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RESEARCH ARTICLE

Relative Expression of Apocarotenoid Biosynthetic Genes in Developing Stigmas of *Crocus sativus* L.

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Abstract

Saffron, the desiccated stigmas of *Crocus sativus*, is recognized for its attractive color, flavor, and aroma which are due to the accumulation of crocin, picrocrocin, and safranal, respectively. HPLC analysis demonstrated maximum apocarotenoid accumulation during the fully developed scarlet stage of stigma development followed by the orange and yellow stages of stigma development. Reverse Transcription-PCR analysis revealed a concurrent expression pattern of *CsZCD* and *CsLYC* genes in a developmental stage-specific manner. However, *CsBCH* and *CsGT2* genes were specifically expressed during the mature, scarlet stage of stigma development. Real-Time PCR analysis showed a sharp increase in gene expression of *CsLYC* gene during stigma development indicative of its possible regulatory role in apocarotenoid biosynthesis or stigma development. Results suggest that genetic manipulation of this gene can help to improve the quality of stigma in saffron; besides highlighting its potential to monitor stigma development during *in vitro* experimentation.

Key words: *Crocus sativus*, *CsBCH*, *CsGT-2*, *CsLYC*, *CsZCD*, stigma development

Introduction

Crocus sativus stigma is the world's most expensive spice rich in carotenoid compounds (Bouvier et al. 2003; Moraga et al. 2004; Rubio et al. 2009). Three important constituents of saffron stigma are crocin, safranal, and picrocrocin responsible for its color, aroma, and taste, respectively. Biosynthesis of apocarenoids occurs in saffron stigmas and involves two different pathways: (1) Mevalonic acid (MVA) pathway that occurs in the cytoplasm (Castillo et al. 2005; Wang et al. 2009) and (2) Non-mevalonic acid (MEP) pathway (2-Cmethyl- D-erythritol 4-phosphate pathway) takes place in plastids that provide the precursors for carotenoids (Arigoni et al. 1997). The MVA pathway starts with the synthesis of mevalonate through three molecules of acetyl CoA and then continues with the production of isopentenyl diphosphate (IPP) molecules, geranyl geranyl pyrophosphate (GGPP), colorless phytoene, colored lycopene, ß-catrotene, (Britton et al. 1998), and zeaxanthin (Bouvier et al. 2003). Beta-carotene with two rings is built up via cyclization of lycopene with lycopene-ß, cyclase (*LYC*) (Britton et al. 1998). The hydroxylation of ß-carotene in the MVA pathway is catalyzed by ß-carotenoid hydroxylase that is coded by the *BCH* gene to yield zeaxanthin (Castillo et al. 2005). The biogenesis of the color- and odor-active compounds of saffron are derived by bio-oxidative cleavage of zeaxanthin (Pfander and Schurtenberger 1982) at the points 7, 8 (7', 8') by zeaxanthin cleavage dioxygenase (*CsZCD*) to produce crocetin dialdehyde and picrocrocin. In *C. sativus* stigmas, the final step involves glucosylation of the generated zeaxanthin cleavage products by glucosyltransferase 2 enzyme which is coded by the CsUGT2 gene in the chromoplast of stigmas (Teale et al. 1992) and then sequestered into the central vacuole of the fully developed stigmas (Bouvier et al. 2003; Dufresne et al. 1997). The quantitative and qualitative changes in the carotenoid and the apocarotenoid profile in *C. sativus* stigmas have been previously studied (Ahrazem et al. 2010; Castillo et al. 2005; Mir et al. 2012; Rubio et al. 2009) and it has been shown that transcriptional regulation of a ß-hydroxylase, *CsZCD*, and lycopene cyclase genes are

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involved in the observed changes. In this study, the relative quantification of apocarotenoids and their genes at three different stages of stigma development in *C. sativus* is described in order to study the involvement of these genes in the transition of immature to mature stage of stigma development in *C. sativus* and, therefore, in apocarotenoid accumulation. Eight stages of development have been defined for *C. sativus* stigmas based on the length of the tissue, pigmentation, and apocarotenoid content (Himeno and Sano 1987). Since sharp shifts in some key apocarotenoid biosynthesis gene expression have been found during stages II, IV, and VIII of stigma development (Mir et al. 2012), these three stages of stigma development were taken in the present study: stage II corresponds to a yellow, undeveloped stigma, stage VIII represents a scarlet, fully developed stigma, and stage IV represents an orange, undeveloped stigma. The apocarotenoid accumulation and expression patterns of apocarotenoid biosynthesis genes were studied during these three stages of stigma development.

