S.; Meeks, W.D.R. A Mutation in the *Arabidopsis TFL1* Gene Affects Inflorescence Meristem Development. *Plant cell* 1991, 3, 877–892.
37. Gaston, A.P.J.; Lerceteau, K.E.; Rousseau, G.M.; Petit, A.; Hernould, M.; Rothan, C.; Denoyes, B. *PFRU*, a single dominant locus regulates the balance between sexual and asexual plant reproduction in cultivated strawberry. *Journal of experimental botany*. 2013, 64, 1837–1848.
38. Weebadde, C.K.; Wang, D.; Finn, C.E.;, Lewers, K.S.; Luby, J.J.; Bushakra, J.; Sjulin, T.M.; Hancock, J.F. Using a linkage mapping approach to identify QTL for day-neutrality in the octoploid strawberry. *Plant Breeding 2008*, 127, 94–101.
39. Sun, J.; Wang, H.; Ren, L.; Chen, S.; Chen, F.; Jiang, J. *CmFTL2* is involved in the photoperiod- and sucrose-mediated control of flowering time in chrysanthemum. *Horticulture Research* 2017, 4, 17001–17006.
40. Ruiz, S.J.L.; Ruiz, M.R.; Montes, H.M.D.C.; Agreda, L.K.A.; Xoconostle, C.B. Vascular expression of trehalose phosphate synthase1 (*TPS1*) induces flowering in *Arabidopsis*. *Plant Omics.* 2016, 9, 344–351.
41. Du, L.; Qi, S.; Ma, J.; Xing, L.; Fan, S.; Zhang, S.; Li, Y.; Shen, Y.; Zhang, D.; Han, M. Identification of *TPS* family members in apple (*Malus x domestica Borkh*.) and the effect of sucrose sprays on *TPS* expression and floral induction. *Plant Physiology & Biochemistry* 2017, 120, 10–23.
42. Xing, L.B.; Zhang, D.; Li, Y.M.; Shen, Y.W.; Zhao, C.P.; Ma, J.J.; An, N.; Han, M,Y. Transcription Profiles Reveal Sugar and Hormone Signaling Pathways Mediating Flower Induction in Apple (*Malus domestica Borkh*.). *Plant & Cell Physiology* 2015, 56, 2052–2068.
43. Ruan, Y.L. Sucrose Metabolism: Gateway to Diverse Carbon Use and Sugar Signaling. *Annual Review of Plant Biology 2014*, 65, 33–67.
44. Matsoukas, I.G.; Massiah, A.J.; Thomas, B. Starch metabolism and antiflorigenic signals modulate the juvenile-to-adult phase transition in *Arabidopsis*. *Plant Cell & Environment* 2013, 36, 1802–1811.
45. Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G. The *Arabidopsis* trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signaling. *Plant Physiology* 2014, 136(3):3649–3659.

46. Aaron, B.; Cecilia, C.C.; Georgina, E.N.; Reyes, J.L.; Juarez, V. M.A; Nelson, A.; Carmen, Q.; Claudia, D.C.; Federico, S. The Class II Trehalose 6-phosphate Synthase Gene *PvTPS9* Modulates Trehalose Metabolism in *Phaseolus vulgaris* Nodules. *Frontiers in Plant Science* 2016, 7, 1589–1601.
47. Ionescu, I.A.; Mller, B.L.; Sanchez, P.R. Chemical control of flowering time. *Journal of Experimental Botany* 2016, 68, 369–382.

