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Full Length Article

Prohexadione-calcium Modified Color Characteristics and Flavonoid Biosynthetic Genes Expression in Herbaceous Peony (*Paeonia lactiflora*) Flowers

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Abstract

Prohexadione-calcium (Pro-Ca), originally developed as a plant growth retardant, has recently been discovered it could modify floral color in several ornamental plants like petunia and rose. However, the underlying mechanism of Pro-Ca on the regulation of floral color is still elusive. In this study, a purple-red herbaceous peony (*Paeonia lactiflora* Pall.) cultivar with control and Pro-Ca treatment was used as the material. Their color indices were measured, flavonoid compositions and contents as well as its biosynthetic genes expression were analyzed. After Pro-Ca treatment, a remarkable rise in the color parameter lightness (L^*) value along with an obvious fall in the color parameter red/green (a^*) value indicated that the loss of red color was occurred. In addition, total anthocyanin, anthoxanthin and flavonoid contents were all decreased significantly after Pro-Ca treatment, which was in accordance with the color indices. Furthermore, the expression patterns of most relative genes in flavonoid biosynthetic pathway expressed an overall decline after Pro-Ca treatment. Among the 2-oxoglutaric acid-dependent dioxygenase genes involved in the pathway, flavanone 3-hydroxylase gene (*F3H*) and anthocyanidin synthase gene (*ANS*) had the highest levels of expression, suggesting that the loss of red color might be the reason of the synergistic inhibitory effect of these enzymes especially *F3H* and *ANS* in response to Pro-Ca. These results would contribute to a theoretical foundation for the regulation of floral color in field *P. lactiflora* by Pro-Ca treatment. © 2017 Friends Science Publishers

Keywords: Herbaceous peony; Prohexadione-calcium; Flavonoids; Gene expression

Introduction

Prohexadione-calcium (Pro-Ca), originally developed as a plant growth retardant, has recently been used as a 2-oxoglutaric acid-dependent dioxygenase (2-ODD) inhibitor. It could lead to the inhibition of the activity of 2-ODDs for the reason that this competitive Pro-Ca to the 2-ODDs on the combination of substrates. Several 2-ODDs are involved in the late steps of gibberellins biosynthesis (Rademacher, 2000) and in the flavonoid biosynthesis (Halbwirth *et al.*, 2006). Thus Pro-Ca could bring about inhibition in these biosyntheses and contribute to multiple positive biochemical effects. Besides, Pro-Ca is an environmental-friendly and eco-toxicological bioregulator with no health risk for users and consumers (Rademacher and Kober, 2003) and would be easily absorbed and utilized by plants though spraying. Until now, the studies on Pro-Ca have been mainly about fruits and vegetables. For example, Pro-Ca had been used to take control of vegetative growth in apples (Duyvelshoff and Cline, 2013; Çetinbaş *et al.*, 2015) and eggplants (Ozbay and Ergun, 2015), induce resistance to fire blight in apples (McGrath *et al.*, 2009) and mitigate postharvest chilling injury in tomato fruits (Aghdam, 2013). In ornamental plants, Pro-Ca could promote resistance to root parasitic weed in sunflowers (Fan *et al.*, 2007) and affect the growth and gibberellins contents in *Chrysanthemum morofolium* (Kim *et al.*, 2010). Otherwise, Pro-Ca would modify the floral color in petunia, impatiens and rose (Ilias and Rajapakse, 2005; Schmitzer *et al.*, 2012). For example, after Pro-Ca treatment, the two rose cultivars with orange-red and dark-red colors turned into light pink and white. Also, the color loss was directly related to a decrease in anthocyanin content (Schmitzer *et al.*, 2012). Bizjak *et al.* (2012, 2013) found that the inhibition of Pro-Ca on enzyme activities of 2-ODDs flavanone 3-hydroxylase gene (*F3H*), flavonol synthase gene (*FLS*) and anthocyanidin synthase gene (*ANS*) in flavonoid biosynthesis pathway in the skin of apples might lead to a decrease in anthocyanin content. Up to now, few studies have focused on the effect of Pro-Ca treatment in ornamental plants, yet the detailed mechanism of how Pro-Ca would modify floral color is still not clear. Hence, a further study on the effect of Pro-Ca to explore its mechanism in flower coloration of ornamental plants is necessary.

