Biochemical and Biophysical Research Communications 459 (2015) 450–456

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of flavonoids and expression of flavonoid biosynthetic genes in two coloured tree peony flowers

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ARTICLE INFO

Article history:
Received 18 February 2015
Available online 3 March 2015

Keywords:
Tree peony
Flavonoids
Anthocyanins
Red
Gene expression

ABSTRACT

Tree peony (*Paeonia suffruticosa* Andr.) has been named the "king of flowers" because of its elegant and gorgeous flower colour. Among these colours, the molecular mechanisms of white formation and how white turned to red in *P. suffruticosa* is little known. In this study, flower colour variables, flavonoid accumulation and expression of flavonoid biosynthetic genes of white ('Xueta') and red ('Caihui') *P. suffruticosa* were investigated. The results showed that the flower colours of both cultivars were gradually deepened with the development of flowers. Moreover, two anthoxanthin compositions apigenin 7-*O*-glucoside together with apigenin deoxyheso-hexoside were identified in 'Xueta' and 'Caihui', but one main anthocyanin composition peonidin 3,5-di-*O*-glucoside (Pn3G5G) was only found in 'Caihui'. Total contents of anthocyanins in 'Caihui' was increased during flower development, and the same trend was presented in anthoxanthins and flavonoids of these two cultivars, but the contents of these two category flavonoid in 'Caihui' were always higher than those in 'Xueta'. Furthermore, nine structural genes in flavonoid biosynthetic pathway were isolated including the full-length cDNAs of phenylalanine ammonialyase gene (*PAL*), chalcone synthase gene (*CHS*) and chalcone isomerase gene (*CHI*), together with the partial-length cDNAs of flavanone 3-hydroxylase gene (*F3H*), flavonoid 3'-hydroxylase gene (*F3′H*), dihydroflavonol 4-reductase gene (*DFR*), anthocyanidin synthase gene (*ANS*), UDP-glucose: flavonoid 3-O-glucosyltransferase gene (*UF3GT*) and UDP-glucose: flavonoid 5-O-glucosyltransferase gene (*UF5GT*), and *PAL*, *UF3GT* and *UF5GT* were reported in *P. suffruticosa* for the first time. Their expression patterns showed that transcription levels of downstream genes in 'Caihui' were basically higher than those in 'Xueta', especially *PsDFR* and *PsANS*, suggesting that these two genes may play a key role in the anthocyanin biosynthesis which resulted in the shift from white to red in flowers. These results would provide a better understanding of the underlying molecular mechanisms of flower pigmentation in *P. suffruticosa*.

1. Introduction

Tree peony (*Paeonia suffruticosa* Andr.) is a rare ornamental plant native to China with more than 1600 years history of cultivation until now. As an outstanding representative of Chinese traditionally famous flowers, *P. suffruticosa* is elegant and its flower colour is gorgeous, which is very popular both at home and abroad [1]. Moreover, as the main ornamental characteristics of *P. suffruticosa*, the flower colour is rich in diversity which can be divided into nine categories including white, pink, red, purple, black, blue, green, yellow and double colour. Among these colours, white is crystal clear, noble and elegant which can particularly win the hearts of girls, and it is also a excellent material for transgenic breeding of *P. suffruticosa* flower with other colour varities. In addition, red is festive and auspicious and its varieties are in the majority [2]. Until now, lots of studies have been thoroughly performed concentrating on *P. suffruticosa* germplasm resources [3], system evolution [4,5], plant disease [6,7] and cultivation physiology [8,9]. And some researches also have been conducted on the qualitative and quantitative analysis of pigment in *P. suffruticosa* white petals [10–12], but the underlying molecular mechanisms especially how white turns to festive red have not been fully elucidated yet till now.

For ornamental plants, flower colour is an important quality determinant which not only affects the ornamental merit but also directly influences their commercial values. In *P. suffruticosa*, the

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http://dx.doi.org/10.1016/j.bbrc.2015.02.126
0006-291X/

chemical constituents of petal colour had been studied systematically, and the colour was determined by flavonoids including anthocyanins and multiform glycosides of flavones and flavonols [2,10–13]. Wang et al. [11] analysed anthocyanins of white Zhongyuan of *P. suffruticosa* cultivars, six anthocyanins including peonidin 3-*O*-glucoside (Pn3G), peonidin 3,5-di-*O*-glucoside (Pn3G5G), cyanidin 3-*O*-glucoside (Cy3G), cyanidin 3,5-di-*O*-glucoside (Cy3G5G), pelargonidin 3-*O*-glucoside (Pg3G) and pelargonidin 3,5-di-*O*-glucoside (Pg3G5G), and six anthoxanthin glycosides were identified. Whereas Fan et al. [12] found only few anthocyanins, and their composition could not be characterized. Also flavonoids in red series *P. suffruticosa* cultivars were analyzed in more detail. All these informations provided a physiological and biochemical basis to investigate the molecular mechanisms of white formation and how white turns to red in the flower petal of *P. suffruticosa*.

