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ORIGINAL ARTICLE

Physiological Mechanism and Developmental Events in Differentiating Floral Buds of *Sophora tonkinensis* Gagnep

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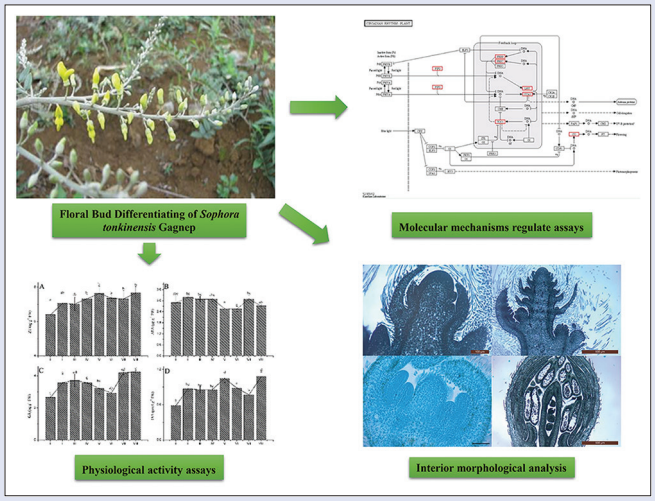
ABSTRACT

Background: *Sophora tonkinensis* Gagnep is an important medicinal plant in China. Previous research has focused on the rapid propagation and quality analysis of *in vitro* tissue culture plantlets. Few studies have focused on floral bud differentiation. Physiological and molecular mechanisms regulating floral bud differentiation have not been elucidated. **Objective:** We used paraffin sections and RNA-seq and then analyzed changes in physiological, biochemical, and endogenous hormones to examine floral bud differentiation. **Materials and Methods:** Buds were harvested in mid-March or early April. After sorting and cleaning, flower buds physiological and biochemical properties were measured and hormones were determined. Morphological observation of *S. tonkinensis* was carried out using paraffin sections. **Results:** The transition from the meristematic to the reproductive phase included changes in the shoot apical meristem morphogenesis and cell differentiation. The process includes floral buds initial differentiation and flower primordium differentiation stages; we defined eight stages in total. There was no significant change in soluble content, but soluble protein content gradually decreased. Activities of antioxidant enzymes changed significantly. Total chlorophyll (Chl a + b) content increased significantly. Endogenous hormone levels changed differently during floral bud differentiation. We identified 104,519 expressed genes and 24 were involved in flowering. MADS-box and *AP* family genes are involved in flower formation and 40 differentially expressed genes associated with floral bud differentiation were identified. **Conclusion:** The higher soluble sugar, protein, and chlorophyll content and the higher peroxidase activity were beneficial to floral bud differentiation of *S. tonkinensis*. The dynamic changes in hormone content contribute to differentiating floral buds.

Key words: Endogenous hormone, floral bud differentiation, morphological observations, physiological mechanism, RNA-seq

SUMMARY

- High levels of nutrition, photosynthesis, and enzyme activity were beneficial to floral bud differentiation of *Sophora tonkinensis* Gagnep
- The dynamic changes in plant hormone can be beneficial for floral bud differentiation of *Sophora tonkinensis* Gagnep
- The flowering of *Sophora tonkinensis* Gagnep is mainly determined by photoperiod pathway.



Abbreviations used: FAA: Formalin-acetic acid-alcohol; TO: Turpentine oil; BSA: Bovine serum album; EDTA: Ethylenediaminetetraacetic acid; PVP: Polyvinylpyrrolidone; POD: Peroxidase; SOD: Superoxide dismutase; NBT: Nitrotetrazolium blue chloride; Chl a: Chlorophyll a; Chl b: Chlorophyll b; IAA: Indole-3-acetic acid; ABA: Abscisic acid; GA: Gibberellic acid; ZT: Zeatin; RIN: RNA integrity number; DEGs: Differentially expressed genes; FPKM: Fragments per kilobase of exon per million fragments mapped; FDR: False discovery rate.

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INTRODUCTION

The transition from vegetative to reproductive growth is an irreversible process, which involves changes in buds apical meristem morphology and cell differentiation patterns. It is an important life-history event for flowering plants.[1] This process can be divided into two main stages as follows: (1) the induction period involves biochemical modification of buds to form reproductive structures, and (2) the differentiation period

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involves the differentiation of floral tissue. As the reproductive phase develops, the cell division rate of the shoot apical meristem increases.[2]

Sophora tonkinensis Gagnep (Leguminosae) is an important traditional medicinal plant. The roots and rhizomes are known as "Shandougen" in China and are mainly distributed in China and northern Vietnam.[3] The first description of *S. tonkinensis* was in the *Kaibao Bencao* (Song Dynasty 973-974 A. D.).[4] The roots and rhizomes have long been used to treat inflammatory disease conditions such as gingivitis, colitis, acute pharyngolaryngeal infection, sore throat, acute dysentery, and gastrointestinal hemorrhage.[5] A previous study indicated that aqueous extracts of *S. tonkinensis* roots have an inhibitory effect on human hepatoma SMMC-7721 cells.[6] *S. tonkinensis* is a perennial plant, and it takes 3 years from seed germination to harvest as a medicinal material. However, its low seed yield makes it difficult to cultivate and limits our ability to augment wild resources.[7]

Floral bud differentiation has a direct effect on the quantity and quality of flowering and fruit set success rates, thus affecting the yield. There have been no detailed studies on these processes in this species. Therefore, the present study was undertaken to investigate the physiological and biochemical changes associated with different stages of floral bud differentiation in *S. tonkinensis*. The study modulated the differentiating floral buds process to improve the seed setting rate and thus increase the yield of this important traditional medicinal plant.