Material and Methods

Research material

Three stages of stigma development (scarlet, orange, and yellow) were collected from September to November 2012 (Fig. 1). Freshly cut stigmas were quickly immersed in liquid nitrogen and then stored at -80°C for RNA isolation. However, dried stigmas were used for apocarotenoid extraction.

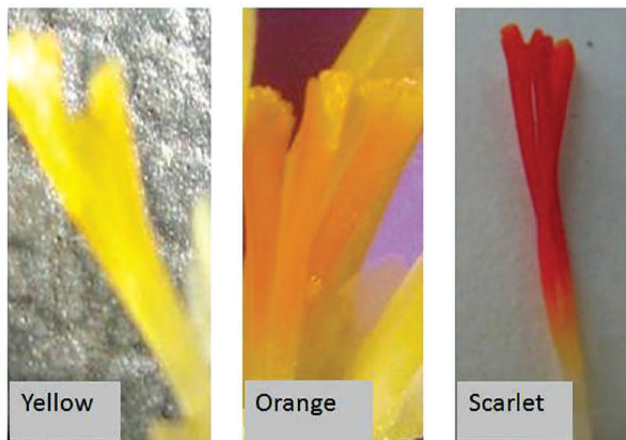

Fig. 1. Different stages of stigma development in *Crocus sativus* L.

Apocarotenoid extraction and HPLC analysis

For the analysis of apocarotenoids, saffron stigma carotenoids were extracted in a microcentrifuge tube by grinding 1 g dried stigma with a micropestle in methanol (100 mL) and incubated for 5 min on ice. Tris-HCl (50 mM, pH 7.5; containing 1 M NaCl) was then added (100 mL) and a incubated for 10 min on ice. The precipitate was collected by centrifugation at 3,000 g for 5 min at 4°C. The pellet was then reground in acetone (400 mL) and incubated on ice for 10 min. The mixture was centrifuged at 3,000 g for 5 min at 4°C. This step was repeated until no color was detected in the pellets. The supernatant was dried and the solid extract was dissolved in HPLC grade methanol. The analysis was carried out in a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps, degasser coupled to a photo-diode-array detector, and injection valve with a 20 µL loop. Separation was carried out with an injection volume of 20 µL, a flow rate of 1 mL min^{-1} with 35-40 min of run time. The analyses were triplicated for each sample. Safranal was detected at 310 nm and crocin at 440 nm, whereas the internal standards for respective apocarotenoids (crocin and safranal) as positive controls and quantitative estimation were detected at the above-mentioned wavelengths (Lozano et al. 1999). Chromatographic separations were performed on C18 (250 × 4.6 mm), 5 µm column using a solvent system consisting of 75% acetonitrile and 25% methanol in an isocratic mode. The mobile phase was filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA) before analysis. Class WP software (version 6.1) from Shimadzu was used for instrument control, data acquisition, and data processing. Quantitative determinations were made by taking into account the respective peak areas of standards at particular retention time versus concentration and expressed in milligrams per gram of saffron stigmas.

