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Full Length Research Paper

The potential role of B-function gene involved in floral development for double flowers formation in *Camellia changii* Ye

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***Camellia changii* Ye, a rare and endangered species, has a phenotype that sepals frequently transform into petals. We assumed that this change would cause single *C. changii* Ye turned double flowers and this was confirmed by the double flowers we found in grafted *C. changii* Ye. The microstructure of floral organs showed that: in perfect petals, the anthocyanin is distributed in the upper and lower epidermis; in petaloid sepals, anthocyaninin had both sepal and petal identities and sepals gradually transformed into petals; in spot-petaloid sepals, anthocyaninin is only distributed in upper epidermis. B-function gene *GLOBOSA1* (*GLO1)* and *GLOBOSA2* (*GLO2*) had high expression at the part with petal identity and had low or no expression at or near the part with sepal identity and these kinds of expression showed that gene played an important role in determining petal identity. B-function gene *DEFICIENS* (*DEF*) and *TOMATO MADS BOX GENE6* (*TM6*) had high expression at the fused part of the stamens and this implied the importance of gene when stamen is transformed into petal. Thus, B-function gene is very important when *C. changii* Ye evolved into double flowers.**

Key words: Petaloid phenotype, double flowers, B-function gene, *Camellia changii* Ye, real-time polymerase chain reaction (PCR).

INTRODUTION

The multiple origins of double flowers and the complexity of genetic rules make it difficult to research the molecular mechanism of ornamental plants' double-flower formation. Only by specifying the position and type of floral organ on the floral meristem can a flower arise. Without these molecular instructions for making a flower some beautifully strange mutants are formed. In these, 'homeotic' mutants one organ is substituted by another organ, prompting researchers to postulate the ABC model of floral development. This simple model proposes 3 classes of function genes (A, B and C) with overlapping expression in whorls so that A alone encodes sepals, B overlapping with A encodes petals, B overlapping with C encodes stamens, while C alone encodes the carpel. A and C expression is confined to distinct whorls since these genes negatively regulate each other's expression (Schwarz-Sommer et al., 1990; Coen and Meyerowitz, 1991). In the years following the formulation of the ABC theory, genes have been identified in several plants that correspond to genes A, B and C. Recent studies on the molecular mechanism of double flowers have focused on the mutation of C-function genes which affect downstream genes or on the interaction of C-function genes with other genes (Bowman et al., 1989; Schwarz-Sommer et al., 1990; Mizukami et al., 1995; Mandel and Yanofsky, 1995; Theissen et al., 2000; Lenhard et al., 2001; Lohmann et al., 2001; Pelaz et al., 2001) and on B-function genes and the interaction between B- and C-function genes (Jack et al., 2001).

Presently, wild *C. changii* Ye has only been found in a valley in Yangchun, China and is in danger of extinction.

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Table 1. Samples information.

Age (year)	Height (cm)	Crown diameter (cm)	Stock diameter (cm)	Stock height (cm)	Amount (plant)
3	93.5±11.3	39.3±8.0	2.9±0.3	41.9±9.2	99
5	94.1±14.0	54.8±5.6	3.5±0.4	51.4±8.9	79
6	137.7±8.1	69.1±7.5	3.7±0.5	57.0±7.5	45

Table 2. Sequence of the primers for real-time PCR.

Gene name	Forward sequence(5'-3')	Reverse sequence(5'-3')
GLO1	GTTGGAGGAGGAGAACAAGCAC	CAAAAGGCATCTGGGACTGG
GLO2	GTCTGGGAAGAGGTTGTGGGA	GTTATGTCTTCTCCTTTCAAGTGCC
DEF	AGGCAAGTCACGTACTCCAAGC	CGTTGAAGTGGAGGGACTGATG
TM6	ATGGGGCGTGGAAAGATAGAG	GACCTTGGCATCGCACAGAA
18S (Control)	GACTCAACACGGGGAAACTTACC	CAGACAAATCGCTCCACCAAC