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Herbaceous peony (*Paeonia lactiflora* Pall.) is a perennial herb belonging to the family Paeoniaceae. It is a traditional famous flower in China, endowed with relatively high ornamental and landscape value mainly for its colorful blossom. The expression of floral color was primarily determined by the production of pigments, especially by flavonoids which included anthocyanins and anthoxanthins (Zhao and Tao, 2015). Also, flavonoid compositions and contents are different in varied flowers in *P. lactiflora* (Jia *et al.*, 2008) and the accumulation of flavonoids is determined by a series of genes in the flavonoids biosynthesis pathway (Nakatsuka *et al.*, 2005). Previously, Zhao *et al.* (2012a) predicted and summarized the putative flavonoid biosynthesis pathway in *P. lactiflora*, including phenylalanine ammonialyase gene (*PAL*), chalcone synthase gene (*CHS*), chalcone isomerase gene (*CHI*), *F3H*, flavonoid 3'-hydroxylase gene (*F3'H*), dihydroflavonol 4-reductase gene (*DFR*), *ANS*, *UDP*-glucoside (*UGT*): flavonoid 3-*O*-glucosyltransferase gene (*F3GT*) as well as flavonoid 5-*O*-glucosyltransferase gene (*F5GT*) and methyl transferase gene (*MT*). In addition, *FLS* was also influenced the accumulation of flavonoids (Zhao *et al.*, 2016). At present, few studies have been focused on the regulation of *P. lactiflora* floral color and its formation mechanism in the field. In order to explore the effect of Pro-Ca on floral color of *P. lactiflora*, in this study, firstly, a purple-red cultivar 'Luhong' with control and Pro-Ca treatment was used as the material. Next, their color indices were measured. Then, qualitative and quantitative analysis of flavonoids was performed. Finally, the expression patterns of *PlPAL, PlCHS, PlF3H, PlF3'H, PlFLS*, *PlDFR*, *PlANS* and *PlF3GT* were analyzed. These results might establish a theoretical foundation for the regulation of floral color in *P. lactiflora* by Pro-Ca treatment.

Materials and Methods

Experimental Materials and Treatments

Experimental materials: Experiments were performed in the fields of the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, China (32°23′31" N, 119°24′50" E) under nature state. A purple-red *P. lactiflora* cultivar 'Luhong' was selected. It was transplanted into eight rows (three plants per row) in the germplasm repository in 2009 and grown in a natural state ever since. The petals separated from the flowers were used as the experiment material.

Treatments: The pretest study was performed in 2014 and we received the most effective concentration at a 1,000 ppm solution of Pro-Ca (90% BR; Shanghai yuanye Bio-Technology Co., Ltd). In 2015, within all eight rows, four rows of plants were given solutions of Pro-Ca at 1000 ppm. Another four rows of plants received a spray of distilled water. Both two groups were sprayed weekly from March 30th to April 27th at dusk in total of five times. The flowers of each group at three developmental stage: stage 1 (S1), flower-bud stage; stage 2 (S2), initiating bloom stage; stage 3 (S3), bloom stage bloom stage were collected from May 8th to May 20th respectively. Petals were separated from the flowers as the samples after each collection. Subsequently, ten random of petals were selected from each group in three developmental stages to measure the color indices – the color parameter lightness (L^*) and red/green (a^*) by a TC-P2A colorimeter (Beijing Optical Instrument Factory, China), and each petal were measured in the middle part to reduce errors. Then samples were quickly frozen with liquid nitrogen and stored at -80°C for further study.

Qualitative and Quantitative Analysis of Flavonoids

The method of qualitative and quantitative analysis of flavonoids was the same as the report of Zhao *et al.* (2014) with some modifications. In brief, the petal powder (1.0 g fresh weight) of each sample was 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. The qualitative and quantitative analysis of flavonoids were performed by high-performance liquid chromatography-electrospray ionization-mass spectrometry (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). Cyanidin-3-*O*-glucoside (Cy3G) and rutin were used as reference for the relative quantitative analysis. Additionally, total content of flavonoids was the sum of anthocyanins and anthoxanthins. Three extractions were made for each biological sample.

