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Full Length Article

Time Course Morphological and Histological Changes in Differentiating Floral Buds of *Styrax tonkinensis*

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Abstract

This study was aimed to reveal the time dependent exact differentiation process of *Styrax tonkinensis* in order to demarcate appropriate stages for regulation of flower development. The external morphology and internal structure of floral buds of *S. tonkinensis* in different differentiation stages were observed by using stereo microscope and making paraffin sections. The method of BBCH (Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie) scale was adopted to describe the external morphology of flower buds and leaves of *S. tonkinensis*. The flower bud differentiation of *S. tonkinensis* began in early March and ended in mid-to-late May. According to the anatomical observation on internal structure, the flower bud differentiation of *S. tonkinensis* could be divided into six stages, including the initiation of flower bud, the appearance of inflorescence primordium, the formation of sepal primordium, the formation of petal primordium, the formation of stamen primordium and pistil primordium. The apical growth point becoming flat marked the transition from vegetative growth to reproductive growth. The order of flower bud differentiation of *S. tonkinensis* was centripetal. The experimental results can provide references for forest managers to understand the flower bud differentiation law of *S. tonkinensis* and take actions to regulate it. © 2019 Friends Science Publishers

Keywords: *Styrax tonkinensis*; Floral bud differentiation; Bud histology; External morphology

Introduction

Flower bud differentiation is an important indicator of transformation from vegetative growth to reproductive growth, which is one of the most important stages in the development of flowering plants. Growers pursue for the yield and quality of fruit directly, which are affected by flower bud differentiation. Short-day photoperiod treatment promoted flower bud differentiation of *Vigna angularis* (Dong *et al*., 2016). Factors affecting flower bud differentiation, such as carbohydrates and hormones (Chen, 1991; Li *et al*., 2003) had been extensively studied. Some studies have shown that GAs inhibited the flower bud initiation of fruit trees (Prang *et al*., 1998), while ABA promoted the flower bud differentiation (Zhu *et al*., 2015). It is thought that the impact of GAs on flower bud differentiation is related to the species and varieties. Xiao *et al*. (2018) confirmed that plant hormones signal transduction pathways were involved in the formation of flower buds. By observing the internal structure of flower buds continually, we can master when and how flower bud differentiation happens.

The flattening of the apical growth point marks the transformation from vegetative growth to reproductive growth (Ryugo, 1986). However, in the scanning electron microscopy study by Engin and Ünal (2007), during the formation and differentiation of *Prunus avium* flower buds, the mark of transformation from vegetative growth to reproductive growth was related to change in the apical growth point from the shape of flat to hemispherical. This was attributed to be caused by the environment, especially the temperature (Westwood, 1978). Benko (1967) divided the flower buds' morphological differentiation of ten varieties of apple (*Malus pumila*) into five phases, including the formation of the growing point primordium, the formation of the cup on sepal primordia, the formation of petal primordium, the formation of carpel and stamen primordia as well as completion of the differentiation. He attributed this differentiation in relation to the earliness of these varieties. The appearance of stamen and carpel primordia contemporaneously was in accordance with the study of Hořavka (1961). Likewise, the flower bud differentiation of *Curcuma kwangsiensis* cultured at 30℃ was divided by Sheng *et al*. (2013) into five phases: initial differentiation phases, inflorescence and bract primordium differentiation phases, flower primordium differentiation phases, small floral primordium differentiation phases and flower organ differentiation phases. Sheng *et al*. (2013) concluded that the expression of FLOWERING LOCUS T (*FT*) was associated with flowering differentiation and the

expression of *FT* at 30℃ and was significantly higher than that at 25 or 20℃. They attributed this phenomenon to high temperature treatment in the initial differentiation phase. Han *et al*. (2018) had recorded detailed changes in the external morphology of flower buds of Pecan (*Carya illinoensis*) in southeastern China. In the study of morphological changes of pistillate flower buds of protogynous individual of *Cyclocarya paliurus*, Fu *et al*. (2011) found that the flower buds were red-spotted at the beginning and gradually turned into the shape of spindle after two weeks. The morphological differentiation finished when the inflorescences were elongated and expanded and the flower stalks and bracts were visible. Understanding the rules and mechanisms of flower bud differentiation helped regulate it and improve the quality of flowers and fruits.