Since *C. changii* Ye blooms every season, it has attracted the attention of the International Camellia Society (ICS). However, only single flowers with 5 to 6 petals but no double flowers were reported. As we observed in the grafted *C. changii* Ye, some flowers became double with 14 to 16 petals, sepal number decreased from 8 to 9 to 4 to 5 and some stamens transformed into petals. Some scholars suggested that the formation of double flowers in camellia was a stamens-carpels origin (Zhao and Liu, 2009), but such formation in *C. changii* Ye might be related to the B-function gene which affected the development of petals and stamens in core eudicots and influenced the second and third whorls of floral organs. Thus, the formation in *C. changii* Ye might be a sepal origin.

In camellia, B-function gene have three sub-families (Viaene et al., 2009): *PI*(*GLOBOSA/PISTILLATA*, including *GLO1* and *GLO2* gene members), *AP3* (*DEFICIENS/APETALA3*, including a *DEF* gene member) and *TM6*-like (including a *TM6* gene member), Viaene et al. (2009) studied the semi-quanti-tative RT-PCR expression and the protein interactions of B-function gene in *Camellia japonica* subs. *rusticana*, but there was no change from sepals or stamens to double flowers. We studied the phenotype and the microstructure of petaloid sepals and the expression of B-function gene in *C. changii* Ye to discuss the relationship between petaloid organs and the formation of double flowers.

MATERIALS AND METHODS

Species sampling

C. changii Ye (scion) was grafted by *C. japonica* 'Hongluzhen' (stock), the total were 223 plants and was planted in the experimental greenhouse of Research Institute of Subtropical Forestry, Chinese Academy of Forestry and nature conditions but >60% relative humidity. Table 1 lists the samples information in this experiment. The phenotype based on these samples was for investigation. Fresh flowers were send to laboratory immediately for free-hand sections for microstructure analysis (OLYMPUS, Shinjuku, Japan). Every part of the flower were put in a silver paper bag separately to gather the total RNA for real Time PCR, frozen in liquid nitrogen immediately and stored at -80°C before use.

Measuring spatial expression by relative-quantitative real-time PCR analysis

Total RNA was extracted from each organs separately, using the plant pillar RNAout kit (TIANDZ, Beijing, China) and each RNA samples was treated by DNase, using pillar DNA-free (TIANDZ, Beijing, China). Total RNA was reverse transcribed into cDNA using avian myeloblastosis virus (AMV) reverse transcriptase (Fermentas, Ontario, Canada) and the included random primer and oligo-dT primers (both primers can ensure the reverse transcription action was efficient). Gene-specific primers were designed based on homology and listed in following Table 2. Ribosomal 18S RNA (GenBank: U42815.1) was used as an endogenous control. Two-step methods was applied with SYBR green fluorescent dye (TARAKA, Dalian, China) and the program for amplification included 30 s polymerase activation at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. The instrument is ABI PRISM 7300 real-time PCR system (ABI, Carlsbad, United States). The results of the relative expression level were related to the tendency of gene expression when the petaloid sepal was "1".

RESULTS

Characteristics of petaloid phenotypes, discovery of double *C. changii* Ye and speculation of its origin

The flowers of normal *C. changii* Ye (Figure 1A) had green sepals without anthocyaninin deposits and red petals with heart-shaped tip. The flowers of petaloid *C. changii* Ye (Figure 1B, short yellow arrow) had anthocyaninin deposits in sepals and larger sepals than those of the normal flowers. According to their appearance