Figures

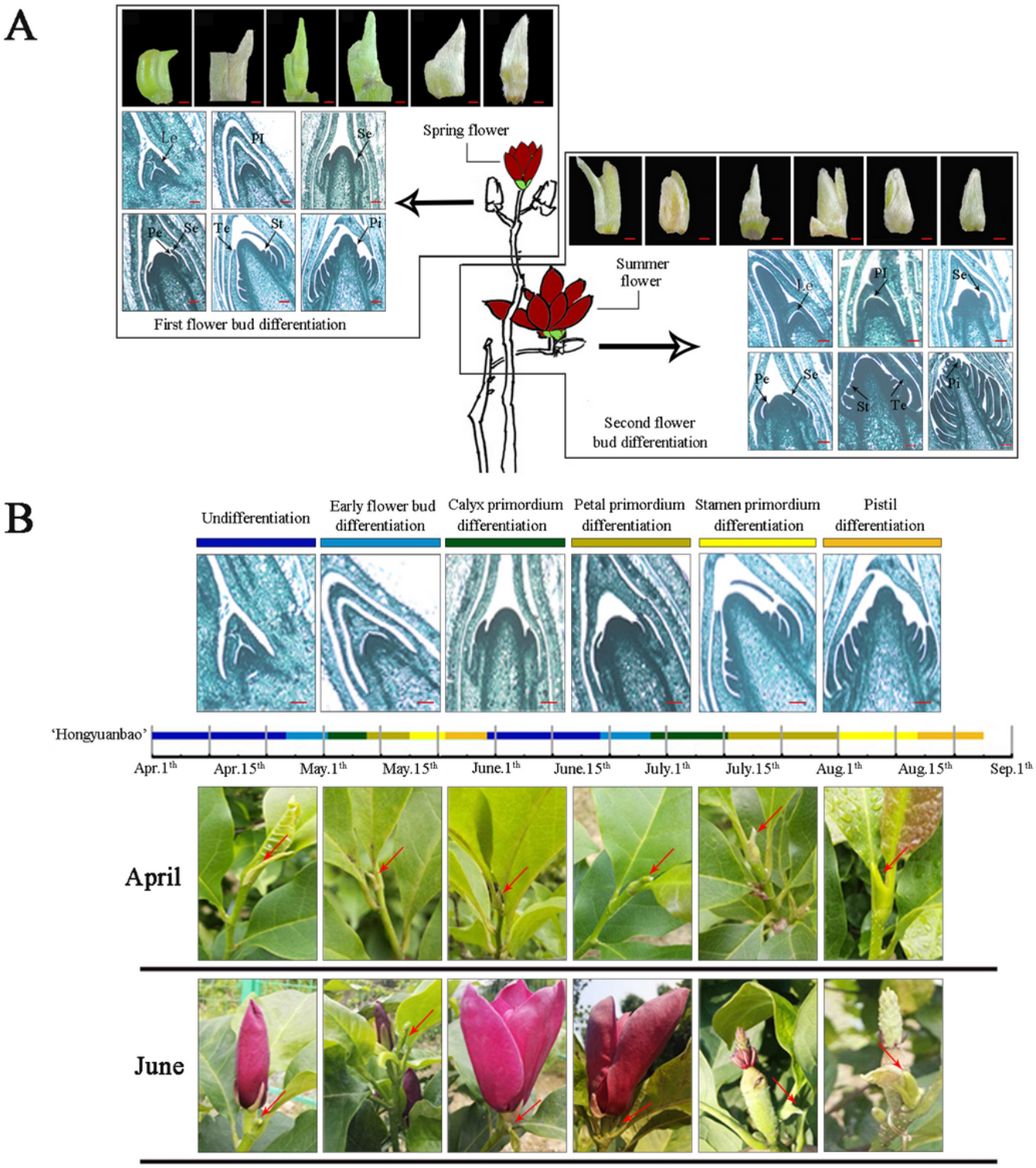

Figure 1

Observation of flower buds and plant phenotypes in Magnolia lifiiflora 'Hongyuanbao'. (A) The morphological change of flower bud differentiation in April and June. White bars, 2 mm; Red bars, 50 μm. (B) The time course of flower bud differentiation. Red bars, 50 μm.