MATERIALS AND METHODS

Plant material

S. tonkinensis was collected from the cultivation field of Guangxi Botanical Garden of Medicinal Plants (22°51′ 1 and 108°19′ 9). The original plant was identified by the Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement at Guangxi Botanical Garden of Medicinal Plants. Three-year-old *S. tonkinensis* of the raceme phenotype were selected for use in the present study. Floral buds were harvested from the field in mid-March or in early April. After sorting and cleaning, the initiation of a new leaf and the floral buds were collected every 3 days and were observed after the floral buds began to differentiate. In addition, when the process of floral bud differentiation began, the initiation of floral buds were collected every day until the end of differentiation.

Interior morphological analysis

Paraffin-sectioning

The floral buds were fixed in formalin–acetic acid–alcohol (8:1:1 ratio of 50% ethanol: Formaldehyde: Glacial acetic acid) for 24–48 h at 25°C. Paraffin embedding was carried out with ethanol concentrations as follows: 60% for 2 h, 70% for 2 h; 2 h; 85% for 2 h; 90% for 1 h; 95% for 1 h; 100% for 1 h, changed twice. Turpentine oil (TO): Ethanol (1:1) for 1 h; TO, changed twice, for 1 h and each; paraffin-saturated TO solution at 37°C for 48 h; paraffin wax (58°C–60°C), changed twice for 2 h and each; then, tissues were embedded into paraffin blocks. Paraffin blocks were trimmed to 10 μm using a microtome. A paraffin ribbon was placed in the slides and baked at 45°C–50°C for 2 h. Safranin O and fast green-stained sections, preserved by neutral balsam, were observed under the microscope (Leica Dm2000 microscope, Leica, Wetzler, Germany).[8]

Transmission electron microscopy

During the floral buds developmental period, freshly cut 3 mm^3 segments of floral buds were fixed in 2.5% glutaric acid in 100 mM cacodylate buffer for 24 h at 4°C, then eluted three times with 50 mM cacodylate buffer, each time for 5 min. They were postfixed in 1% OsO_4 in 50 mM cacodylate buffer for 4 h at 4°C and then eluted three times with cacodylate buffer. The samples were dehydrated in an ethanol series and then acetone once and then embedded in epoxy resin. 60-nm ultra-thin sections were stained with uranyl acetate and lead citrate[9] and were observed under a HTACHI H-7650 transmission electron microscope (HTACHI, Microsystems GmbH, Tokyo, Japan).

Physiological activity assays

Determination of osmolytes

Soluble sugar was determined by the anthrone method.[10] The absorbance at 640 nm was measured using methanol as a blank. The concentration of soluble sugar was calculated using a glucose solution as a standard.[10]

Determination of protein content

The total protein content of each enzyme extract was measured by the dye-binding method[11] using a protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China) and bovine serum albumin solution for the standard.

Determination of enzyme activity

Floral buds were frozen in liquid nitrogen immediately after harvesting and stored at −80°C until enzyme assays. Approximately 0.3 g of leaf material was homogenized in an ice bath in 3 mL 0.05 M phosphate buffer (pH 7.8) with 0.1 mM ethylenediaminetetraacetic acid and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was used for enzyme activity assays at 4°C. Peroxidase (POD) activity was measured in the presence of 16 mM guaiacol and 10 mM H_2O_2 by monitoring the increase in absorbance at 470 nm in phosphate buffer.[12] The activity of superoxide dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of nitrotetrazolium blue chloride (NBT). One unit of SOD activity was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction at 560 nm.[13]

Determination of pigment content

Chlorophyll (Chl a and Chl b) were extracted using 80% acetone. The absorbance of the extracts was measured using a Model UV-752N spectrophotometer (Inesa, Shanghai, China) at 649 and 665 nm. The content of Chl a, Chl b and total chlorophyll (Chl a + b) were determined according to the method of Wellburn.[14]

Quantification of plant hormones by enzyme-linked immunosorbent assay

Plant hormone concentration during floral bud differentiation was measured by enzyme-linked immunosorbent assay (ELISA). The plant hormones ELISA kit (IAA, abscisic acid [ABA], GA_3, and Zeatin [ZT]) used here was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. Floral buds from different periods were treated according to the manufacturer's instructions. The experiment was repeated three times.

Molecular mechanisms assays

RNA extraction and cDNA library construction

Total RNA was extracted from floral buds at the same stages as those observed above using a HiPure HP Plant RNA Mini Kit (Magen, Guangzhou, China), with three biological replicates of each stage. The quality and purity of the RNA samples were assessed using an RNA 6000 Nano LabChip Kit and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), using an RNA integrity number (RIN) of >7.0. Poly-(A)-containing mRNA was purified using a NEBNext® Poly (A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). Fragmentation buffer was added to disrupt the mRNA strands into short

fragments, which were used as templates to synthesize the first-strand cDNA using reverse transcriptase and random hexamer primers. The second-strand cDNA was synthesized using buffer, dNTPs, RNase H, and DNA polymerase I. The double-stranded cDNA fragments were subjected to end-repair and adapter ligation. A uracil base in the adapter was excised with a USER enzyme. Adapter-modified fragments were selected using gel purification, and PCR products were amplified to create the final cDNA library. The cDNA library was achieved by means of NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs).