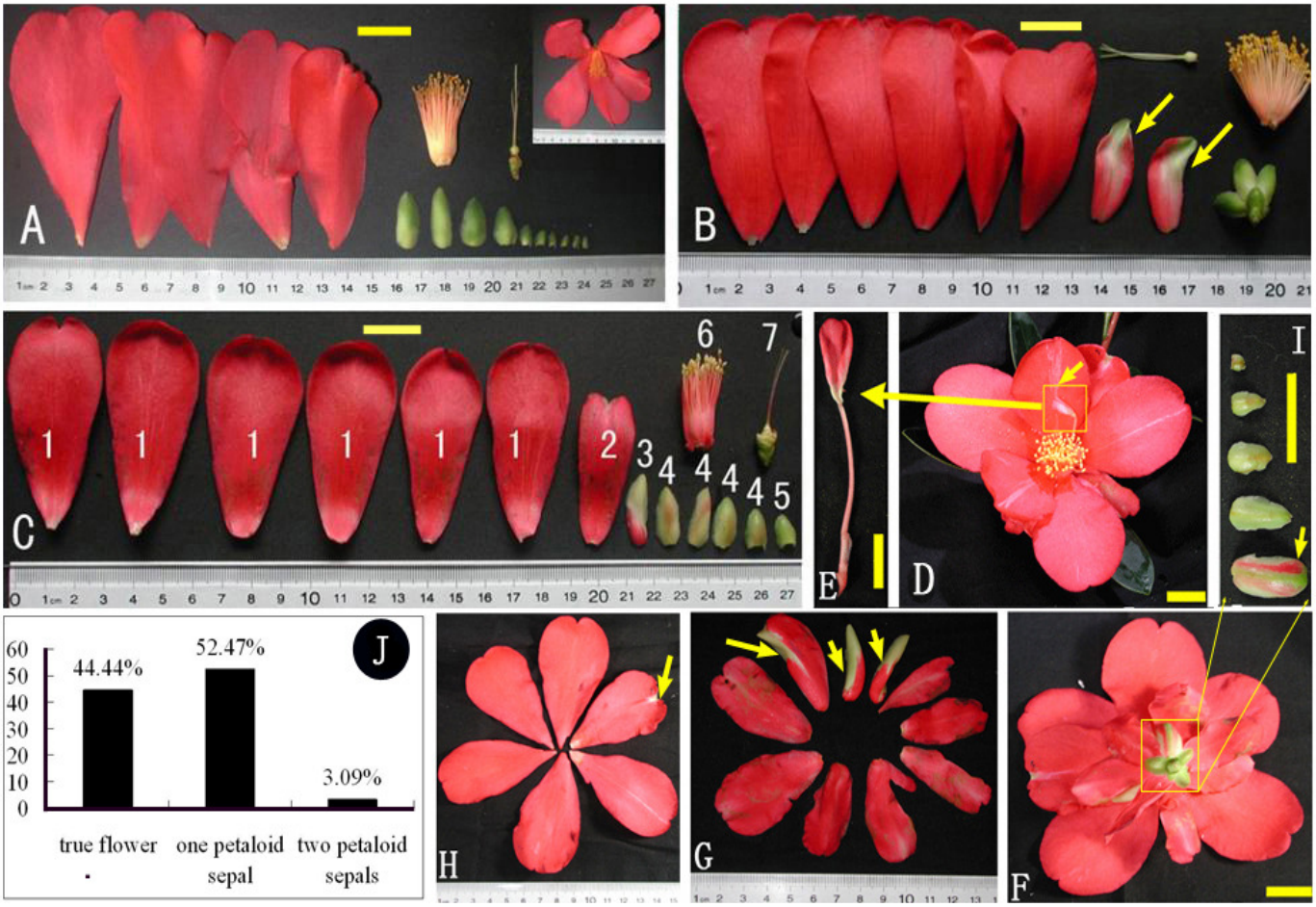

Figure 1. Flower organ phenotypes in *C. changii* Ye. A represent true flower of five petals. Two petaloid sepals of a flower represented with B (the petaloid site start at the bottom of the sepals); C represent every part of a petaloid sepals flower (perfect petals is showed by arabic number 1, imperfect petals is Arabic number 2, arabic number 3 represent petaloid sepals, Arabic number 4 represent some petaloid of a sepal, normal sepal represent arabic number 5, arabic number 6 represent androecium, gynoecium represented with arabic number 7); D represent double flower of *C. changii* Ye; E represent a petaloid stamen from double flower of *C. changii* Ye. F represent the back of double flower; G represent the first whorl petals of double flower and H represent the second whorl petals; I represent the sepals of double flower (including a pelatiod part of a sepal); J represent proportion of petaloid and true flowers (the results showed the petaloid flower proportion was 53.56%). Petaloid part represented with shortly yellow arrowhead, and scale bar is 2 cm.