Styrax tonkinensis, a fast-growing tree species with strong adaptability, is widely distributed in sub-tropical areas including Vietnam, Laos and southern China (Zhang *et al*., 2018). This species is one kind of ideal ornamental tree with white flowers, which blooms quickly in late April. The flowers of *S. tonkinensis* are in strings with light fragrance and can be used as medicine to relieve pain. The substance contained in flowers acts as an important pharmacological ingredient that protects the cardio-cerebral organs. *S. tonkinensis* enters the buds induction stage in early March. The appearance of flower primordium marks the beginning of morphological differentiation, which is from mid-late March to May. Few researches referring to flower bud initiation of *S. tonkinensis* could be found. Therefore, mastering the flower bud differentiation in *S. tonkinensis* was essential for artificial adjustment of its production season.

In this study, the internal structures at different time points of *S. tonkinensis* flower bud differentiation were obtained by anatomy and the differentiation period of *S. tonkinensis* could be determined through the combination of the external morphological changes, which could provide a substrate for the study of the flower bud differentiation of *S. tonkinensis*.

Materials and Methods

Plant Material

At the end of November, 2016, five 4a *S. tonkinensis* trees with similar height, which grew well with no pest attacks, were selected and tagged from the planting base of Jiangsu Guoxing Biotechnology Co. Ltd., located in Luhe district, Nanjing (32°54' N, 118°84' E). Since then we took photos of buds in these trees. From 15th March, 2017, the photos of the external morphology of buds on five mother trees were taken with a single-lens reflex camera every three days to observe the change of buds color, floss condition and the spread of leaf during the period between the sprout of the winter bud and the end of flower bud differentiation. In total forty buds (eight buds from each tree) were collected each time, out of which twenty five buds were used for the observation of flower bud differentiation and fifteen buds were used for the observation of structure. The tree structure chart of *S. tonkinensis* was drawn according to the conditions of flower buds and leaf buds in different period.

BBCH Scale and Experimental Methods

Biologische Bundesanstalt, Bundessortenamt and Chemische Industrie (BBCH) scale is a way of describing the developmental period of plants, which uses number 0-9 to represent the different stages of plant development (Lancashire *et al*., 1991). In this experiment, the morphology of flower buds and leaves was represented by 0 and 1, since the flower bud differentiation of *S. tonkinensis* was the only objective of the study.

Fifteen buds were used to observe the flower bud structure by Olympus stereo microscope to take photos. Twenty five buds were cut both sides of the flattened parts and fix them with 70% FAA. Method of Zheng (1978) and Li (2000) was followed for making conventional paraffin sections. After dehydration, clearing, wax penetration process, embedding, slicing, flatting and plastering and staining with safranine and fast green, the tissues were observed under DM5000B light microscope and then microphotographed.

Results

Process of Flower Bud Differentiation in *S. tonkinensis*

Initial stage of flower bud differentiation: In early March, winter buds sprouted and on 19th March, there were 1-2 primordia sprouted on the inner base of the leaves of *S. tonkinensis* winter buds. The cells were small and dense, forming conical protrusions. With the division of apical meristem cells, the apical growth point gradually became flat and wide. The growing tip changed from vegetative growth to reproductive growth (Fig. 1A).

Appearance of inflorescence primordium: On 23th March, the primordium elongated gradually with the anticlinal division of apical meristem. Meanwhile, several enations formed and stretched around the base of the primordium and the inflorescence was visible in this stage (Fig. 1B).

Formation of sepal primordium: The inflorescence primordium continued to stretch and the flower primordium divided furtherly with the top becoming wide and smooth on 27th March. At the same time, a ring of sepal primordium was differentiated on the top edge of flower primordium, which bent inward (Fig. 1C).

Formation of petal primordium: On 4th April, the flower primordium continued to stretch with cells division. The sepal primordium bended inward slightly when stretching with the top being sharp gradually. On the inner side of the sepal primordium, five petal primordia enations were formed, which curled inward and were thicker than the sepal primordium (Fig. 1D).