Flavonoids formation and accumulation originated from flavonoid biosynthetic pathway has been well characterized, and extensive studies have also been carried on related enzymes and genes [14]. Up till now, this metabolic pathway has been well studied in *Helianthus annuus* [15], *Chrysanthemum grandiflorum* [16], *Paeonia lactiflora* [17] and other ornamental plants. In *P. suffruticosa*, Zhou et al. [18–21] firstly isolated *PsCHS*, *PsCHI* and *PsDFR*, and investigated their functions. Subsequently, the full-lengths of six structural genes including *PsCHS*, *PsCHI*, *PsF3H*, *PsF3′H* and *PsDFR* were cloned and the putative flavonoid biosynthetic pathway was predicted and summarize by Zhang et al. [22], which were used to reveal the molecular mechanism of colour fading of in-vase *P. suffruticosa* 'Luoyang Hong' flowers comparing with on-tree ones in field conditions. This report clarified the molecular mechanism of red degree transfromation from dark to light, but not shift from red to white in *P. suffruticosa*. Unlike the model plant *Arabidopisis thaliana*, it was very difficult to find a flower colour mutation in *P. suffruticosa*. Therefore, in order to clarifiy the molecular mechanisms of white formation and how white turns to red in *P. suffruticosa*, two representative cultivars 'Xueta' (white) and 'Caihui' (red) were selected to measure flower colour variables, determine flavonoid accumulation with high-performance liquid chromatograph-electrospray ionization-mass spectrometry (HPLC-ESI-MS^n), isolate nine structural genes involved in flavonoid biosynthetic pathway by rapid amplification of cDNA ends (RACE) and reverse-transcription polymerase chain reaction (RT-PCR) technologies, and investigate their expression patterns using real-time quantitative polymerase chain reaction (Q-PCR). These findings could enrich the understanding of flower pigmentation in *P. suffruticosa*.

2. Materials and methods

2.1. Plant materials

Tree peony was grown in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32°30′ N, 119°25′ E). Two cultivars based on their flower colour phenotypes, including a white cultivar 'Xueta' and a red one 'Caihui' were used as the plant materials. The young leaves were used for gene isolation and the petals were used for flavonoid and gene expression analysis. After measurement of flower quality and colour indices, all samples were immediately frozen in liquid nitrogen, and then stored at −80 °C until analysis.

2.2. Colour indices measurement

The colour of fresh petals were measured on a TC-P2A chroma meter (Beijing Optical Instrument Factory, China) using three colour parameters including L^*, a^* and b^* values. The hue angle (H° = arctangent (b^*/a^*)) were calculated according to the methods reported previously [23,24].

2.3. Qualitative and quantitative analysis of flavonoids

Flavonoid analysis was performed according to the method of [25] and He et al. [26] with some modifications. The petals of each sample (1.0 g fresh weight) were extracted with 6 mL of acidic methanol solution (70: 0.1: 29.9; v/v/v, CH_3OH: HCl: H_2O) at 4 °C for 24 h. Qualitative and quantitative analysis of flavonoids was performed using HPLC-ESI-MS^n (LCQ Deca XP MAX, Thermo) coupled with photodiode array and mass spectrometry detectors (HPLC-PDA-MS, Thermo company) with a three-dimensional quadrupole ion trap mass spectrometer. The HPLC column was TSK gel ODS-80Ts QA (4.6 mm × 250 mm) (Tosoh, Japan). The specific conditions were the same as the report of Zhao et al. [27]. Each peak area of anthocyanins and anthoxanthins detected under 525 nm and 350 nm was recored. Additionally, total contents of flavonoids were the sum of anthocyanins and anthoxanthins.