Gene Expression Analysis

The relative expression levels of genes were analyzed using real-time quantitative polymerase chain reaction (Q-PCR) with a BIO-RAD CFX Connect ™ Real-Time System (Bio-Rad, USA). Total RNA was separately extracted from each sample under using Trizol Regent Kit (TaKaRa, Japan) according to the manufacturer's instructions with some modifications. All RNA samples purified with DNase I kit (TaKaRa, Japan). The cDNA was transcribed using a reverse transcription kit (TaKaRa, Japan) for real-time quantitative PCR. Finally, Q-PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems, USA) with three replicates. All gene-specific primers in this study are shown in Table 1. The Q-PCR efficiency of each gene was obtained by analyzing the standard curve of a cDNA serial dilution. Relative abundance was calculated with the $2^{-\Delta\Delta CT}$ method using *P. lactiflora* Actin (JN105299) for template normalization (Zhao *et al.*, 2012b).

Statistical Analysis

The comparison were made between the two group plants

(foliar sprayed with 1,000 ppm Pro-Ca solution and distilled water). Primers were designed using a Primer 5.0 program. Design of the experiments was completely randomized with three replications. All data were average values of three replicates at least with standard deviations to determine comparative responses of floral color with or without Pro-Ca spray. The results were statistically analyzed for variance using the SAS/STAT statistical analysis package (version 9.4, SAS Institute, Cary, NC, USA).

Results

Color Indices

The petals of 'Luhong' in plants with control and Pro-Ca treatment were selected as the materials in this study. Through comparative eyes, the floral color was turned lighter in plants with Pro-Ca treatment compared to the control (Fig. 1). To check the results more scientifically, a chroma meter was used. By measuring their color indices, the result of floral color was expressed as the color parameter L^* and the color parameter a^* values. As shown in Fig. 2, the parameter L^* value of both petals with control and Pro-Ca treatment presented a gradually upward trend during flower development, while the parameter a^* value presented a downward trend. In addition, after Pro-Ca treatment, the parameter L^* value was significantly increased by nearly 50% compared to the control in each developmental stage. Correspondingly, the parameter a^* value was markedly decreased after Pro-Ca treatment, especially in S3 that decreased to 55% compared to the control.

Identification of Flavonoid Compositions

Depending on the color indices, qualitative and quantative analysis of flavonoids was performed by HPLC-ESI-MS^n. Flavonoid compositions could be detected unambiguously from their ultraviolet-visible absorption characteristics. Anthocyanin and anthoxanthin compositions were separated by their different absorption bands at 525 nm and 350 nm, respectively. According to the HPLC profiles (Fig. 3), similar compositions of flavonoid were found in both petals with control and Pro-Ca treatment, but the peak area of each composition was not the same. Comparing with the control, all the peaks were obviously lower after Pro-Ca treatment, except a few peaks of anthoxanthins. Subsequently, five anthocyanins and seven anthoxanthins compositions were separated and characterized (Fig. 4). At 525 nm, the maximum absorption wavelength (λ_{max}) of the obvious peak a3 was 275 and 515 nm, $[M+H]^+$ was at m/z 625.22 with fragment ions at m/z 463.01 and 301.24. Fragments with an m/z of 301 represented a peonidin derivative and it was formed by m/z 625 that lost two fragment ions of 162 mass units. From the mass number of glucose 162, it was known that it was a peonidin derivative with two residues.

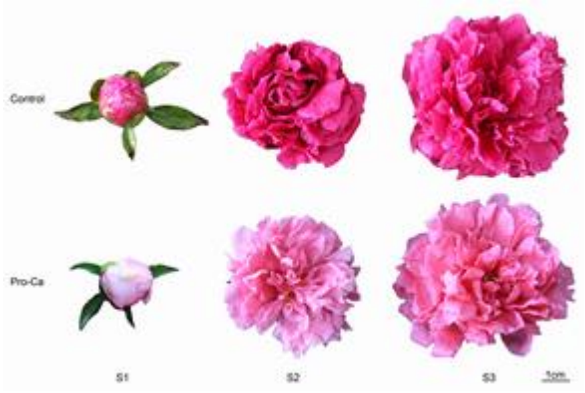

Fig. 1: *P. lactiflora* flowers with control and Pro-Ca treatment during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage