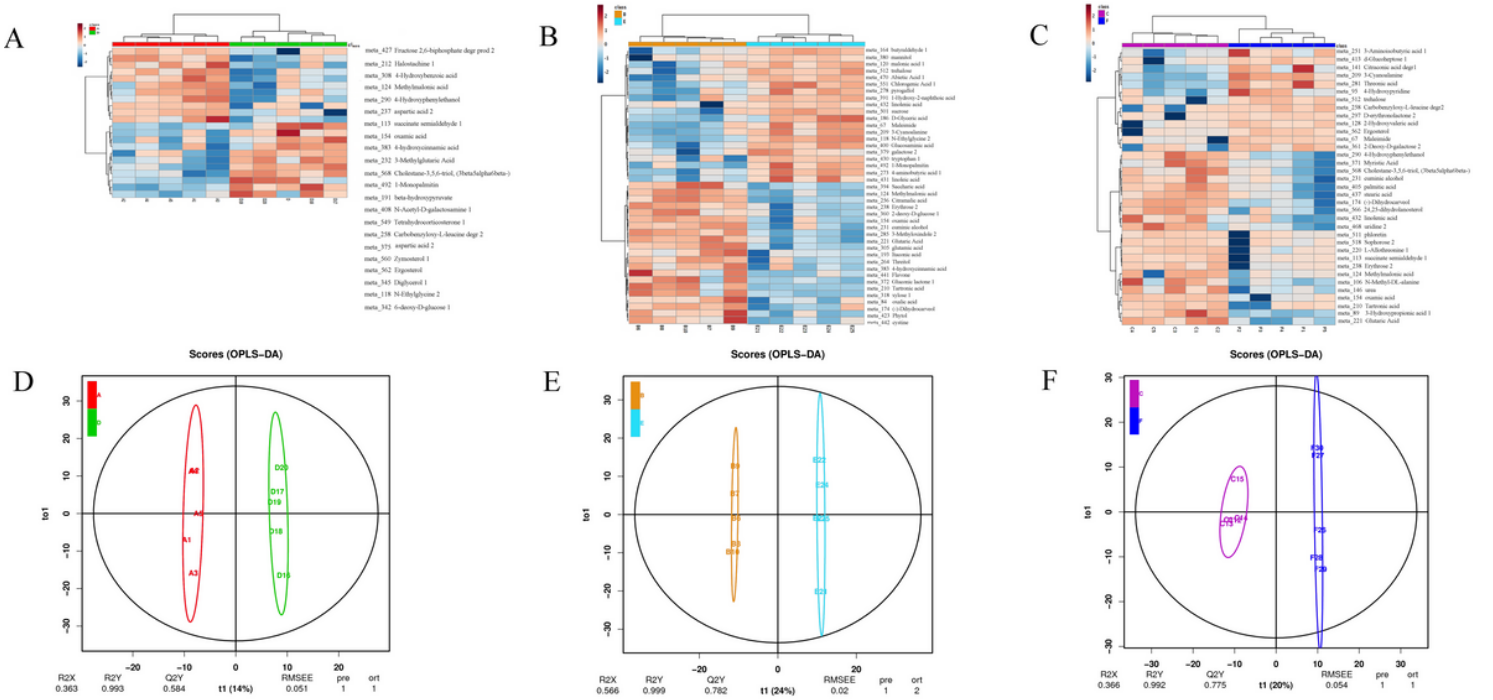

Figure 2

Comparisons of metabolite levels in spring and summer flower bud differentiations. (A-C) Heatmap of the classification in the different stage (respectively represent the early, middle and later stage) comparing between spring and summer flower bud differentiations on metabolite profiles. (D-F) orthogonal projection to latent structures discriminant analysis (OPLS-DA) score plot for the different stage (respectively represent the early, middle and later stage) comparing metabolite between spring and summer flowering.