2.4. Isolation of flavonoid biosynthetic genes and sequencing

Total RNA was extracted using a modified CTAB extraction protocol [31]. *PAL*, *CHS* and *CHI* genes were isolated by RACE technology, which was performed using 3′ and 5′ full RACE Core Set Ver. 2.0 (TaKaRa, Japan) with specific primers (Supplemental Table 1). *F3H*, *F3′H*, *DFR*, *ANS*, *UF3GT* and *UF5GT* genes were isolated by RT-PCR technology, which was performed using a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) with specific primers (Supplemental Table 1). PCR products were separated by 1% agarose gel electrophoresis and sequenced.

2.5. Gene expression analysis

The transcript levels of genes were analysed using real-time quantitative polymerase chain reaction (Q-PCR) with a BIO-RAD CFX96™ Real-Time System (Bio-Rad, USA). The cDNA was synthesized using PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan). The *P. suffruticosa β-Tubulin* (EF608942) was used as an internal control. All gene-specific primers for Q-PCR were shown in Supplemental Table 2. Q-PCR was performed using the SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan), and relative expression levels were calculated by the $2^{-\triangle\triangle Ct}$ comparative threshold cycle (Ct) method [28], and the expression level of *PAL* in S1 of 'Xueta' was used as control.

2.6. Sequence and statistical analysis

Sequences splicing and analysis were performed by DNAMAN 5.0 software. Homology search was carried out using the GenBank BLAST. All data were average values of three replicates at least with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA).

3. Results

3.1. Flower quality and colour indices

Two cultivars of *P. suffruticosa* with two different flower colours (white, 'Xueta'; red, 'Caihui') were selected as the materials, and their flower diameter and flower fresh weight between 'Xueta' and 'Caihui' were shown in Fig. 1. During flower development, these two indices both gradually increased and reached maximum values in