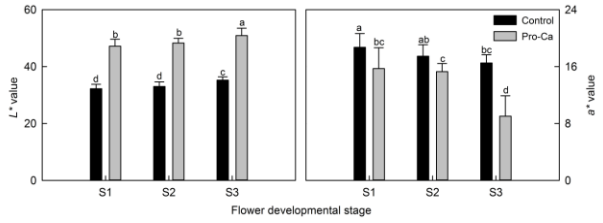

Fig. 2: Color indices of *P. lactiflora* petals with control and Pro-Ca treatment during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

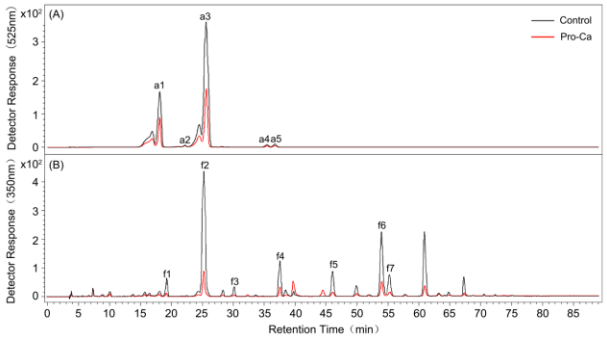

Fig. 3: HPLC chromatograms of *P. lactiflora* petals with control and Pro-Ca treatment in S1 (Stage 1 = flower-bud stage). (A) Anthocyanins, detected at 525 nm. (B) Anthoxanthins, detected at 350 nm. a1-a5 indicate identified anthocyanins; f1-f7 indicate identified anthoxanthins

By comparing the HPLC retention time, spectral and reported mass spectrum data, it was identified as peonidin-3,5-di-*O*-glucoside (Pn3G5G). Similarly, at

350 nm, the obvious peak f2 with λ_{max} of 265 and 345 nm, $[M+H]^+$ at m/z 611.15 with fragment ions at m/z 448.96 and 287.37, was detected as a kaempferol derivative. The neutral loss of two 162 mass units corresponded to two glucose molecules. By comparing the HPLC retention time, spectral and mass spectrum data with reported literatures, it was identified as kaempferol-3,7-di-*O*-glucoside (Km3G7G). Using the same method, peaks a1 (λ_{max} 275 and 515 nm, $[M+H]^+$ (m/z) 611.18, MS^2 (m/z) 448.99 and 287.29), a2 (λ_{max} 275 and 500 nm, $[M+H]^+$ (m/z) 595.21, MS^2 (m/z) 432.99 and 271.28), a4 (λ_{max} 275 and 515 nm, $[M+H]^+$ (m/z) 463.21, MS^2 (m/z) 301.27) , a5 (λ_{max} 275 and 515 nm, $[M+H]^+$ (m/z) 711.21, MS^2 (m/z) 549.03, 463.05 and 301.22), f1 (λ_{max} 260 and 355 nm, $[M+H]^+$ (m/z) 465.01, MS^2 (m/z) 303.43), f3 (λ_{max} 265 and 345 nm, $[M+H]^+$ (m/z) 697.18, MS^2 (m/z) 534.99, 448.96 and 287.33), f4 (λ_{max} 265 and 355 nm, $[M+H]^+$ (m/z) 465.28, MS^2 (m/z) 303.15), f5 (λ_{max} 265 and 355 nm, $[M+H]^+$ (m/z) 465.04, MS^2 (m/z) 303.11), f6 (λ_{max} 270 and 350 nm, $[M+H]^+$ (m/z) 601.29, MS^2 (m/z) 449.03 and 286.95), f7 (λ_{max} 265 and 365 nm, $[M+H]^+$ (m/z) 449.12, MS^2 (m/z) 287.29) were characterized as cyanidin-3, 5-di-*O*-glucoside (Cy3G5G), pelargonidin-3, 5-di-*O*-glucoside (Pg3G5G), peonidin-3-*O*-glucoside (Pn3G), peonidin-3-*O*-malonylglucoside-5-*O*-glucoside (Pn3MloG5G), quercetin-3, 7-di-*O*-galactoside (Qu3G7G), kaempferol-3-*O*-malonylglucoside-7-*O*-glucoside (Km3MloG7G), quercetin-3-*O*-galloyglucoside (Qu3GloG), quercetin-3-*O*-galactoside (Qu3G), kaempferol-3-*O*-galloyglucoside (Km3GloG) and kaempferol-7-*O*-glucoside (Km7G), respectively.