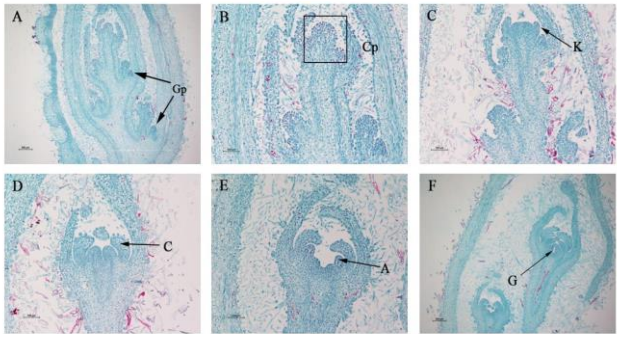


Fig. 1: The flower bud anatomical structure of *S. tonkinensis*. (A) Gp: growing tip, 2017.3.19 (B) Cp:inflorescence primordium, 2017.3.23 (C) K:sepal, 2017.3.27 (D) C: petal primordium, 2017.4.4 (E) A: stamen, 2017.4.12 (F) G: pistil, 2017.4.16

Formation of stamen primordium: On 12th April, following the formation of the petal primordium, there was a round of stamen primordia formed on the inner side of the petal primordium, and the cells were smaller and denser. With the periclinal division of the flower primordium, the primordium grew wider and the interior was recessed inward (Fig. 1E).

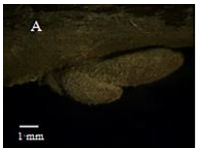
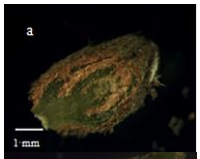
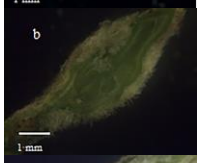
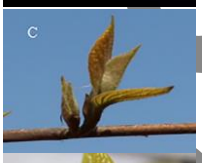
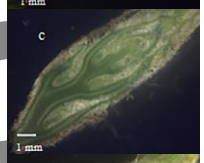
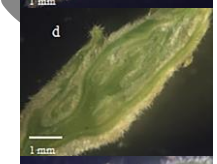
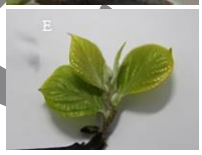
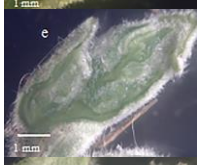
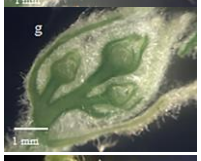
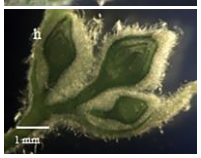
Formation of pistil primordium: The petals continued to stretch and curled inward on 16th April. The stamen primordium stretched upward and the center of the growing tip inside the bud gradually uplifted upward, developing into a larger enation to form the pistil primordium. The pistil primordium was conical and hollow inside (Fig. 1F).

The pistil primordium continued to develop and the top extended upward into a stigma with a concave top and a hollow inner core. The base of the pistil primordium expanded to form an ovary and the base of ovary continued to develop, forming irregular enation as ovule primordium, which can form pollen tube with the connection between the top and the stigma.

External Morphology of *S. tonkinensis* Buds

At the end of November, 2016, there were 2-3 winter fascicular buds and the winter buds on the inner side of *S. tonkinensis* branch were larger and longer. The winter buds were rich in internal layers with structures of multilayer leaf-like and branch, which were mixed buds (Table 1a). Among the three winter buds, the winter buds on the inner side of the branch would sprout in the next year, forming new shoots; some buds in the middle would form new shoots and some would not sprout; the outermost winter buds would not sprout and eventually fell off. The winter buds of *S. tonkinensis* began to sprout in early March of the next year, gradually forming new shoots and young leaves. The winter bud developed into a new shoot and four leaves formed. During the flower bud differentiation, there were different colors of leaves in each period, which underwent transition from red-green→yellow-green→green→dark green.