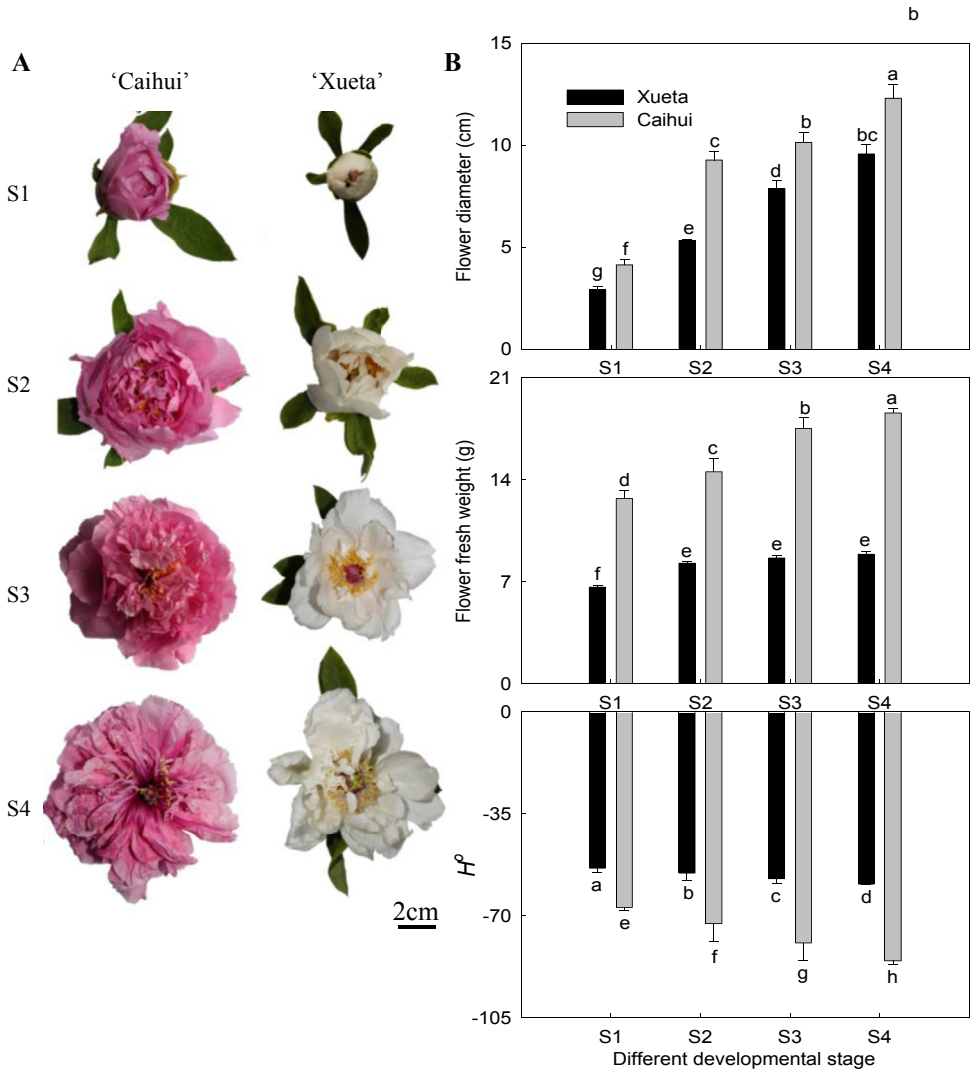

Fig. 1. Flowers of *P. suffruticosa* (A) and their flower qualitative traits (B). S1 (Stage 1): flower-bud stage; S2 (Stage 2): initiating bloom stage; S3 (Stage 3): bloom stage; S4 (Stage 4): wither stage. Different letters indicate significant differences ($P < 0.05$).

S4. and 'Caihui' was always larger than that of 'Xueta' in each stage. Moreover, their colour variables were also measured, which were expressed as H°. The value of H° was as follows: 0° for reddish-purple, 90° for yellow, 180° for bluish-green and 270° for blue [23,24]. During flower development, H° of 'Xueta' and 'Caihui' both decreased from −53.67° and −67.16° to −59.14° and −85.46°, respectively, and the former was always higher than the latter. These data showed that flower colour trends were consistent with the visual results. As flower development proceeded, the flower colour gradually deepened for plants.

3.2. Qualitative and quantitative analysis of flavonoids

Subsequently, composition of flavonoids in *P. suffruticosa* was detected and identified using HPLC-ESI-MS^n and their contents were analyzed. On the basis of the ultraviolet–visible absorption characteristics, anthocyanins and anthoxanthins were detected under the wavelength of 525 nm and 350 nm, respectively. As shown in Fig. 2, there were some differences in the chromatographic peaks of 'Xueta' and 'Caihui'. At 525 nm, no anthocyanin was found in 'Xueta', while there was an obvious peak (a1) was identified in 'Caihui'. Its maximum absorption wavelength (λ_{max}) was 287 and 515 nm, $[M+H]^+$ m/z was 625, where its fragment ions were m/z 463 and 301. In addition, m/z 301 was a peonidin derivative, which was formed by m/z 625 losing neutral fragments of two glucoses (mass number 162), indicating that this compound was a peonidin derivative with two glucoses. A comparison of the spectral and mass spectrum data reported in the literatures identified peak a1 as Pn3G5G [12]. At 350 nm, there were many same and different peaks were detected in these two cultivars, but only two peaks (f1 and f2) in them were identified. Using the same method, peaks f1 (λ_{max} 265 and 340 nm, $[M+H]^+$ (m/z) 433, MS^2 (m/z) 271) and f2 (λ_{max} 267 nm, $[M+H]^+$ (m/z) 338, MS^2 (m/z) 433 and 271) were characterized as apigenin 7-*O*-glucoside [29] apigenin deoxyhesohexoside [12].

Mv3G5G and rutin were used as the references for the relative quantitative analysis of anthocyanins and anthoxanthins, respectively. The results showed that there were significant differences in both two different cultivars and also their different developmental stages (Fig. 4). For anthocyanins, its content in 'Xueta' was basically under the limit of detection, in contrast, its content of 'Caihui' in S1 was 0.20 mg/g and gradually increased along with the development of flower, as well as reached to 0.31 mg/g in S4 which was increased 55% than that in S1. Concerning