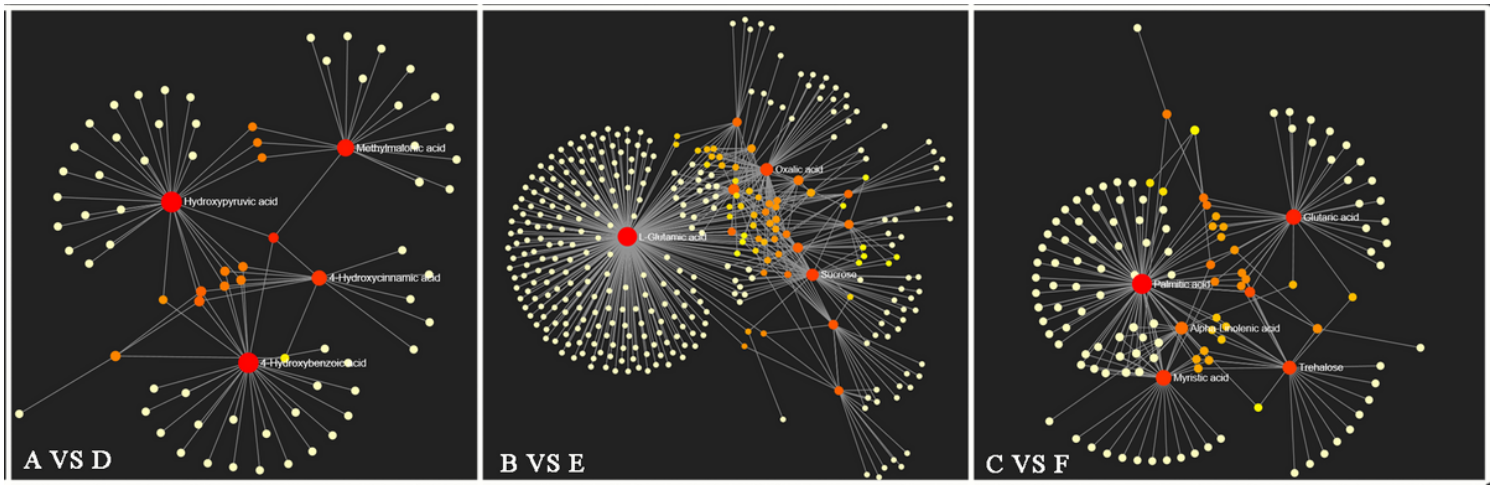

Figure 3

Metabolite-Metabolite Interaction Network in different stages. A, B, C represent the early, middle and later stage of flower bud differentiation, respectively. S1, S2, S3 represent the early, middle, and later stage of the first flower bud differentiation, respectively. S4, S5, S6 represent the early, middle, and later stage of the secondary flower bud differentiation, respectively.