Table 1: Observation on the external morphology of flower bud differentiation of *S. tonkinensis*

Date	External morphology	Longitudinal section of the mixed buds
2016.11.29		
2017.03.01		
2017.03.19		
2017.03.23		
2017.03.27		
2017.04.04		
2017.04.12		
2017.04.16		

Spatial Distribution of *S. tonkinensis* Flowers

The winter buds of *S. tonkinensis* were born on the branches of the year. The sprout of each winter bud was visible with 2-3 leaves unfolded (Table 1B and Table 2). After 8 days, flower buds and leaf buds could be distinguished. With the elongation of the new shoots, some winter buds differentiated only 4-5 leaves and some differentiated into leaves in which there were 1-2 racemes at the base of the new leaves. The multiple racemes at the top of the new shoots formed panicle (Fig. 2a). There were 1-6 racemes and a panicle at the top of the new shoots and each new shoot had more than 20-30 flowers. For each lateral branch,

Table 2: BBCH scale to describe the morphology of flower buds and leaves of *S. tonkinensis*

Code	Stage	Description
Principal growth stage 0: bud development		
00	Dormancy (Table 1A and 1a)	Three small fascicular buds appeared and the inner buds were larger. They were brown with rust-colored floss outside and rich layers in the buds
01	Beginning of bud swelling (Table 1B and 1b)	The lateral buds were bulky with split bud scales and the leaf structure was visible from the outside. The inside of the buds were green and the branch structure was visible.
03	End of bud swelling	Light green buds emerged.
07	Beginning of bud burst (Table 1c)	The inside of the bud was green and the growing point was visible inside the leaf.
09	Bud showed green growing point (Table 1d)	The growing point inside the leaf was clearly visible and the undifferentiated part was densely covered with white floss.
Principal growth stage 1: leave development		
10	leaves separated	Leaves began to separate.
12	Two leaves unfolded (Table 1c)	2-3 leaves were unfolded with reddish-green and rusted-color floss.
13	Three leaves unfolded (Table 1d)	3-4 leaves were unfolded with yellow-green and the rusted-color floss began to fall off.
15	All leaves unfolded (Table 1f)	The leaves were obviously enlarged and the multilayer structure of the flower buds was macroscopic.
19	Leaves matured (Table 1h)	The leaves were darker in color and the petal, stamen and pistil were macroscopic.

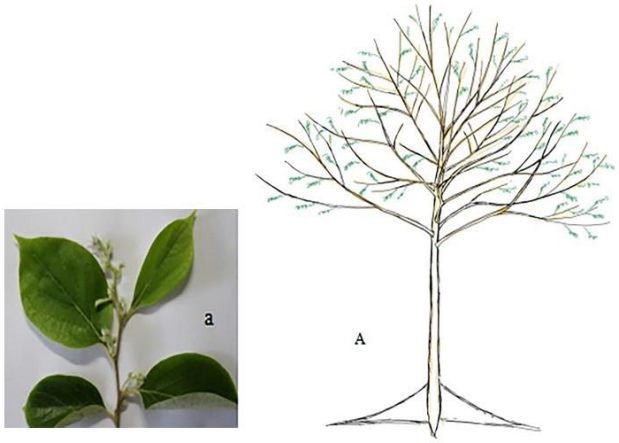


Fig. 2: The spatial distribution of *S. tonkinensis* flowers. (A) tree of *S. tonkinensis* green part: new flowering shoots (a) new flowering shoots

the new shoots with inflorescences were alternate in the upper middle part of the annual branches, sagging slightly. For the whole tree, the flowering new shoots were concentrated in the middle and lower part of the tree and the winter buds on the upper branches of the tree mostly developed into leaves, which were larger, wider and greener than the leaves around the inflorescences (Fig. 2A).