Reverse transcription PCR analysis

Frozen stigmas were ground in a cold and sterilized mortar and pestle into fine powder and total RNA was extracted using RNA isolation kit (Roche Applied Science) following the manufacturer's protocol. Quality of the extracted RNAs was checked by measuring the absorbance at 260 and 280 nm by a Nano-Drop and RNAs with ratio of OD 260/280 ranging from 1.2 to 1.5 were used for cDNA synthesis. For each sample, 5 µg of total RNA as template and 18 bp oligo dT primer and first strand cDNA synthesis kit (Roche Applied Science) were used for first-strand cDNA synthesis as described by the manufacturer. The synthesized cDNA was stored at -20°C for the gene expression study. Reverse transcription was carried for amplification of *CsZCD*, *CsLYC*, *CsBCH*, *CsGT2*, and Cs*TUB* gene as internal control, using AMVRT cDNA kit (Roche Applied Science, Penzberg, Germany) according to the user's manual. Forward- and reverse-primer sequences were used for amplification of these genes and the expected length of amplicons are shown in Table 1. PCR reactions were performed in thermocycler (Takara, Japan) with 2-5 µg of cDNA. Initial denaturizing at 95°C for 5 min

Table 1. Primer sequences for amplification of apocarotenoid biosynthesis genes in saffron with expected amplicons lengths

Primer	Forward Primer (5'to 3')	Reverse Primer (5' to 3')	Amplicon size (bp)
CsZCD	GTCTTCCCCGACATCCAGATC	CTCTATCGGGCTCACGTTGG	241
CsLYC	AGATGGTCTTCATGGATTGGAG	ATCACACACCTCTCATCCTCTTC	247
CsBCH	TCGAGCT TCGGCATCACATC	GCAATACCAAACAGCGTGATC	495
CsGT2	GATCTGCCGTGCGTTCGTAAC	GATGACAGAGTTCGGGGCCTTG	400
CsTUB	TGATTTCCAACTCGACCAGTGTC	ATACTCATCACCCTCGTCACCATC	225

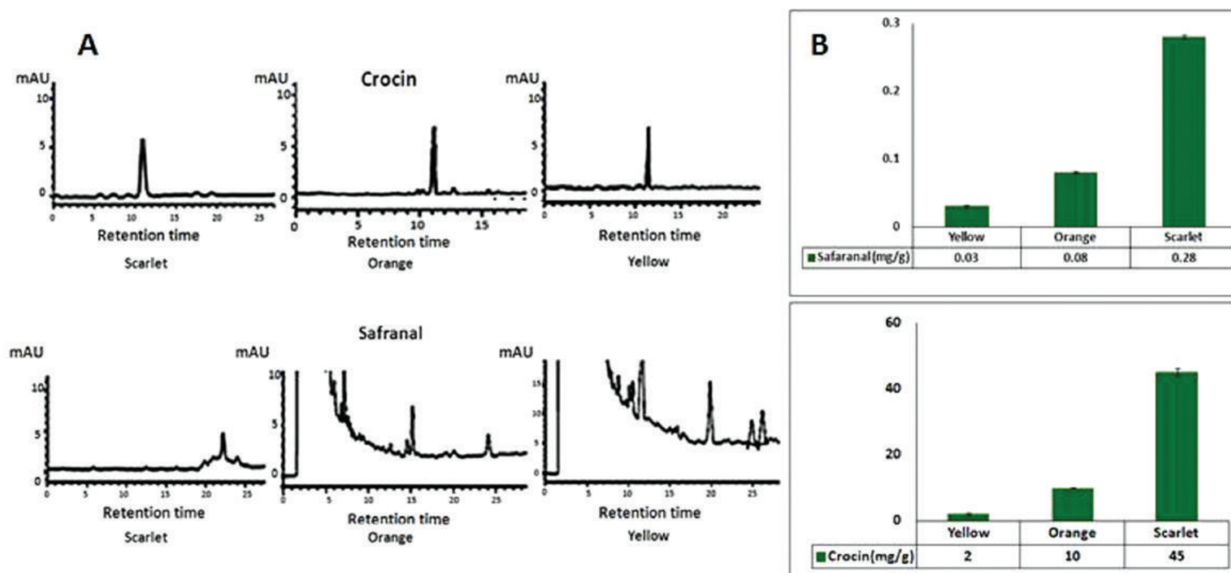

Fig. 2. Relative apocarotenoid quantification through HPLC during different stages of stigma development. HPLC chromatogram (A) for crocin and safranal during scarlet, orange, and yellow stages of stigma development (X and Y axis showing mAU and retention time, respectively) and relative quantification (B) of crocin and safranal during scarlet, orange, and yellow stages of stigma development