Illumina sequencing, assembly, and annotation

The cDNA library was sequenced on an Illumina HiSeq 2500 sequencing platform (Illumina) to yield 2 × 150-bp paired-end raw reads. The adapter sequences were read with a ratio of ambiguous N nucleotides >5%, and low-quality sequences (quality score of <30) were removed from the raw reads using Trimmomatic.[15] Sequencing reads were *de novo* assembled using pooled reads from all replications and stages in the Trinity software package[16] with default parameters, and the result was further clustered and assembled using TIGR Gene Indices clustering tools under default parameters.[17] The assembled transcriptome sequences were named "unigenes." All unigenes were searched against Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and TrEMBL databases using BLASTp with a cutoff E-value of 10^{-5}. Gene Ontology (GO) annotation was acquired using InterProScan.[18]

Differential gene expression analysis

To analyze the differentially expressed genes (DEGs) during floral bud differentiation, RSEM[19] estimated the fragments per kilobase of exon per million fragments mapped (FPKM) value of each unigene based on the length of the gene and mapped read count. The detection of DEGs was performed using edgeR program[20] with the rigorous algorithm method. The threshold *P* value in multiple tests and analyses was determined by the false discovery rate (FDR). The DEGs were deemed significant by the following criteria: FDR <0.05 and the absolute value of Log2 (Ratio) and KEGG and GO enrichment was carried out with an in-house R script using the hypergeometric test and *P* values were corrected against FDR. Significance was determined with a cutoff corrected $P = 0.05$. The expression data of the MADS-box gene were analyzed using the software package of the heatmap tool on the BMKCloud platform (www.biocloud.net).

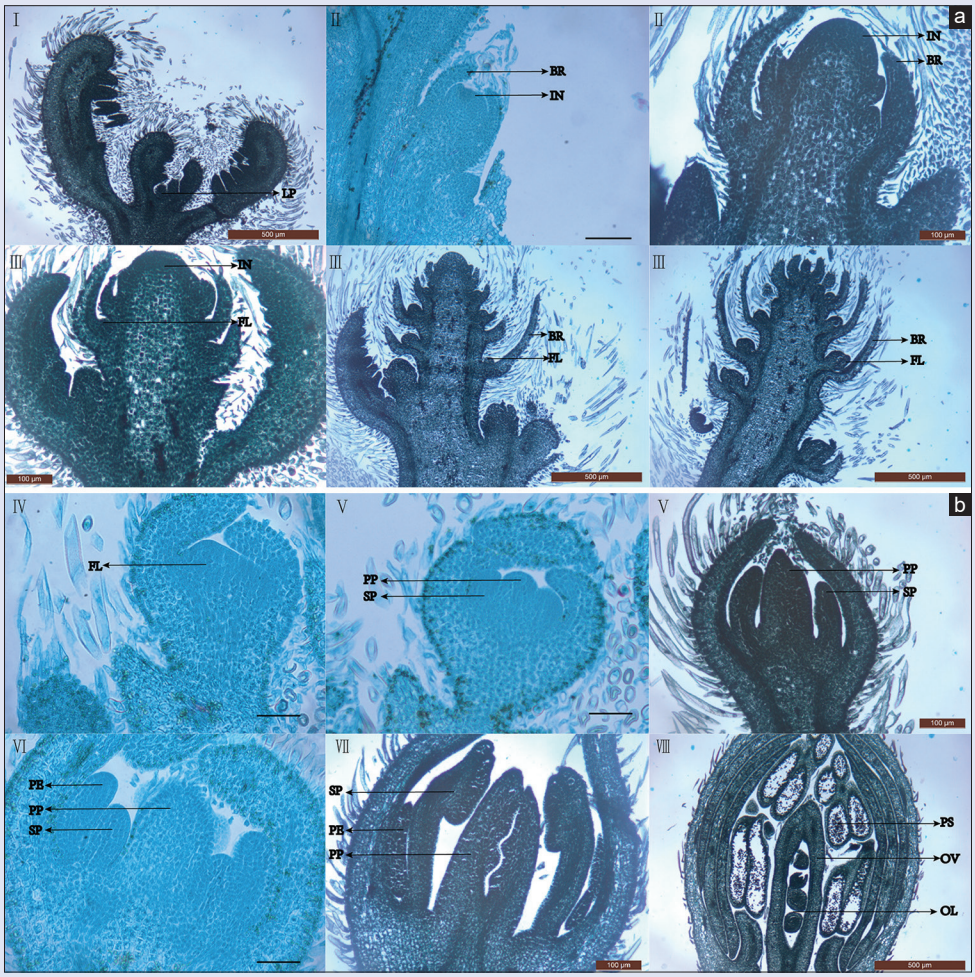

Figure 1: Anatomical changes in differentiating *Sophora tonkinensis* floral buds. (a) Illustrates the developmental course of *Sophora tonkinensis* in the "induction period" (I-III): Floral buds undifferentiated stage (i); preliminary stage of floral bud differentiation (II); Inflorescence differentiation stage (III). (b) Illustrates the developmental course of *Sophora tonkinensis* "differentiation period" formed by floral tissue (IV-VIII): Calyx differentiation stage (IV); corolla and pistil differentiation stage (v); stamen differentiation stage (VI); completion stage (VII, VIII). LP: Leaf primordium; IN: Inflorescence primordium; FL: Flower primordium; BR: Bract primordium; PE: Petal primordium; PP: Pistil primordium; SP: Stamen primordium; PS: Pollen sac; OL: Ovule; OV: Ovary

Statistical analysis

Multiple comparison testing was performed using one-way ANOVA followed by a least significant difference (LSD) test. Statistical analyses were performed using the SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The results are expressed as means ± standard errors of the means. $P < 0.05$ was considered statistically significant.