Quantitative Analysis of Flavonoids

Based on the references of Cy3G and rutin, the relative quantity of anthocyanin anthoxanthin compositions were analyzed. As shown in Fig. 5, the content of tentative anthocyanin and anthoxanthin compositions in petals with control and Pro-Ca treatment all basically showed a download trend during flower development and reached their minimum in S3. Meanwhile, compared to control, the content of each composition was basically lower in each stage with Pro-Ca treatment, except for a2 in S2. Besides, Cy3G5G and Pn3G5G were identified as the two major anthocyanin compositions, which were averagely accounted for 18.85 and 51.90% of total anthocyanin contents in control, 21.91 and 54.66% in Pro-Ca treatment. Concerning total contents, total anthocyanins, anthoxanthins and flavonoids in both were all in decline during flower development (Fig. 5). What's more, total contents, whether anthocyanins, anthoxanthins, were much lower in petals with Pro-Ca treatment, nearly fell up to average 50 and 70%. Taken together, total flavonoid content in control was approximately about average 2.8 times as much as those in Pro-Ca treatment.

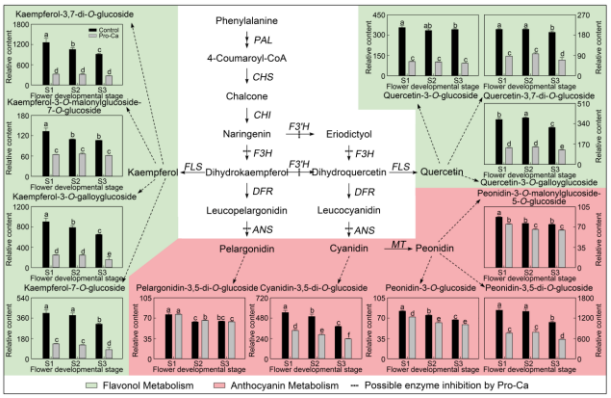

Fig. 4: Simplified schematic overview of the major flavonoid biosynthetic pathway in *P. lactiflora* and the contents ($\mu g \cdot g^{-1}$ FW) of tentative flavonoid compounds of petals with control and Pro-Ca treatment in three developmental stages. The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

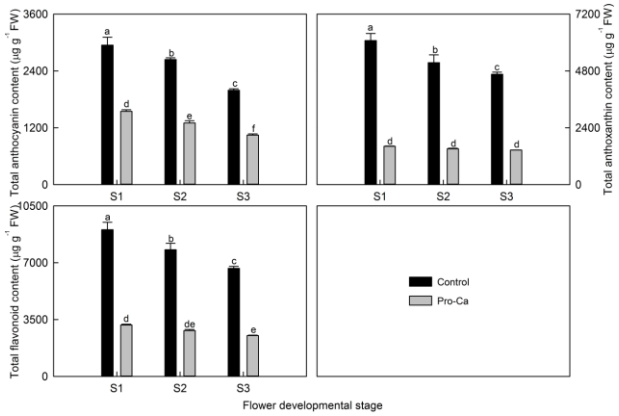

Fig. 5: Total anthocyanins, anthoxanthins and flavonoids content of *P. lactiflora* petals with control and Pro-Ca treatment during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

Expression Analysis of Flavonoid Biosynthetic Genes

To explore major genes influencing the flavonoids formation due to Pro-Ca treatment, the expression patterns of its biosynthetic genes were analyzed by Q-PCR. The samples were the same in qualitative and quantative analysis of flavonoids, and the result was shown in Fig. 6. From the point of the overall expression level, the expression level of upstream *PlCHS* was the highest, upstream *PlF3'H* and downstream *PlF3GT* were the lowest and the rest were in between.