followed by 35 cycles of amplification according to the subsequent scheme: denaturizing 1 min at 94°C, annealing at 56.2°C for 30 s, and extension at 72°C for 40 s and final extension at 72°C for 7 min. The experiments were repeated twice. Subsequently, 5 μL of the PCR products were used on 1.2% (w/v) agarose (Sigma-Aldrich, St Louis, MO, USA).

Real-Time PCR analysis

The Real-Time PCR was performed in 96-well plates with a LightCycler 480 real-time PCR instrument (Roche Diagnostics) using the LightCycler 480 SYBR Green I Master kit. Reactions were performed in triplicate and contained 5 μL SYBR Green I Master, 2 μL PCR-grade water, 2 μL cDNA, and 0.5 μL of each of the 10 μm forward- and reverse gene-specific primers in a final volume of 10 μL. Tubulin gene was taken as reference gene. Gene expression at the scarlet stage of stigma development was taken as positive control. The reactions were incubated at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 56.2°C for 15 s, and 72°C for 20 s. The specificity of the PCR reaction was confirmed with a heat dissociation protocol (from 60°C to 95°C) following the final PCR cycle. This ensured the resulting fluorescence originated from a single PCR product, and did not represent primer dimers formed during PCR or a non-specific product. Primers for real-time PCR assay were evaluated by performing real-time PCR on serially diluted cDNA to make sure that concentrations of the primers used in the assay generate CT values consistent with the dilution. LightCycler 480 software (version 1.5, Roche Diagnostics) was used to collect the fluorescence data. Advanced relative quantification between the genotypes and three stages of stigma development was done through $2^{-\Delta\Delta cp}$ method (Livak and Schmittgen 2001).

Results

Relative quantification of apocarotenoids

Crocin and safranal contents were estimated during the yellow, orange, and scarlet stages of stigma development in saffron. Both components were identified as previously described in the literature (Caballero-Ortega et al. 2007; Li et al. 1999; Lozano et al. 1999; Tarantilis et al. 1995). Fig. 2 shows the representative HPLC chromatograms and concentration of each detected compound in three stages of stigma development. Crocin content was found to increase from 2 ± 0.4 mg g^{-1} in the yellow stage to 10 ± 0.2 mg g^{-1} in the orange stage of stigma development. It further increased to 40 ± 1.15 mg g^{-1} of dry stigma during the scarlet stage of stigma development. Safranal biosynthesis also showed a drastic increase from the yellow to the scarlet stage of stigma development. Safranal increases from 0.03 ± 0.002 to 0.08 ± 0.001 mg g^{-1} of dry stigma from the yellow to orange stage and further increases to 0.28 ± 0.003 mg g^{-1} of dry stigma in the scarlet stage of stigma development.

Expression of apocarotenoid biosynthesis genes

Expression of key genes responsible for biosynthesis of apocarotenoids in *C. sativus* was investigated during different stages of stigma development in saffron through semi-quantitative (Reverse Transcription PCR) or quantitative (Real-Time PCR) analysis.

Semi-quantitative gene expression analysis

The scarlet stage of stigma development showed the highest expression of *CsLYC*, *CsBCH*, *CsZCD*, and *CsGT-2* genes. *CsLYC* and *CsZCD* genes were also expressed in the orange and yellow stages of stigma development (Fig. 3);