RESULTS

Anatomical changes in differentiating floral buds

According to the morphological changes in stem tips and the characteristics of floral bud differentiation, the floral bud differentiation of *S. tonkinensis* was divided into eight stages as follows. Floral buds undifferentiated stage (I): The growth phase; mainly differentiated vegetative organs such as a leaf, stem, and axillary buds. The preliminary stage of floral bud differentiation (II): Shift from vegetative to reproductive growth from mid-April and formation of inflorescence primordium growth cones in the leaf axilla with a distinct tunica-corpus structure. The longitudinal division of growth cones is larger than in the transverse division, gradually extending into the inflorescence primordia axis. Inflorescence differentiation stage (III): with the continuous upward differentiation of the inflorescence primordia axis, the leaf-like bracts primordia appears on both sides from the base upward in accordance with the spiral line. The floret primordia gradually mature from bottom to top. Calyx differentiation stage (IV): the apex of the hemispherical floret primordia gradually becomes wide and flat, forming small protrusions around the growth cone and only two protrusions were observed in the longitudinal section, which is the calyx primordia. Corolla and pistil differentiation stage (V): the apical tissue growth cone of the inner buds of the sepal continues to divide unequally. There were three protrusions in the longitudinal section. The middle protrusion was the pistil primordia, and the two sides were the corolla primordia. At this stage, the pistil primordia divide faster than the petal primordia. Stamen differentiation stage (VI): during the process of pistil primordia differentiation, small protuberances (stamen primordia) are formed on both sides of the pistil primordia. The completion stage of floral bud differentiation (VII, VIII): The division of floral bud differentiation is completed gradually. It was observed that the calyx and corolla were elongated, covering the internal organs of the flower and forming floral buds; the mature stamen anthers and filaments begin to differentiate; mature pistil stigma, style, and ovary begin to differentiate, and the style is hollow [Figure 1].

Observation of floral bud differentiation by transmission electron microscopy

During the undifferentiated stage of floral buds, it was observed that there were large vacuoles and more starch grains in the cells. After floral buds began to differentiate, the volume of cells gradually decreased owing to the decrease in vacuoles and the thickening of the cytoplasm. In this process, vacuoles and starch grains gradually reduced [Figure 2].

Osmolytes

The concentrations of soluble sugar and protein were analyzed during floral bud differentiation. The changes in soluble sugar content were basically the same. Soluble sugar increased slightly in the early stage (Stage I–III). At Stage IV, soluble sugar peaked at 18.57 ± 1.21 mg/g, which was 27.76% lower than that of stage VII. After that, the soluble sugar content in the buds returned to the previous level. This indicates that before floral bud differentiation, *S. tonkinensis* needs a certain accumulation of soluble sugar. During Stage VII, some soluble sugar was consumed to ensure the smooth process of floral bud differentiation [Figure 3a].

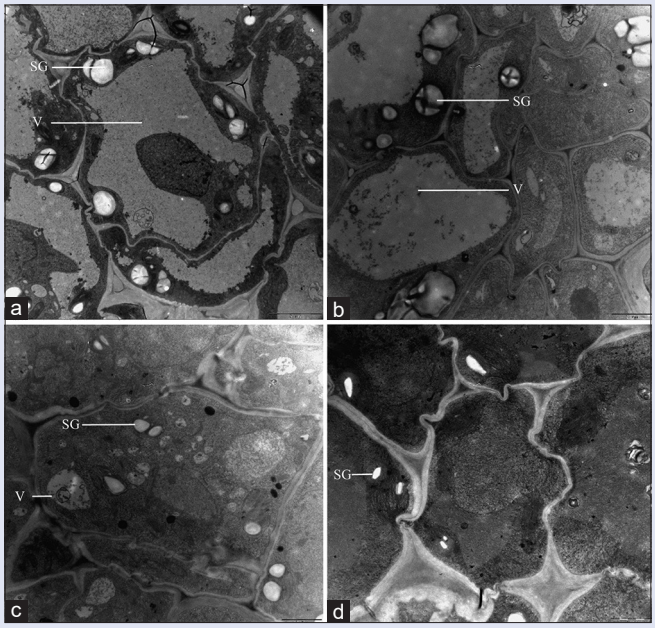

Figure 2: Morphological analysis of differentiating *Sophora tonkinensis* floral buds. (a) Floral buds undifferentiated stage: A large nucleus and vacuoles, an obvious nucleolus and many starch granules (bar = 5 μm); (b-d) the change of the vacuole and starch granules as differentiating floral buds progresses: (b) vacuole and starch granules reducing (bar = 2 μm); (c) small vacuoles and starch granules (bar = 2 μm); (d) volume was reduced and vacuoles and starch granules gradually disappeared (bar = 2 μm). SG: Starch granules; V: Vacuoles

The protein content during the floral bud differentiation period of *S. tonkinensis* gradually decreased. In the early stage of floral bud differentiation, protein content decreased to a low level. During inflorescence primordia differentiation, the protein content increased by 17.75%. Then, the protein content in the stamen and primordia differentiation stage and the stamen and pistil differentiation stage increased significantly ($P < 0.05$). The protein content of *S. tonkinensis* was at a high level before floral bud differentiation began and it decreased rapidly at the beginning of the differentiation process [Figure 3b].