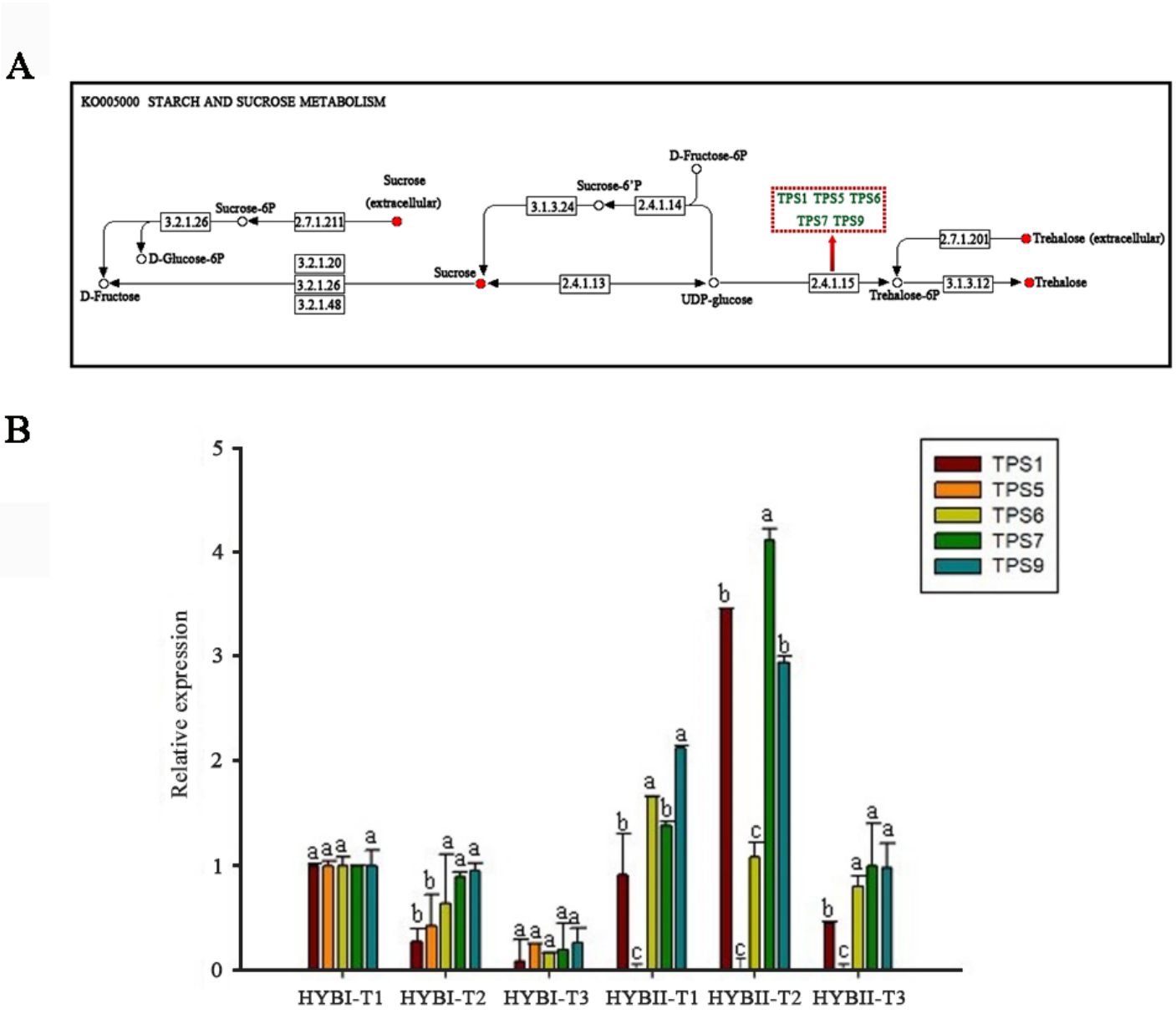

Figure 4

Important metabolite pathways during the spring and summer flower bud differentiations. (A) Enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Relatively high numbers of metabolites were annotated as starch and sucrose metabolism with rich factor. (B) The levels of expression of MlTPS genes in the three stages of the first and secondary flower bud differentiation processes in Magnolia lifiiflora 'Hongyuanbao'.