Table 1: Gene-specific primers used in Q-PCR analysis

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
PAL	TGAGTCAAGTTGCCAAGAG	GCATTAAAGGGTAAGTAGCG
CHS	ATGACTGGAACTCGATACTCT	CTCACTCAAAACATGCCTAG
CHI	CAACTTTCCGAGGCTGTG	CTCCTTTGACCTTATCCATC
F3H	AAGGAAGCACTCACAAACG	GAAGACCACCCACCTGAT
F3'H	GGCGAGAAACCAAACATT	CCATCCGTAGCCCTAAACT
FLS	GCTATCTCATCCGCACAA	CTTCAAACTTTCCTCTACCAG
DFR	GCACTTTCTCCAATCACAG	CAAATGTAGCGACCCTCT
ANS	AGATTAGAACAAGAAGTCGGTG	GGAGGATGAAAGTGAGGG
Actin	GTTGCCCTTGATTACGAG	CAGCTTCCATTCCGATTA

Table 2: Retention time, ultraviolet-visible spectral properties, mass spectrometric data and tentative identification of the compounds detected in *P. lactiflora* petals with control and Pro-Ca treatment

Peak	Retention time	Mass spectrometry information			Tentative identification
	(min)	λ_{max} (nm)	$[M+H]^+$ (m/z)	MS^2 (m/z)	
a1	18.01	275; 515	611.18	448.99; 287.29	Cyanidin-3,5-di-*O*-glucoside
a2	22.10	275; 500	595.21	432.99; 271.28	Pelargonidin-3,5-di-*O*-glucoside
a3	25.47	275; 515	625.22	463.01; 301.24	Peonidin-3,5-di-*O*-glucoside
a4	35.23	275; 515	463.21	301.27	Peonidin-3-*O*-glucoside
a5	36.50	275; 515	711.21	549.03; 463.05; 301.27	Peonidin-3-*O*-malonylglucoside-5-*O*-glucoside
f1	19.19	260; 355	627.14	465.01; 303.43	Quercetin-3,7-di-*O*-glucoside
f2	25.12	265; 345	611.15	449.21; 287.37	Kaempferol-3,7-di-*O*-glucoside
f3	29.99	265; 345	697.18	534.99; 448.96; 287.33	Kaempferol-3-*O*-malonylglucoside-7-*O*-glucoside
f4	37.30	265; 355	617.21	465.28; 303.15	Quercetin-3-*O*-galloyglucoside
f5	45.73	265; 355	465.04	303.11	Quercetin-3-*O*-glucoside
f6	53.54	270; 350	601.29	449.03; 286.95	Kaempferol-3-*O*-galloyglucoside
f7	54.81	265; 365	449.12	287.29	Kaempferol-7-*O*-glucoside

a1-a5 indicate identified anthocyanins; f1-f7 indicate identified anthoxanthins

Besides, the expression level of upstream *PlF3H* and downstream *PlDFR*, *PlANS* also achieved a higher level. In terms of the overall trend, the expression patterns of *PlPAL*, *PlCHS*, *PlF3H*, *PlF3'H*, *PlDFR*, *PlANS* and *PlF3GT* exhibited a similar downward trend during flower development and reached the lowest level, except *PlF3GT* in S2 in the control. Unlike the other genes, the expression patterns of *PlFLS* showed firstly a rise from S1 to S2 followed by a decline from S2 to S3 in both. After Pro-Ca treatment, the expression level of *PlF3H*, *PlF3'H*, *PlDFR*, *PlANS* and *PlF3GT* were significantly reduced compared to the control, except for *PlF3H* and *PlF3GT* in S2 which were not obviously changed. Also, the expression level of *PlF3'H*, *PlDFR* and *PlANS* fell down to nearly half or less in petals with Pro-Ca treatment. Although the expression levels of *PlF3H* and *PlFLS* in S2 were a little higher in petals with Pro-Ca treatment than in the control, there was no notable difference between the two.