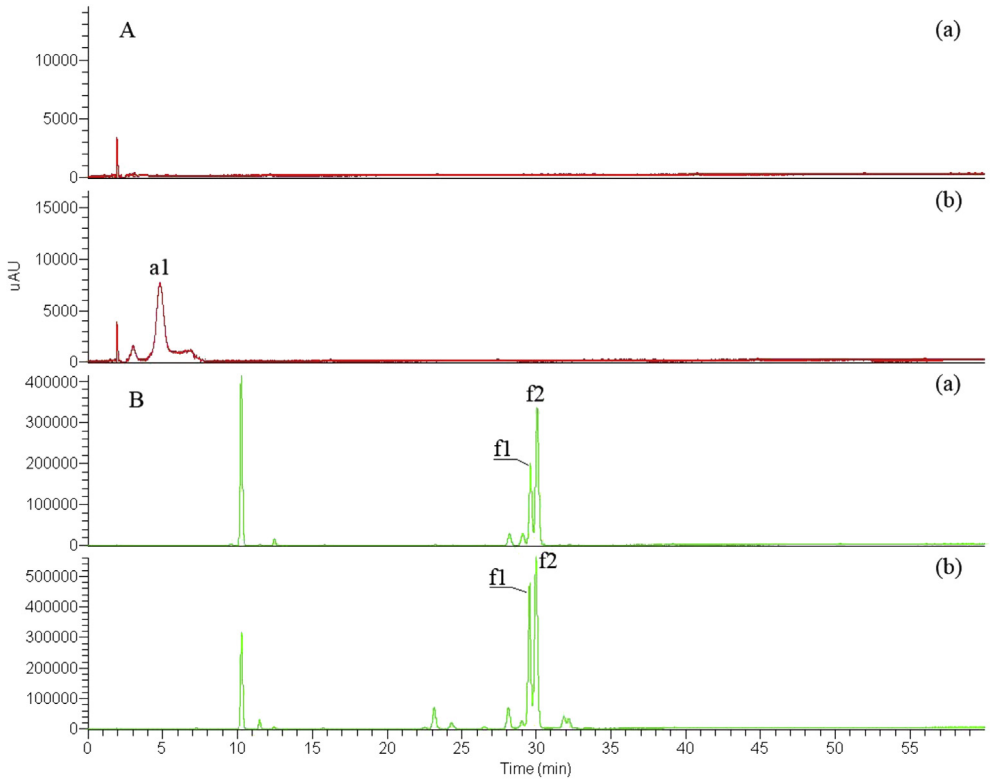

Fig. 2. HPLC chromatograms of *P. suffruticosa* anthocyanins (A, detected at 525 nm) and anthoxanthins (B, detected at 350 nm) in S1. (a) indicates HPLC chromatograms of 'Xueta'; (b) indicates HPLC chromatograms of 'Caihui'. a1 indicates identified anthocyanin; f1and f2 indicate identified anthoxanthins.

anthoxanthins, its content in 'Xueta' and 'Caihui' presented a similarly increasing tendency with flower development, which rose from 2.98 mg/g and 5.30 mg/g in S1 to 3.46 mg/g and 6.08 mg/g in S4, respectively. Moreover, the content of anthoxanthins in 'Caihui' was about 70% higher than that in 'Xueta' during the whole development of flower. As far as flavonoids was concerned, its total content in 'Xueta' and 'Caihui' all presented an upward trend along with flower development,

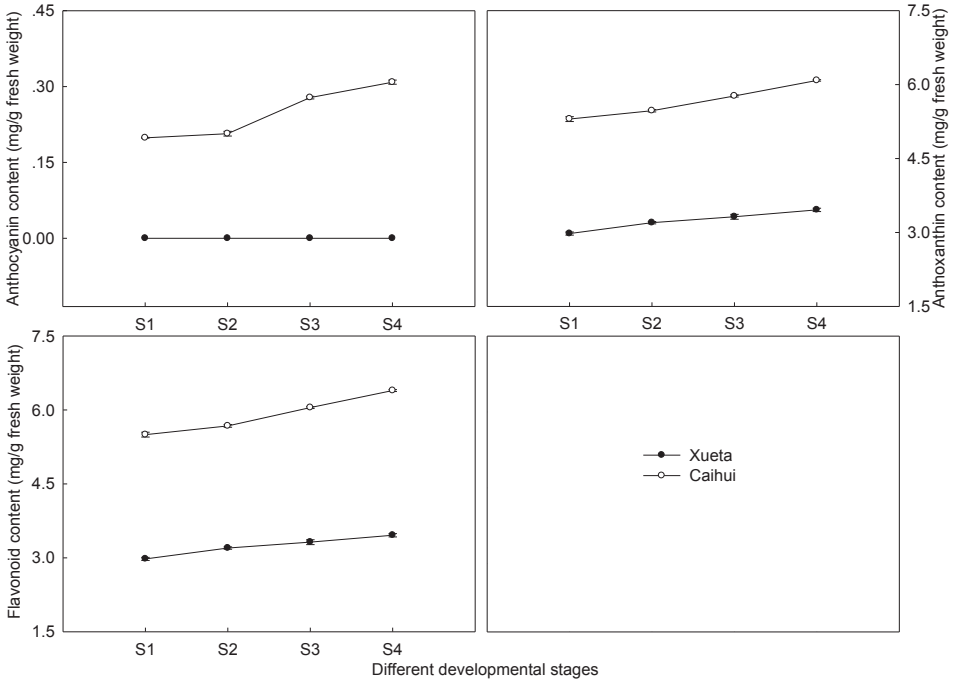

Fig. 3. Total contents of anthocyanins, anthoxanthins and flavonoids in four developmental stages.