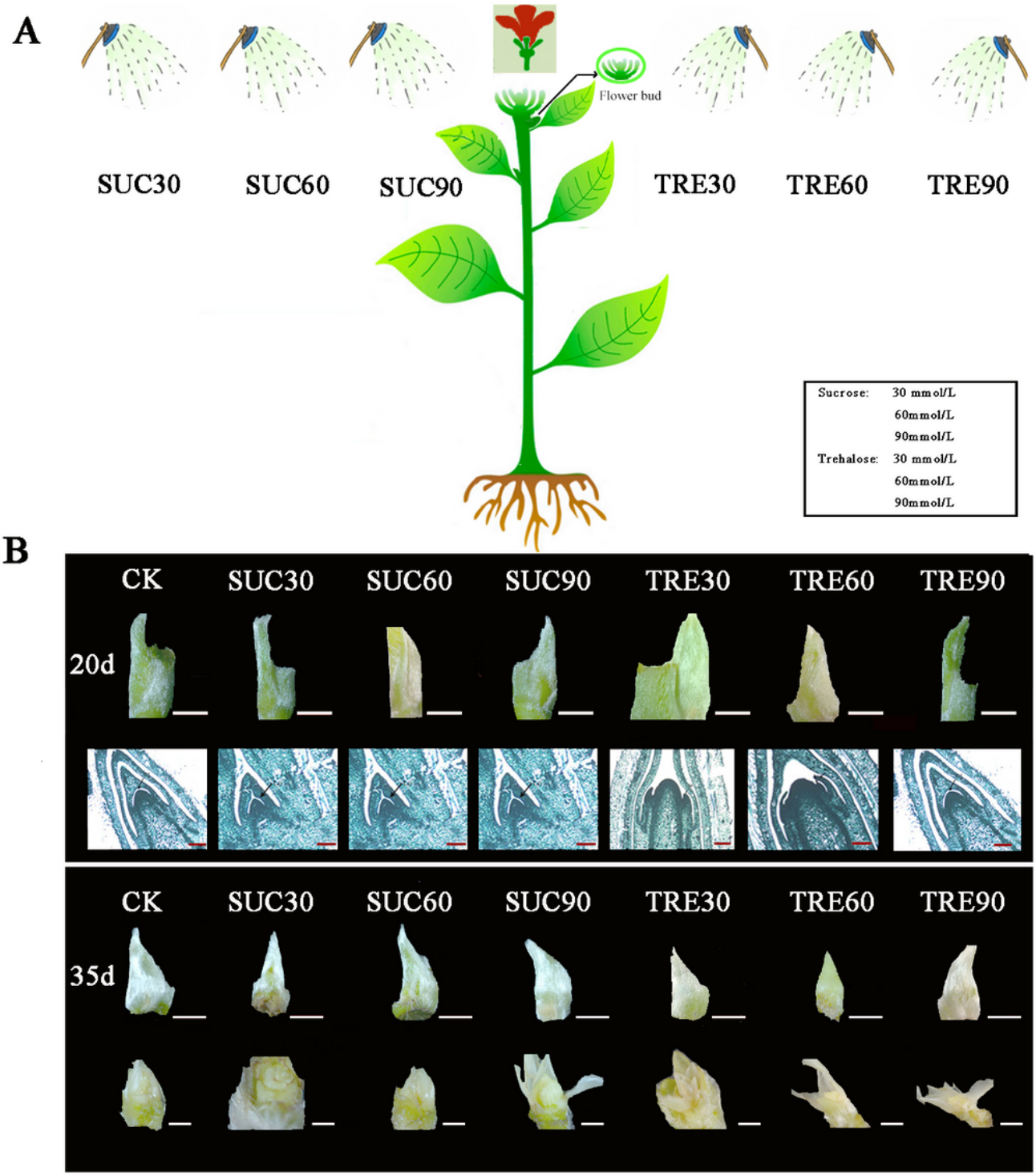

Figure 5

The effect of spraying on the leaves of M. lifiiflora ‘Hongyuanbao’ plants with the solution of trehalose or sucrose. (A) Treatment with different concentrations of trehalose or sucrose solutions. (B) The 20th and 35th day after treatment were chosen to observe the process of flower bud differentiation. Red bars, 50 μm; White bars, 2 mm.