Discussion

In this study, a chroma meter was used to determinate flower color for more accurate results. Also, a uniform color space was applied to chroma meter. In a uniform color space, the color parameter L^* value represented lightness while the color parameter a^* value represented a ratio of red/magenta and green. More specifically, a higher value of the L^* indicated a lighter color but a bigger absolute value, while a^* represented a darker color in red/magenta or green. A positive value of the parameter a^* was for red/magenta while a negative value was noted for green. After Pro-Ca treatment, nearly 50% increase of the parameter L^* value together with an average 10% decrease of the parameter a^* value were detected in petals with Pro-Ca treatment in each developmental stage compared to the control. This was in accordance with the results in petunia, impatiens and rose (Ilias and Rajapakse, 2005; Schmitzer *et al.*, 2012), which could further prove that treatment with Pro-Ca could lead to a loss of red color in ornamental flowers. Moreover, the dilution was reflected not just in the final result, but at each developmental stage, which might indicate that the inhibition brought by Pro-Ca treatment had worked before it came to bloom. In addition, the parameter L^* value was gradually increased along with the parameter a^* value was gradually decreased during flower development in both. This showed that the flower color was slightly faded during flower development, which was consistent with the results in other *P. lactiflora* cultivars by Zhong *et al.* (2012) and Zhao *et al.* (2012a, 2016) reports.

The expression of floral color was the result of internal factors combined with external factors, among these factors, floral color were primarily determined by the production of pigments, especially by the flavonoids which included anthocyanins and anthoxanthins (Jia *et al.*, 2008; Zhao *et al.*, 2012a). Previous study by Zhao *et al.* (2015) showed that the floral colors were related not only to the compositions but also the contents of flavonoid.

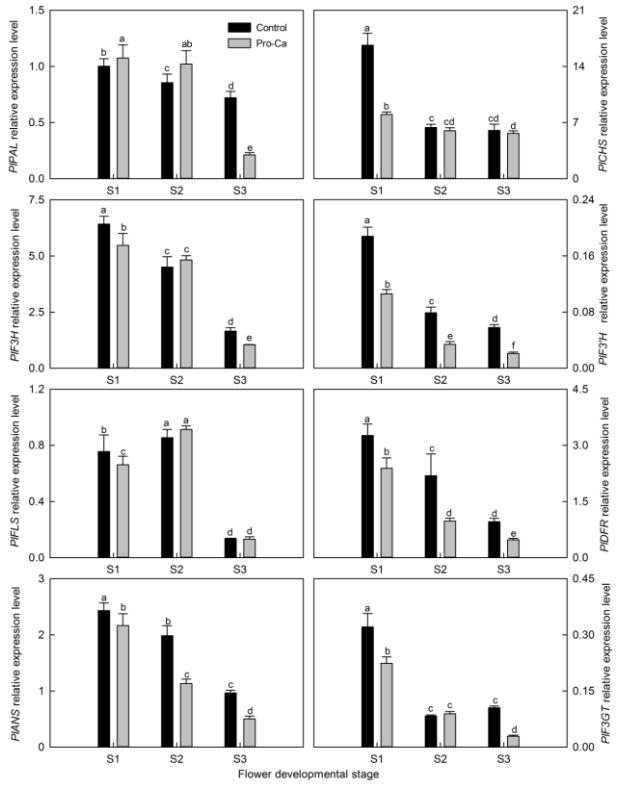

Fig. 6: Flavonoid biosynthetic genes expression analysis of *P. lactiflora* petals with control and Pro-Ca treatment during flower development. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage. The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