rances, the petals were divided into two types: in type one (Figure 1c, marked with arabic numeral 1), they look like perfect petals; in type two (Figure 1C, marked with arabic numeral 2), they were smaller and had chlorophyll deposits and sepal identity and the heart-shaped tip changed. The double *C. changii* Ye had 16 petals (Figure 1G, H), one petaloid stamen (Figure 1E) and only 4 sepals (Figure 1I). Some petals had both sepal and petal identities.

After careful comparison, we found that the petaloid sepals transformed from bottom and then expanded upward until they fully developed into petals (Figure 1C, marked with arabic numeral 3). The bottoms of petaloid petals were exactly like perfect petals, but there were chlorophyll deposits in upper part, exhibiting sepal identity. In the grafted *C. changii* Ye, as many as 55.16% (Figure 1J) of the sepals were found petaloid. Considering the increase of petals and decrease of sepals and the fact that some petals had both petal and sepal identities, we inferred that the formation of double flowers in *C. changii* Ye was a sepal origin.

Petaloid microstructure of sepals in *C. changii* Ye

The different phenotypes between perfect sepals and petaloid sepals lied at the different distributions of chlorophyll and anthocyaninin. Whether the observation cohered to the microscope structure, especially the distribution of spot-petaliod, anthocyaninin was important to find petaloid sepals generation.

Perfect petals had anthocyaninin near upper and lower epidermis (Figure 2A), normal sepals had chlorophyll between upper and lower epidermis (Figure