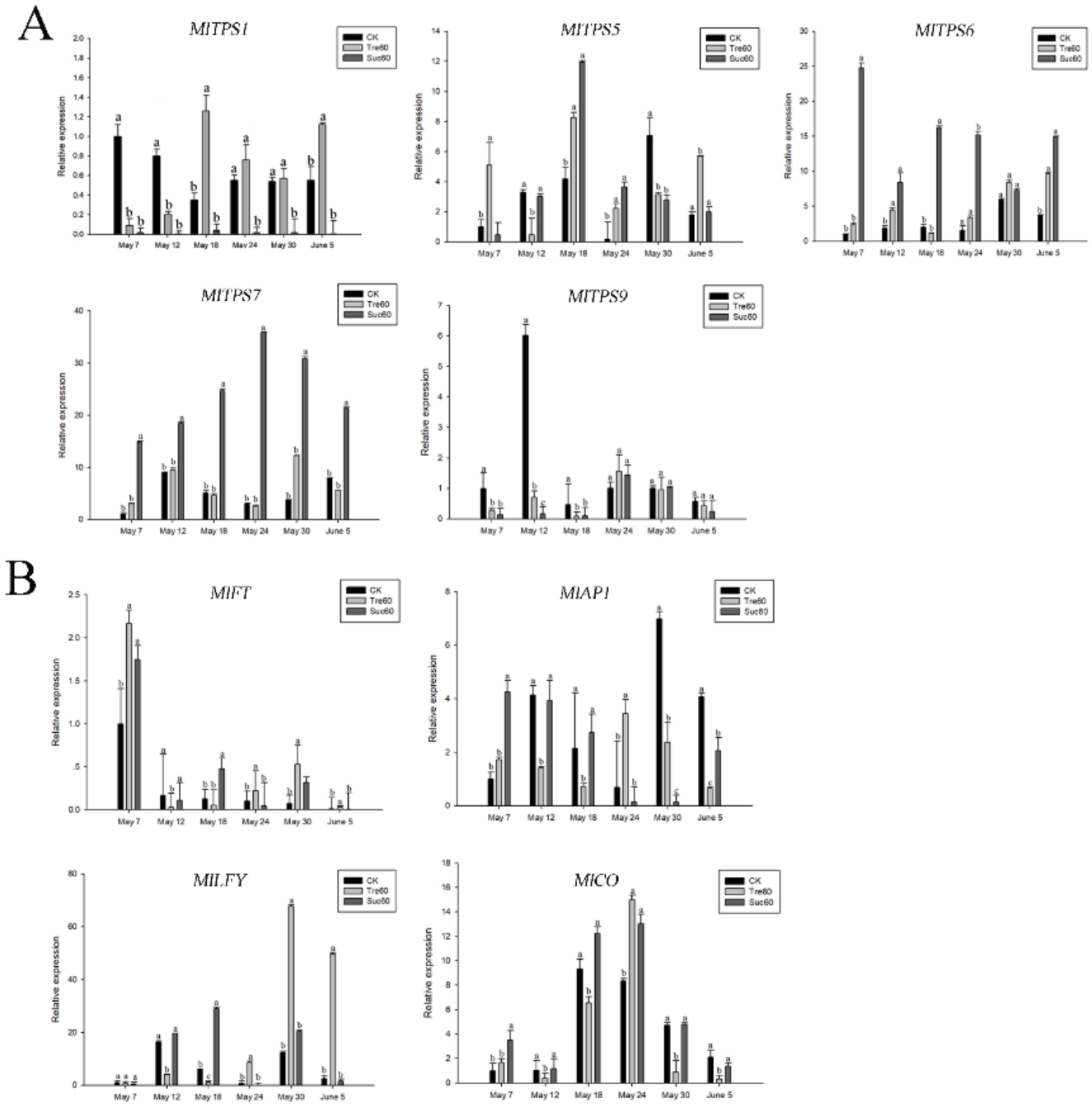

Figure 6

Expression levels of TPS and flowering genes in Magnolia lifiiflora ‘Hongyuanbao’. (A) The relative expression of TPS genes in response to treatment with trehalose or sucrose during the floral induction. (B) The relative expression of flowering genes in response to treatment with trehalose or sucrose during floral induction.

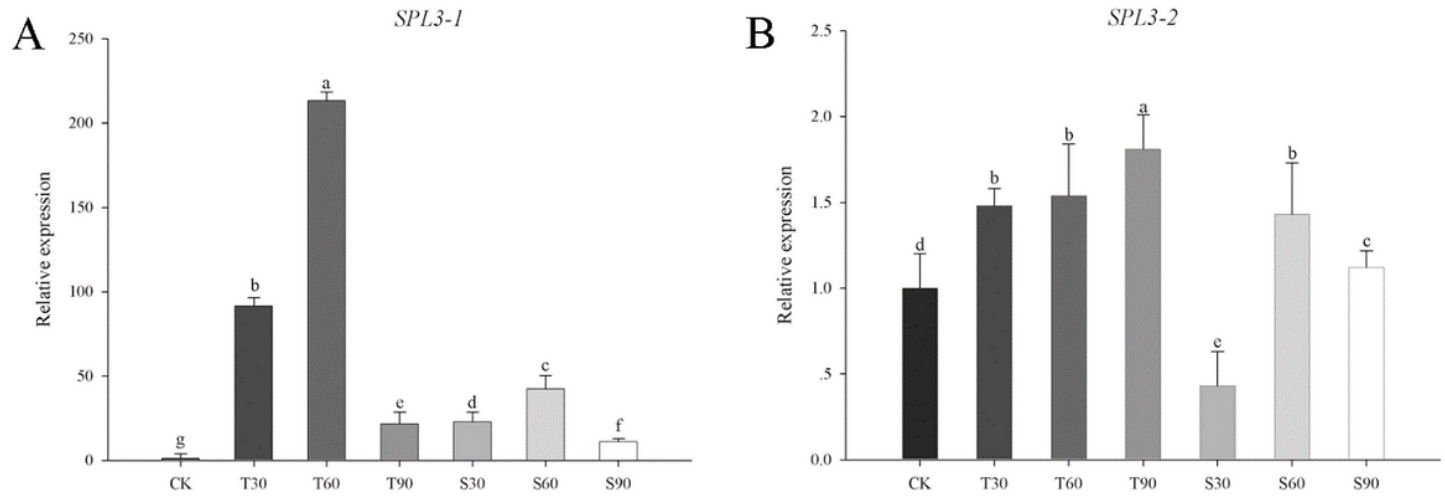

Figure 7

Analysis of the expression of SPL3 gene after treatment with sugars. (A) The relative expression of SPL3-1. (B) The relative expression of SPL3-2.