Antioxidant enzyme activities

As shown in Figure 3, the POD and SOD activities in *S. tonkinensis* significantly changed during floral bud differentiation and those changes were similar. Compared with the early stage (Stage I), at Stage VIII, POD and SOD activities decreased by 40.11% ($P < 0.05$) and 26.01% ($P < 0.05$), respectively [Figure 3c and d].

Pigments

Chlorophyll is an important pigment used in photosynthesis to convert inorganic molecules or ions into organic biological molecules. With the initiation of floral bud differentiation, Chl a + b content increased significantly. Compared with Stage I, Chl a + b concentration increased by 37.90% ($P < 0.05$) and 43.07% ($P < 0.05$) at stages III and VII, respectively [Figure 3d].

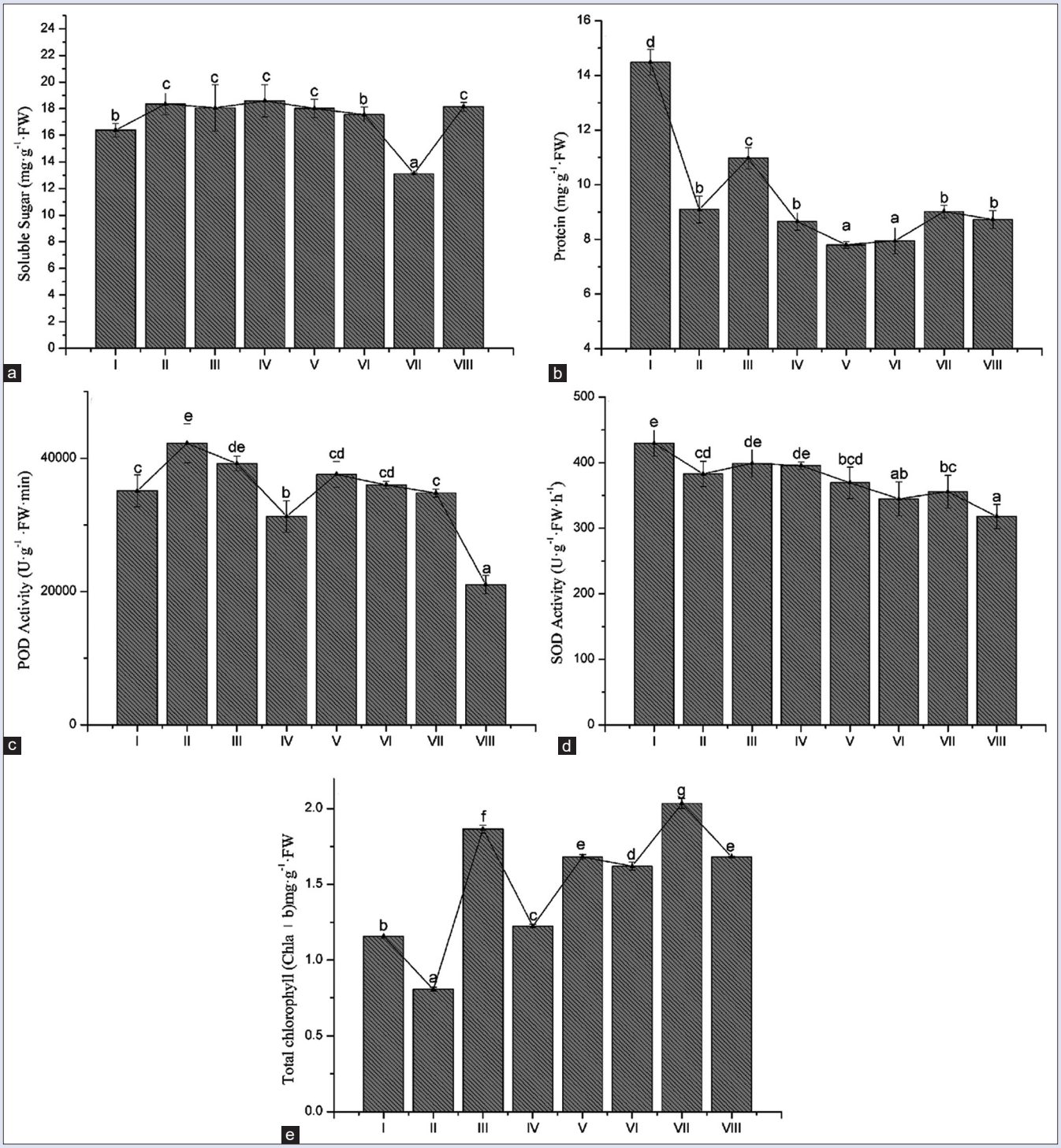

Figure 3: Influence of differentiating floral buds on the (a) soluble sugarin, (b) protein (c) superoxide dismutase, (d) peroxidase and (e) total chlorophyll (Chl a + b) in *Sophora tonkinensis* grown Letters indicate statistical differences ($P < 0.05$) according to an least significant difference test; the same letter denotes no significant difference among treatments, $n = 3$

The dynamic changes in plant hormones

Endogenous hormone levels were determined and compared among the different stages of floral bud differentiation (I–VIII). The levels of ZT increased gradually during floral bud differentiation. Compared with Stage I, ZT concentration increased by 16.52% at the completion of floral bud differentiation (Stage VIII) [Figure 4a].