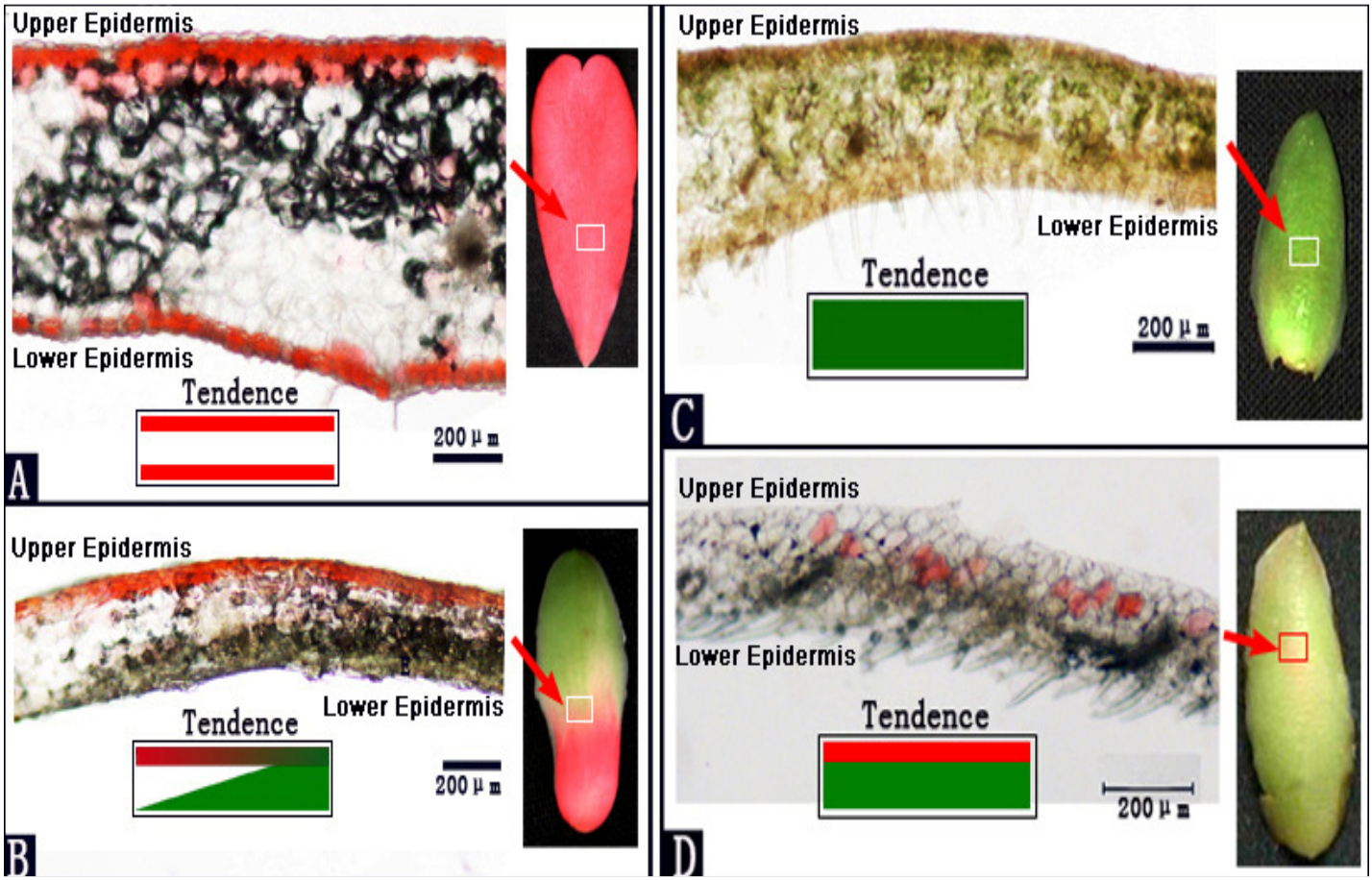

Figure 2. Microstructure of anthocyaninidin location. A represent perfect petal that anthocyaninidin is located upper and lower epidermis mostly; B represent petaloid sepal that anthocyaninidin is gradually transform into perfect petal. Normal sepal is green both upper and lower epidermis that is represented with C; Some petaloid part of sepal represented with D; a few anthocyaninidin is located upper epidermis. Scale bar is 200 μm.

2C) and petaloid sepals had both sepal and petal identities (Figure 2B). At the part with petal identity, anthocyaninin distributed as the same way as that of perfect petals and at the part with sepal identity, chlorophyll distributed as the same way as that of normal sepals. While at the joint part, the distribution of anthocyaninin had transitional features (Figure 2B). Spot-petaloid sepals had only a few anthocyaninin deposits, most of which were in the middle where sepals bumped and the microstructure showed that little anthocyanin deposited near the upper epidermis of the spots (Figure 2D).

Comparing the distributions of anthocyaninin and chlorophyll of petaloid sepals, we found that according to the observed phenotype, the red area with anthocyaninin deposits had petal identity and the area with chlorophyll deposits had sepal identity. The joints of sepals and petals had typical transitional features. Such phenomena suggested that petaloid organs were caused by intrinsic factors. However, the distribution rules of anthocyaninin in spot-petaloid sepals were different from those in sepals and petaloid sepals, which might be caused by extrinsic factors.

Special expression pattern of B-function genes in *C. changii* Ye

In order to explain whether petaloid sepals were caused by environmental influence, selection pressure from grafting or different levels of gene expression, we conducted real-time PCR to verify special expression of B-function gene by using RNA extracted from different part of floral organs in *C. changii* Ye (Figure 3).