Discussion

The flower bud differentiation of *S. tonkinensis* began in early March and ended in mid-to-end May. The flower buds of *Citrus reticulata* and *C. illinoinensis* differentiated only 1-2 months before flowering, while other tree species may last for 4-5 months or even longer (Guimond *et al.*, 1998; Samach and Smith, 2013). According to the observation from the longitudinal sections, the flower bud differentiation of *S. tonkinensis* could be determined. The apical growth point of *S. tonkinensis* was flat surrounded by densely cells. The transition from vegetative growth to reproductive growth of apical point took place. Sheng *et al.* (2013) reached the similar conclusion and this transition was a crucial phase during the development of plants. Gene and environment conditions might lead to this (Koornneef *et al.*, 1998; Simpson *et al.*, 1999; Araki, 2001). On 23th March, the inflorescence was visible in this stage, which was differentiated from inflorescence primordia. Then multiple flower primordia were formed. In *P. cerasus*, flower primordia emerged in axils of bracts (Diaz *et al.*, 1981). Five petal primordia of *S. tonkinensis* were appeared on the inner side of the sepal primordium with large thickness. Reducing buds exposure to high temperature could decrease the formation of abnormal flowers (Beppu *et al.*, 2001). Stamen primordium and pistil primordium were formed in sequence. The top of pistil primordium developed into a stigma and the bottom expanded to form an ovary.

The order of flower bud differentiation of the floral organs of primordium on *S. tonkinensis* was centripetal. It was consistent with the order of differentiation of most plants (Guerriero and Bartolini, 1995), which was in the sequence of sepals, petals, stamens and then pistils (Mert *et al.*, 2013). In classification of Weberling (1989), the determinate inflorescence referred to the main axis of inflorescence ended in a flower and the indeterminate inflorescence was that the inflorescence continued to grow and produced flowers lately. A mutation in *CENTRORADIALIS (CEN)* gene of *Antirrhinum* and in *TERMINAL FLOWER 1 (TFL1)* gene of *Arabidopsis* made indeterminate inflorescence to determinate (Kato *et al.*, 1998). The flower bud differentiation process of *S. tonkinensis* conformed to the differentiation law of determinate inflorescence, that was, the flower primordium on the top of the inflorescence developed into flowers, which had advantages in flowering, pollination and fruiting, comparing with the flower primordium differentiated later.

During the development of *S. tonkinensis*, reproductive growth was later than vegetative growth. The winter buds of *S. tonkinensis* generally sprouted in early March and then developed into new shoots and young leaves. During the development of the new shoots, 2-3 leaves were unfolded firstly and then the inflorescence primordium was formed on the inside of the leaves,

which could develop into inflorescences later (Fig. 1B). Therefore, during the development of mixed buds, small flowers were differentiated in middle-to-late March, which marked the beginning of the reproductive growth. This study indicated that the early-to-middle March was the flower bud induction period of *S. tonkinensis* and the middle-to-late March to May was the morphological differentiation period of the flower bud.

During the flower bud differentiation of *S. tonkinensis*, the external morphology changed significantly which was due to changes in the internal morphology. The flower buds entered the early differentiation stage when 2-3 leaves unfolded on the new shoots with rusted-color floss on the outer part of the leaves. The sepal primordium of flower buds began to form when the rusted-colored floss on the leaves fell off and then turned into yellow-green color. During the petal formation, the structure of the inflorescence could be observed on the inner side of the leaf base. During the differentiation of stamen, rachis elongated obviously and multilayer structure inside the flower buds could be observed. Floral organs could be distinguished through longitudinal section during the differentiation of pistil.

As regards spatial distribution of *S. tonkinensis* flowers, for each lateral branch, the new shoots with inflorescence were alternate in the upper middle part of the annual branches and the new shoots with leaves were in the middle and lower part of the annual branches, which may be due to more nutrients on the upper middle part of the annual branches. It is likely that the type of shoots also had an important influence upon flower bud differentiation (Alburquerque *et al.*, 2003). For the whole tree, the new shoots with flower buds were concentrated in the middle and lower part of the tree, and the winter buds on the upper branches of the tree mostly developed into leaves, which were larger, wider and greener than the leaves around the inflorescence and the growth rate was also faster. The growth of the leaves around the inflorescence might be restricted by the growth of the inflorescence and the leaves in the upper part of the tree could perform photosynthesis to produce sugar to meet the growth demands of the tree.

Conclusions

Six stages could be found during flower bud differentiation of *S. tonkinensis* and each period could be distinguished by changes of external morphology. BBCH was a good method to describe the changes of flower buds and leaves. It hopes that the results of our study can help to understand the flower bud differentiation law of *S. tonkinensis*, providing scientific guidance for controlling florescence and regulating its flowers.

Acknowledgements

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