The ABA concentration in buds was relatively low at Stages V and VI compared with Stages I–III. As shown in Figure 4b, plants maintained a relatively stable ABA content during the entire differentiation process with results showing that no significant difference was observed in each stage compared with Stage I ($P > 0.05$).

As for endogenous GA_3 levels, I observed a progressive increase from Stage I to III ("induction period") from 2.66 to 3.71 ng/g ($P < 0.05$),

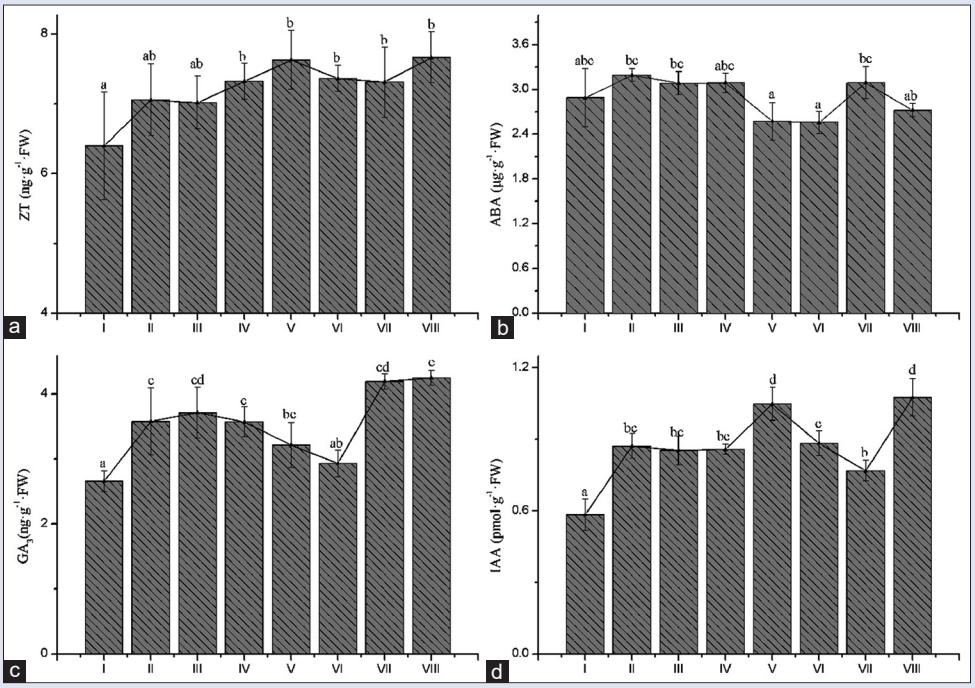

Figure 4: Dynamic changes of hormone content of differentiating floral buds on the (a) zeatin levels; (b) abscisic acid levels; (c) GA_3 levels; (d) indole-3-acetic acid levels in *Sophora tonkinensis* grown. Letters indicate statistical differences ($P < 0.05$) according to an least significant difference test; the same letter denotes no significant difference among treatments, $n = 3$

followed by a progressive decrease from Stage IV to VI from 3.57 to 2.93 ng/g ($P < 0.05$). During the entire differentiation process, GA_3 levels increased by 37.43% ($P < 0.05$) [Figure 4c].

During the entire floral bud differentiation, endogenous IAA levels increased significantly, as shown in Figure 4d. Compared with the early stage (Stage I), at Stages IV and VIII, IAA levels increased by 31.95% ($P < 0.05$) and 45.72% ($P < 0.05$), respectively [Figure 4d].

Differentiating flower buds transcriptome sequencing, assembly, and functional annotation

Flowering pathway

The assembled genes were annotated to determine the functions of the genes and to predict their roles in the determination and development of flower buds in combination with the changes in expression levels. A total of 104,519 unigenes were assembled, of which 34.53% were annotated using the GO database and 69.89% were annotated using the TrEMBL database. Research on genes focuses on genes with well-defined functional annotations. Previous studies on the model plant *Arabidopsis thaliana* (L.) Heynh, have shown that there are five main regulatory pathways that should be considered when studying flowering. The regulatory pathways are photoperiod, vernalization, gibberellin, autonomic flowering, and age list the five factors.[21] The key genes of each pathway were searched for in the transcript data. CONSTANS (CO) encodes the main transcription factor involved in flowering that is controlled by the photoperiod pathway.[22,23] It was found that 24 of the assembled genes were CO based on transcription group. The expression trends of the remaining genes are shown in Figure 5.