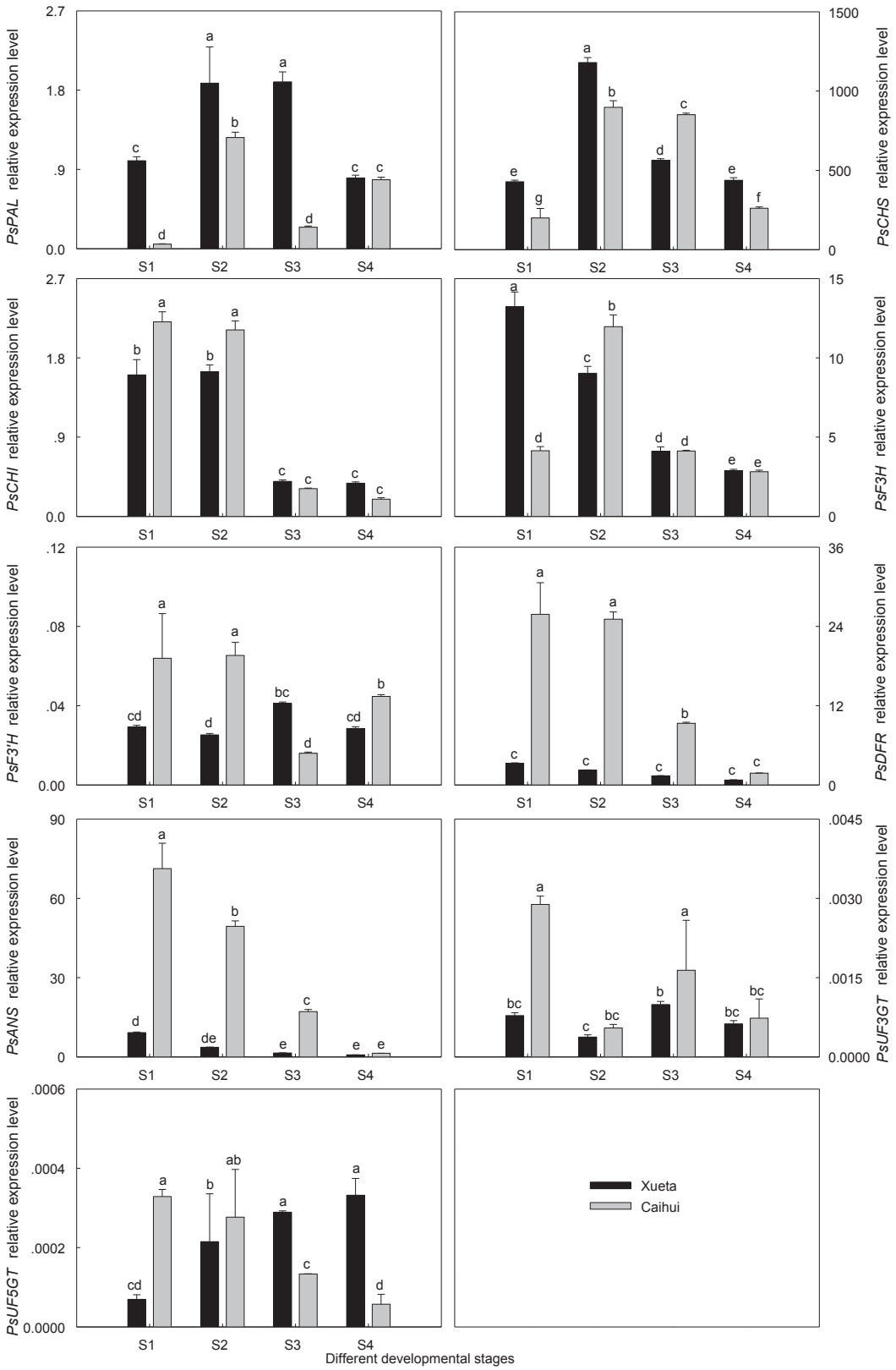

Fig. 4. Expression analysis of flavonoid biosynthetic genes from *P. suffruticosa* in four developmental stages. Different letters indicate significant differences ($P < 0.05$).

and reached their maximum in S4 with 3.46 mg/g and 6.39 mg/g, respectively, which were both approximately 1.16 times of those in S1. Meanwhile, compared with 'Xueta', total content of flavonoids in 'Caihui' was always higher with the average value of 2.67 mg/g.