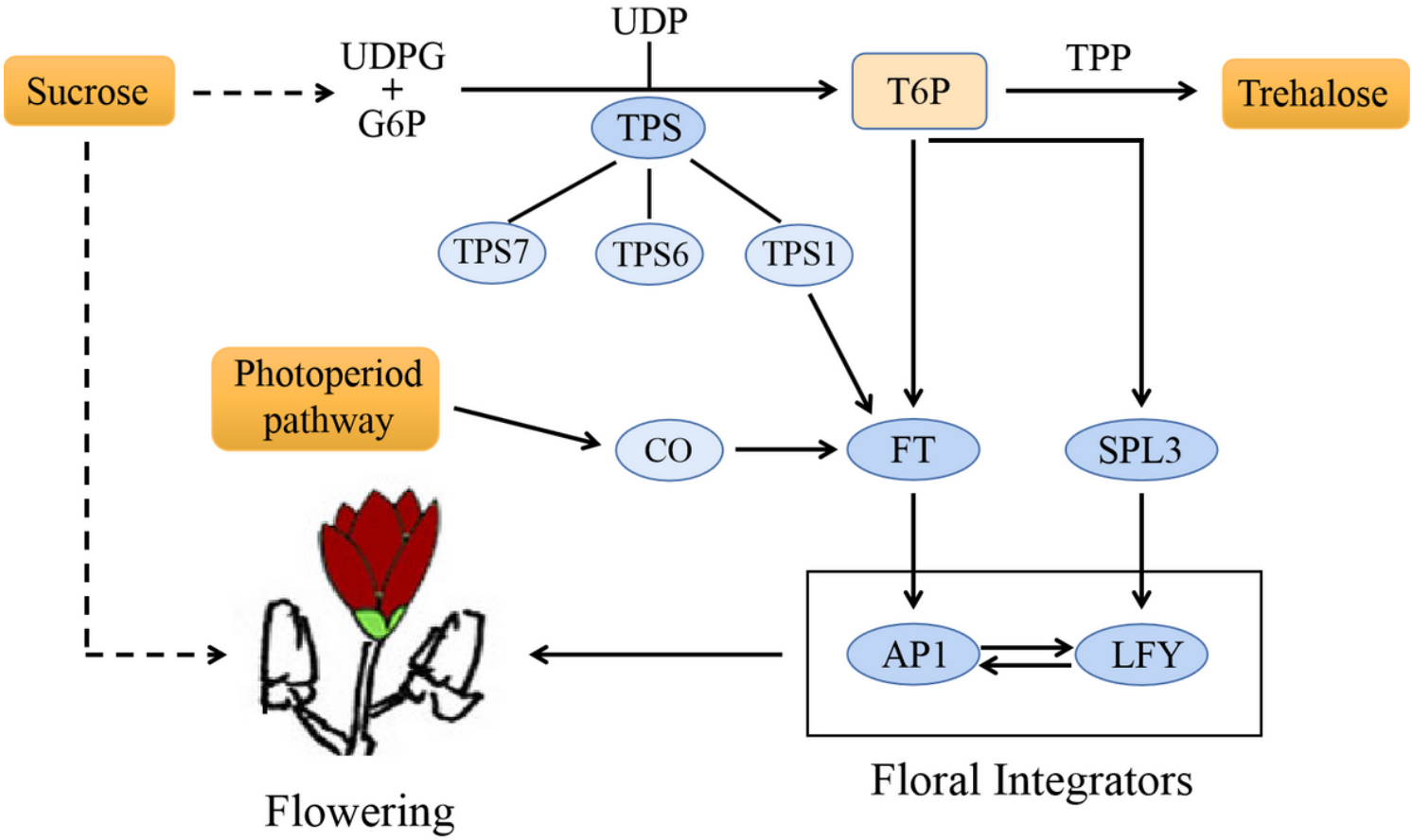

Figure 8

A simple schematic network of sugar metabolism that regulates flowering time in ‘Hongyuanbao

Supplementary Files

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

- Additionalfile1.xlsx