Some CO was highly expressed at stage I, then the expression level began to decrease and then increased at the later stages. Some CO had an obvious increasing trend from Stage I to II. Considering the key stages of flower buds formation and inflorescence formation at Stages I and II, these CO may play a decisive role in the flowering process. Thus, the trends in CO expression in the transcription group showed that the flowering of *S. tonkinensis* was mainly regulated by the photoperiod pathway. We focused on the gene expression in Stages I and II of flower buds formation and flower primordia formation. Through an enrichment analysis of DEGs using the KEGG database, it was also found that several genes were being expressed in the photoperiod pathway [Figure 6].

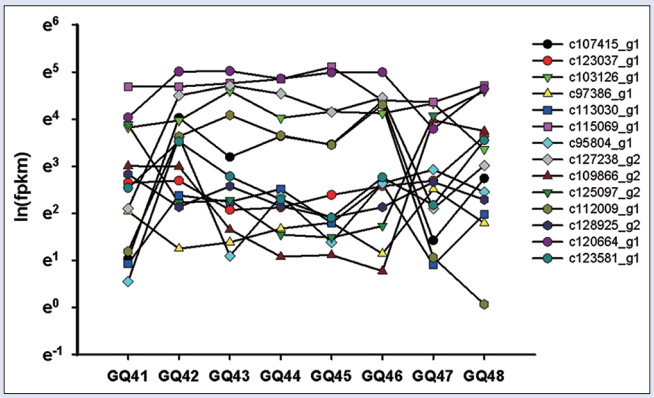

Figure 5: Trends in CO expression

Sequence analysis of flower development genes

Flower development is regulated by a variety of genes, among which the gene that encodes the MADS-box protein plays an important role. MADS-box is a transcription regulator that regulates the expression of genes.[24] Through clustering analysis of MADS-box genes expression patterns, it was found that these genes can be roughly divided into two categories, as shown in Figure 7. In one category, they may play an

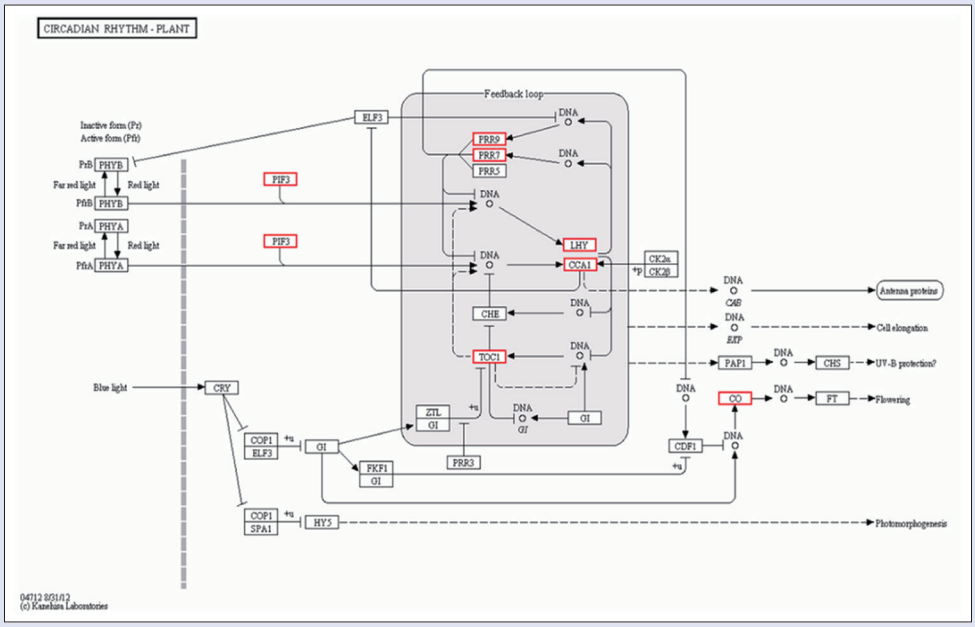

Figure 6: Genes expression in Kyoto Encyclopedia of Genes and Genomes photoperiod pathway (Note: Red indicates increased genes expression)

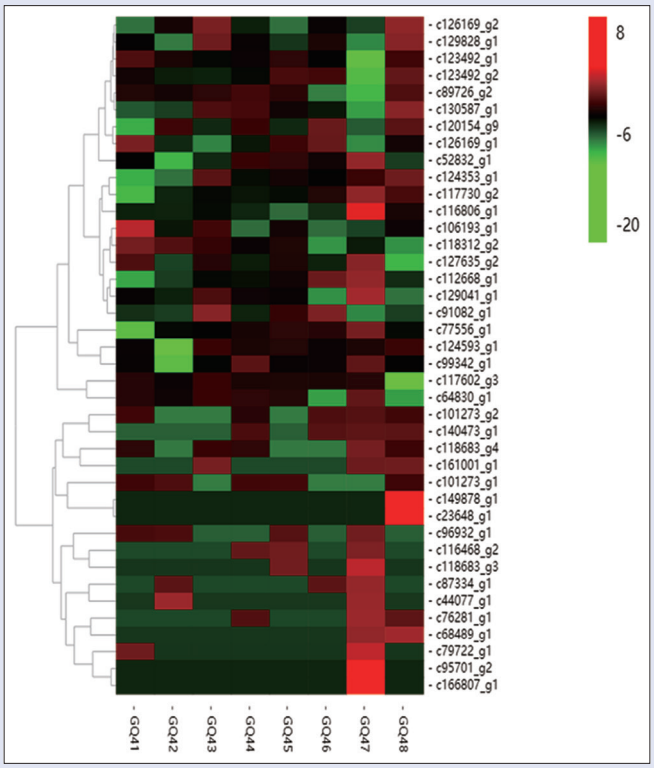

Figure 7: Cluster analysis of MADS-box genes expression

important role in the development of stamens and carpels in anaphase, while the other may play an important role in the development of petals and calyx in anthesis.