3.3. Isolation and sequence analysis of flavonoid biosynthetic genes

To investigate the formation mechanisms of white and red colour of *P. suffruticosa* petals at the molecular level, nine structural genes in flavonoid biosynthetic pathway including *PsPAL*, *PsCHS*, *PsCHI*, *PsF3H*, *PsF3′H*, *PsDFR*, *PsANS*, *PsUF3GT* and *PsUF5GT* were isolated (Supplemental Table 3). Among these genes, the full-length sequence cDNA of *PsPAL* was 2412 bp, and contained an open reading frame (ORF) of 2013 bp, an untranslated region (UTR) of 198 bp in 5′ end, a 3′-UTR of 201 bp and a complete Poly A tail. Similarly, the 1459 bp *PsCHS* cDNA contained an 1185 bp ORF encoding a 394 amino acid protein. The 895 bp *PsCHI* cDNA contained a 654 bp ORF encoding a 217 amino acid protein. These three genes had been deposited in GenBank with the accession numbers KM871191, JN105300 and JN105297, respectively. However, only the partial-length sequence of *PsF3H*, *PsF3′H*, *PsDFR*, *PsANS*, *PsUF3GT* and *PsUF5GT* were obtained. The 783 bp *PsF3H* fragment contained a part ORF of 783 bp encoding a 260 amino acid protein. The 756 bp *PsF3′H* fragment contained a part ORF of 756 bp encoding a 252 amino acid protein. The 284 bp *PsDFR* fragment contained a part ORF of 195 bp encoding a 64 amino acid protein. The 626 bp *PsANS* fragment contained a part ORF of 570 bp encoding a 189 amino acid protein. The 1438 bp *PsUF3GT* fragment contained a part ORF of 1438 bp encoding a 479 amino acid protein. The 1094 bp *PsUF5GT* fragment contained a part ORF of 1094 bp encoding a 364 amino acid protein. These six genes had also been deposited in GenBank with accession numbers KM871192, KM871195, KM871193, KM871194, KM871196 and KM871197, respectively. Their proteins all shared high identity and similarity with related proteins from other plants (Supplemental Table 4).

3.4. Expression analysis of flavonoid biosynthetic genes

Expression patterns of flavonoid biosynthetic genes were performed using Q-PCR with the same samples used in the qualitative and quantitative analysis of flavonoids. The results revealed that all flavonoid biosynthetic genes transcripts were all detected in four different developmental stages, but their expression levels were different from each other (Fig. 4). Among these nine genes, the expression level of *PsCHS* was the highest, downstream *PsUF3GT* and *PsUF5GT* exhibited the lowest expression levels, while those of other genes were in between them. In terms of the combination of genes and flower developmental stages, expression patterns in 'Xueta' and 'Caihui' showed similar evolution tendency except *PsF3′H*, *PsUF3GT* and *PsUF5GT*. For example, the expression levels of *PsCHI*, *PsF3H*, *PsDFR* and *PsANS* in these two cultivars presented a downward trend basically, while *PsPAL* and *PsCHS* reached the maximum level in S2 and then decreased. For *PsF3′H*, *PsUF3GT* and *PsUF5GT*, their expression levels in 'Caihui' also presented a downward trend basically, *PsUF5GT* in 'Xueta' presented an upward trend, but *PsF3′H* and *PsUF3GT* in 'Xueta' presented oscillating state. For white petals in 'Xueta', both *PsDFR* manipulating the formation of leucoanthocyanidin and *PsANS* controlling the formation of anthocyanidin had lower expression levels compared to the 'Caihui' in the four different developmental stages, whereas *PsPAL* and *PsCHS* showed an opposite trend which basically presented higher expression levels than those in 'Caihui'.

4. Discussion

Flower is an important organs in ornamental plants, its quality changes with certain regularity during its development. When colour variable was concerned, the petal colour of red cotton flower deepened dramatically after blooming [30], and in *P. lactiflora*, its petal colour in different blooming stages was measured by the Royal Horticultural Society Colour Chart (R.H.S.C.C.), and its corresponding colour was changed from red-purple to violet [31], which had been proved by Zhao et al. [17]. In this study, the petal colour of *P. suffruticosa* with white and red flowers presented a contrary tendency with *P. lactiflora*, namely its colour deepened gradually as the flower developed which was in line with the report of Zhou et al. [18]. This result may tell us that the law of growth and development was not the same in different plants, and even close relative plants also had certain specificity in themselves.