For example, in *P. lactiflora*, anthocyanins were responsible for red and purple petal color, whereas anthoxanthins were for yellow (Zhao *et al.*, 2014). The content of anthoxanthins was much higher in red flowers than in white flowers (Zhao *et al.*, 2016). Depending on HPLC-ESI-MS^n, the quality and quantity of flavonoids were analyzed that the compositions of flavonoid were basically the same after Pro-Ca treatment, but the contents witnessed remarkable changes. After Pro-Ca treatment, a dramatic reduction was shown in individual and total anthocyanidin contents. It was consistent with the result in rose flowers (Schmitzer *et al.*, 2012), which demonstrated that Pro-Ca treatment brought about a negative effect on individual and total anthocyanin contents in ornamental flowers. Meanwhile, the content of several components belonged to anthoxanthins, such as flavonols, was also reduced after treated with Pro-Ca in young grape flowers (Puhl *et al.*, 2008). It was mainly coincided with the result in our study that most individual and total anthoxanthins content decreased after Pro-Ca treatment. As a result, after Pro-Ca treatment, the accumulation of both anthocyanins and anthoxanthins were inhibited in this study. Also, the reduction of anthocyanin and anthoxanthin contents was due in large part to the dilution of floral color (Zhong *et al.*, 2012). Furthermore, among the anthocyanins, Pn3G5G and Cy3G5G were identified as the two major anthocyanins in 'Luhong'. In *P. lactiflora*, Pn3G5G and Cy3G5G were responsible for red coloration by comparing anthocyanin compositions in 41 *P. lactiflora* cultivars (Jia *et al.*, 2008). In our study, a remarkable decrease in Pn3G5G and Cy3G5G after Pro-Ca treatment would mainly cause a loss especially of red coloration. However, it was worth noticing that compared to anthocyanins like Pn3G5G and Cy3G5G, anthoxanthins presented a negligible part of purple-red color definition as anthoxanthins were mainly responsible for yellow or evenly colorless in *P. lactiflora* (Zhao *et al.*, 2014).

The accumulation of flavonoids was determined by a series of genes in the flavonoids biosynthesis pathway (Nakatsuka *et al.*, 2005). Pro-Ca, a 2-ODD inhibitor, performed by blocking several 2-ODDs involved in the flavonoids biosynthesis pathway (Rademacher, 2000). Earlier, Halbwirth *et al.* (2006) proposed that *F3H* and *FLS*, the two 2-ODDs genes, were inhibited in young grapevine leaves, flowers, and berries after Pro-Ca treatment. The inhibition of *F3H* had been proved in the skin of apples, while the expression levels of *PAL*, *CHS, F3H, DFR*, *ANS* and *FGT* were also reduced in varying degrees after treatment (Bizjak *et al.*, 2013). In our study, we also noted that the expression of *PlCHS, PlF3H, PlF3'H*, *PlDFR*, *PlANS* and *PlF3GT* was lower levels in petals with Pro-Ca treatment. Mostly the expression of rest of the genes remained basically at consistent level. On the whole, a universal reduction of enzyme activities studied in this study was shown in flavonoid pathway except *PlPAL* after Pro-Ca treatment. With the regular expressions of upstream *PlCHS* and downstream *PlFLS*, as well as lower expressions of *PlF3H*, *PlF3'H* and downstream *PlDFR*, *PlANS*, *PlF3GT*, fewer amount of anthocyanins and anthoxanthins was produced after Pro-Ca treatment. Furthermore, *F3H*, *FLS* and *ANS* were the 2-ODDs in the flavonoids biosynthesis pathway (Halbwirth *et al.*, 2006). Lower expression of *PlF3H* and *PlANS* might indicate that *F3H* and *ANS* were the possible enzymes inhibited by Pro-Ca treatment, while *F3'H*, *DFR* and *UGT* might also be suppressed (Fig. 6). Specifically, *ANS* was the major enzyme in anthocyanins biosynthesis. It played an important role in the accumulation of anthocyanins because it catalyzed the colorless leucoanthocyanidins to the colored anthocyanidins. In *P. lactiflora*, *PlANS* was directly associated with red petal pigmentation (Zhao *et al.*, 2014; 2016). For example, a higher expression level of *PlANS* was detected in purple-red flowers than in pink flowers, whereas in white or yellow flowers, it was all in a lower level (Zhao *et al.*, 2016). In our study, after Pro-Ca application, the lower expressions of *PlANS* combined with *PlDFR* and *PlF3GT* were correlated with their anthocyanin contents and might eventually led to a loss of red color in *P. lactiflora* flowers.

Conclusion

A synergistic inhibitory effect of flavonoid biosynthesis genes especially *PlF3H* and *PlANS* after Pro-Ca treatment, might inhibit the accumulation of anthocyanins and anthoxanthins. The contents of anthocyanins, especially Pn3G5G and Cy3G5G, was a major factor in floral color and was mainly responsible for red and purple. A decrease in anthocyanins content eventually contributed to a loss of red color in *P. lactiflora* flowers after Pro-Ca treatment. These results would establish a theoretical foundation for the regulation of floral color in *P. lactiflora* by Pro-Ca treatment.

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