We divided an imperfect petal into two parts and compared it with perfect one. The results showed that both *GLO1* and *GLO2* genes had higher expression at the lower part of imperfect petal than at the upper part, in contrast to the results from perfect petals. *PI* gene had high expression at the part with petal identity and had low or no expression at or near the part with sepal identity. Therefore, the expression levels of *PI* gene could be

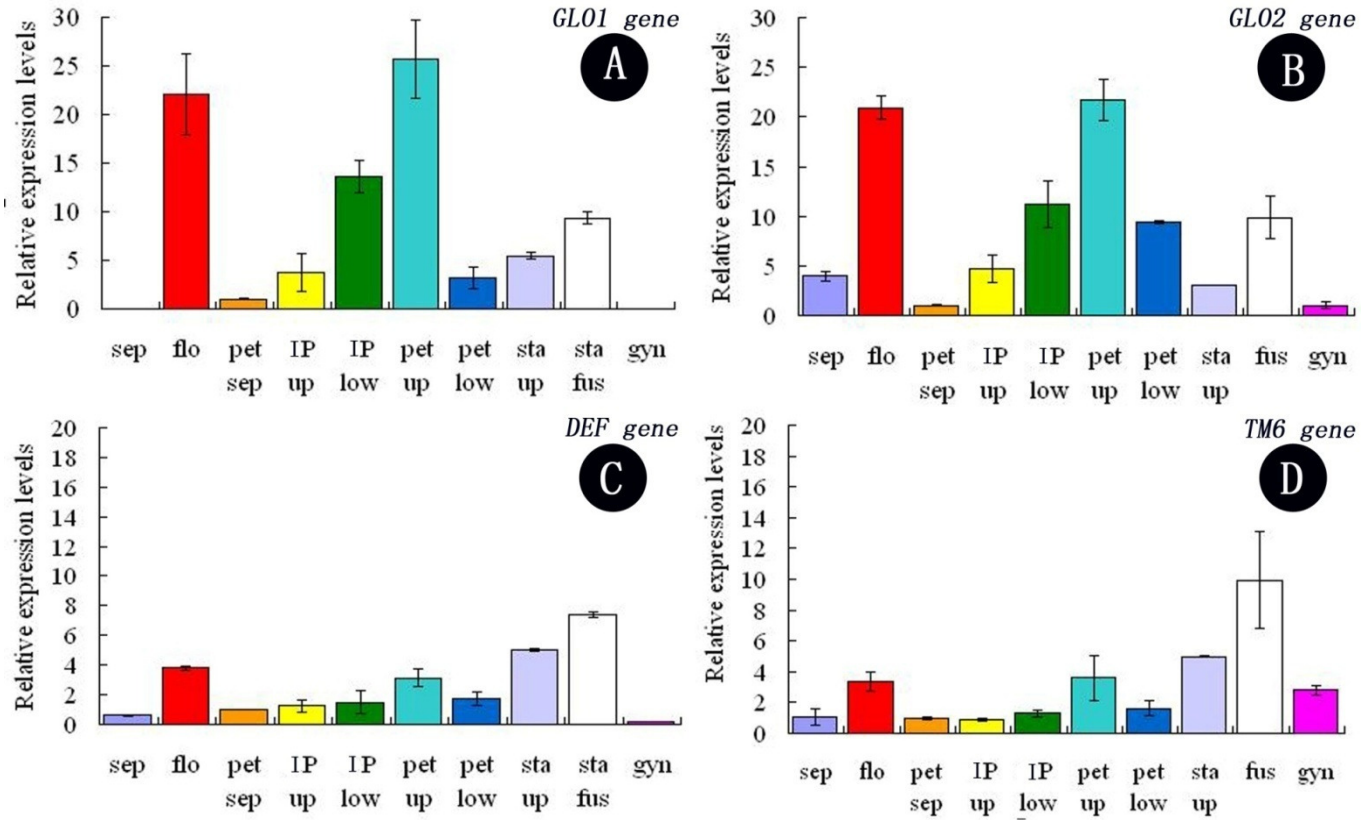

Figure 3. Real-time PCR expression data for B-function genes in floral organ of *C. changii* Ye. Sep = sepals, flo = flower, pet sep = petaloid sepals, IP up = upper part of imperfect petals, IP low = lower part of imperfect petals, pet up = upper part of true petals, pet low = lower part of true petals, sta up = upper part of stamens, sta fus = fused part of stamens, gyn = gynoecium. The relative expression level was related to tendency of gene expression when the pet sep was "1". The relative expression was calculated as: $2^{-\Delta Ct}=2^{-(Ct,t-Ct,r)}$. Ct: cycle threshold; Ct,t: cycle threshold of target gene; Ct, r: cycle threshold of 18S rRNA (endogenous control), and the error bar indicates the SD.