DISCUSSION

As people become more conscious of their health, the demand for Chinese herbal medicine increases; however, this demand cannot be met by wild resources alone. Therefore, the ability to produce high-quality Chinese herbal medicine at high yields is important.[25] Propagation through high-quality seeds is an important way to obtain germplasm resources. The floral bud differentiation process has a direct effect on the quantity, quality, and seed setting rate of plants.[26] Therefore, the ability to manipulate floral bud differentiation could help toward developing breeding strategies that could improve the yield and quality of *S. tonkinensis*.

Vegetative growth and accumulation of nutrients are the basis of floral bud differentiation and large amounts of nutrients are required. Whether the nutrients are sufficient determines the quality of the seed produced at the end of the process. Throughout the floral bud differentiation process, floral buds act as "metabolic sinks," and their development is closely associated with carbohydrate metabolism.[27] Soluble sugar is an indispensable nutrient for floral bud differentiation and is also the main assimilative substance in plants, playing an important role in growth, development, and metabolism.[28] During flower induction, the increase in sucrose supply in the terminal buds of *Vitis vinifera* L. was observed by measuring phloem secretions.[29] As shown in Figure 3a, compared with the undifferentiated stage (Stage I), there was a higher soluble sugar content at the calyx differentiation stage (Stage IV), which corresponded to increased floral buds number [Figure 1]. Interestingly, there were no differences in soluble sugar content at Stages II–V ($P > 0.05$). This indicated that soluble sugar was continuously produced and transformed during floral bud differentiation.

Protein accumulation is important for flower formation, as was demonstrated in a previous study on strawberry plants that found protein accumulation at the beginning of floral bud differentiation.[30] The soluble protein of the chrysanthemum increased rapidly and maintained a high level during the initiation stage of floral bud differentiation.[31] A previous study reported that high protein content is not conducive to the initiation of the floral bud differentiation stage.[32] In the present study, the soluble protein content decreased rapidly at Stages I and II ($P < 0.05$). This indicates that higher protein content at the floral bud undifferentiated stage was not conducive to the initiation of flower bud differentiation. However, higher protein accumulation before flower bud differentiation was important for flower formation [Figure 3b].

In addition to nutrient accumulation, a series of enzymes are involved in flower bud differentiation. During growth, a plant becomes more resistant to adverse conditions and thus more SOD is needed to scavenge radicals.[33] The activity of SOD increased rapidly, which could cause the plant to become senescent before flowering.[34] POD is related to respiration, and a higher level of POD activity is beneficial to vegetative growth and reproductive growth.[32] In the present study, SOD activity gradually decreased throughout the differentiation process, while POD activity maintained a high level during the initiation stage, which were beneficial for floral bud differentiation [Figure 3c and d].

The chlorophyll content of *Rhododendron pulchrum* Sweet was found to be positively correlated with the flowering rate during floral bud differentiation.[35] In the present study, the content of chlorophyll increased gradually throughout the differentiation process and peaked at its completion. This could be explained as a high content of chlorophyll being important for floral bud differentiation of *S. tonkinensis* [Figure 3e].

CTK is an important hormone involved in floral buds induction. The rapid increase in ZT content during floral bud differentiation [Figure 4a] indicates that the process requires a higher level of ZT.[36] Previous studies have shown that the increase in ZT content is beneficial to flower formation of *Vanilla peanigoeia* Ancer.[37] ABA can promote or inhibit flowering and it can cause branches to stop growing and antagonize GAs and thus ensure that the differentiated tissues have a suitable growth rate, which is beneficial to flowering.[38] However, it was observed no significant differences in ABA activity throughout the whole floral bud differentiation process [Figure 4b].

The effect of GA_3 on floral buds is staged. The reduction of GA_3 is necessary before floral bud differentiation. At the end of floral bud differentiation, GA_3 promoted flower development. In the present study, similarly the GA_3 content was lower at Stage I but higher in the stage of floral bud differentiation [Figure 4c]. The effect of IAA on flowering induction is indirect through regulating GA_3, CTK and ABA in floral buds.[39] The results showed that IAA content increased during the whole differentiation process and might promote floral bud differentiation of *S. tonkinensis* [Figure 4d].

In this study, transcriptome information was obtained using high-throughput sequencing of flower buds at eight stages of flower bud differentiation. A large number of gene sequences involved in the development of *S. tonkinensis* flowers can be obtained by analyzing the data of the flower buds transcript group of *S. tonkinensis*. It was concluded that the flowering of *S. tonkinensis* is mainly determined by the photoperiod pathway.

CONCLUSION

On the basis of the present results, increased soluble sugar content, protein, chlorophyll and increased POD activity were beneficial to floral bud differentiation. Furthermore, changes in hormones contribute to floral bud differentiation. Other factors affecting floral bud differentiation and the corresponding cultivation measures to regulate floral bud differentiation deserve further study.

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Conflicts of interest

There are no conflicts of interest.

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