Anthocyanins is the stable form of anthocyanidins, and their differences were one of the important reasons for the development of a variety of colours. In *P. suffruticosa*, the pink and red flower colours were mainly determined by pelargonidin derivatives, while pinkish-blue, reddish-purple, reddish-black and purple flower colours contained mainly peonidin derivatives as the core anthocyanins [12]. Moreover, Cy3G5G, Pn3G5G and Pn3G were found in 'Caihui' categorized as pinkish-blue, and their relative abundance of individual anthocyanins were 4.90%, 90.50% and 4.60%, respectively [2,12]. Our result was consistent with previous studies, but only one main anthocyanin composition Pn3G5G and two anthoxanthin compositions apigenin 7-*O*-glucoside together with apigenin deoxyheso-hexoside were identified, and a few of flavonoid compounds in the HPLC chromatograms (Fig. 3) are not characterized because of low signals in the HPLC-PDA-MS detector or confused mass spectra data in our current analytical method and conditions. However, apigenin 7-*O*-glucoside and apigenin deoxyheso-hexoside also had been found in white petals of 'Xueta', but no anthocyanins were observed which was also in line with previous studies [12], which suggested that anthocyanins were the critical influencing factor for the performance of red petals in *P. suffruticosa*. But in the meantime, the changes of anthocyanin content during the development of flower also affected the formation of flower colour. Zhao et al. [17,27] found that the anthocyanin content of *P. lactiflora* petals was basically in decline with its constantly bleached colour. And in *P. suffruticosa*, previous studies all showed that accumulated amounts of anthocyanins in petals were increased during its development [22,18], which was also proved by our investigation.

Flavonoid biosynthesis is manipulated by a series of structural and regulatory genes, among which, the expression of structural genes is directly associated with flavonoid biosynthesis and accumulation [32]. In this study, nine structural genes in flavonoid biosynthetic pathway including *PsPAL*, *PsCHS*, *PsCHI*, *PsF3H*, *PsF3′H*, *PsDFR*, *PsANS*, *PsUF3GT* and *PsUF5GT* were isolated firstly. And their deduced proteins all had high similarities to those of other plants, especially these nine genes in *P. lactiflora* and five genes including *PslCHI*, *PsF3H*, *PsF3′H*, *PsDFR*, *PsANS* in *P. suffruticosa*. However, an interesting phenomenon was also found as previous studies that the obtained sequences in our study were not exactly the same with those sequences of *P. suffruticosa* deposited in GenBank, which might be caused by cultivar difference [22]. Subsequently, the expression patterns of these nine genes were analyzed, and their transcription levels were different for each other. In *Senecio cruentus*, the difference between white and coloured flowers was mainly in whether *ScCHS* gene expression or not [33]. Meanwhile In *P. lactiflora*, the expression levels of *PlPAL*, *PlCHS*, *PlCHI*, *PlF3H*, *PlF3′H*, *PlDFR*, *PlANS*, *PlUF3GT* and *PlUF5GT* in white flowers were all lower than those in red flowers, especially *PlDFR*, *PlANS* and

PlUF3GT which induced the formation of a large amount of coloured anthocyanins from anthoxanthins [17]. In this study, the expression levels of *PsDFR* and *PsANS* in 'Caihui' were much higher than those in 'Xueta', which induced changing a proportion of anthoxanthins to anthocyanins and displaying red. This result was similar to those in *P. lactiflora* [17]. But for different development stages, the expression levels of *PsCHI*, *PsF3H*, *PsDFR* and *PsANS* in two cultivars all showed the maximum level in the first stage and then decreased basically, which was not always correlated with their flavonoid contents. This result had some similarities with those in previous reports, which was speculated that high expression levels of these genes defined as the early stage genes would provide enough substrates for the later accumulation of flavonoids and anthocyanins during early stages, and in later stages, these genes might act as triggers for flavonoid and anthocyanin biosynthesis whose slight expression levels might be enough to induce flavonoid and anthocyanin accumulation [21,22]. On the whole, the results of this study would provide a better understanding the underlying molecular mechanisms of flower pigmentation in *P. suffruticosa*.

Conflict of interest

None.

Acknowledgments

This work was supported by the Agricultural Science & Technology Independent Innovation Fund of Jiangsu Province (CX[14] 2135) and the Priority Academic Program Development from Jiangsu Government.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.126.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.126.

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