used to identify petal or sepal identities. PI gene had high expression at the upper part of perfect petals, which inferred that they specified petal identity. *GLO1* gene had no expression in normal sepals and high expression in petaloid sepals, which suggested that petaloid sepals had partial petal identity.

DEF and *TM6* gene had lower expression than *PI* genes. Though, they had the same rules as *PI* gene expression in upper and lower part of the imperfect and perfect petals, the expression level in the lower part of imperfect petals was higher than that of upper part and the level in the upper part of perfect petals was higher than that in lower part. In petaloid and normal sepals, the expression levels of *DEF* and *TM6* genes were almost the same, but both levels were high in the fused part of stamens, which implied the gene's importance in the stamens transform into petals.

DISCUSSION

In the Theaceae, sepals frequently changed into petals (Zhang and Ren, 1998) and these phenomenon was also found in other species (Zhao and Liu, 2009), such as *Mirabilis jalapa* 'Ercenglou' (Cheng, 2001). The petaloid sepals in *Aquilegia* do not depend on B-class genes for their identity (Kramer, 2009), but the petaloid sepals and stamens in *C. changii* Ye were the origin of double flowers. The petaloid sepals or stamens were probably a phenotype or transition when sepals or stamens transformed into petals. Petaloid organs started from the bottom of sepals and near the perfect petal. Their color turned from green to red and expanded upward. Anthocyaninin distributed clearly in the middle of some sepals, probably because the middle part bumped and absorbed so much light. The stamens started petaloid that anthers transformed into petals and filaments gradually grew broader and thicker.

This is a preliminary study of B-function gene in *C. changii* Ye. They had the same expression as those in *C. japonica* subs. *rusticana* which had no petaloid organs. In a number of core eudicots, B-function gene had been found to be responsible for the development of petals and stamens in the second and third whorls of flowers

Among other species, mutation in both *PI* and *AP3* genes caused homeotic changes in the second and third whorls, causing petals mutate into sepals and staments into carpels (Jack et al., 1992; Goto et al., 1994). In our research, *GLO1* and *GLO2* genes had high expression at the part with petal identity and *DEF* and *TM6* gene had high expression at the fused part of stamens. It suggested that B-function genes might have a key role in the morphogenesis of sepals and stamens transforming into double flowers in *C. changii* Ye. In addition, the interactions among different functions that controlled the development of floral organs determined the floral organs and other properties such as blooming period (Huala et al., 1992; Tröbner et al., 1992; Ng et al., 2001). Therefore, further researches on B-function gene will help to uncover the reason why *C. changii* Ye blossoms every season.

Wild *C. changii* Ye has single flowers with 5 to 6 petals. Though in the data of Zhang et al. (Zhang and Ren, 1998), there were no petaloid organs in *C. changii* Ye, the reasons might be that firstly, petaloid organs were common in *camellia*; secondly, most of the *C. changii* Ye were single flowers with 5 to 6 perfect petals, so the petaloid sepals were not counted as petals. In our results however, petaloid flowers accounted for the most part (55.16%). Petaloid organs caused by normal sepals have both sepal and petal identities and in most cases, the organs with petal identity were larger than those with sepal identity. Whether such organs should be counted as sepals or petals is yet to be discussed. We reported the discovery of double *C. changii* Ye and the formation of double flowers was a sepal origin. However, as stamens became petaloid in double flowers, it might have other origin mode: stamens-carpels origin. It is exactly this complicated phenotype that forms a splendid and rich camellia world and makes researches on the origin of double-flower camellia more and more difficulty.

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