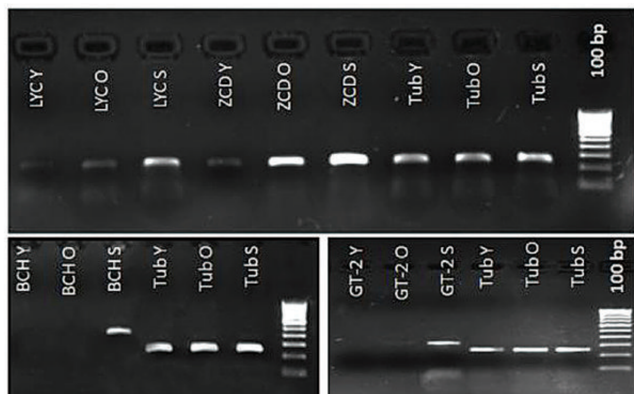

Fig. 3. Semi-quantitative relative expression of apocarotenoid biosynthesis genes (*ZCD*, *LYC*, *GT2*, and *BCH*) and tubulin (Tub) gene at scarlet (S), orange (O), and yellow (Y) stages of stigma development in saffron. *ZCD*: Zeaxanthin Cleavage Dioxygenase; *LYC*: lycopene-ß, cyclase; *GT2*: Glycosyl transferase 2; *BCH*: ß carotene hydroxylase

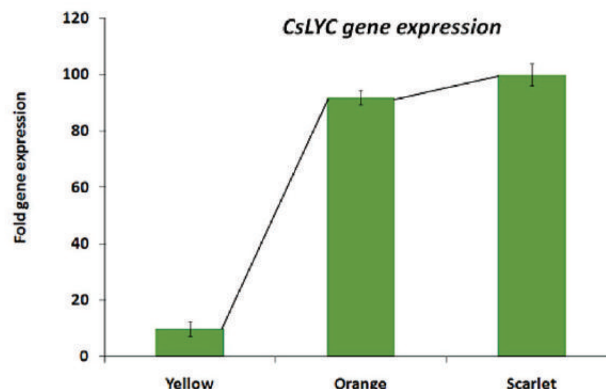

Fig. 5. Change in gene expression of *CsLYC* gene during different stages of stigma development in saffron. *Cs: Crocus sativus; LYC*: lycopene-ß cyclase

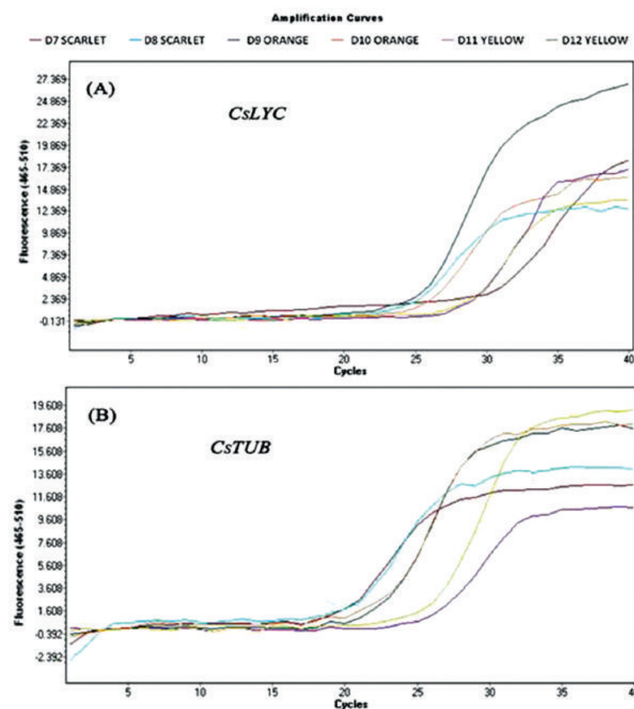

Fig. 4. Amplification curves of *CsLYC* (a) and Tubulin (b) genes at scarlet, orange, and yellow stages of stigma development. *Cs: Crocus sativus*; *LYC*: lycopene-ß cyclase

however, *CsBCH* and *CsGT-2* gene expression was not found during the orange and yellow stages of stigma development.

Quantitative changes in apocarotenoid biosynthetic genes

In the present study, the relative expression of the *CsLYC* gene was studied through real-time PCR analysis. Real-Time PCR amplification of *CsLYC* and tubulin genes at the yellow, orange, and scarlet stages of stigma development is shown in Figs. 4a and 4b, respectively. Real-Time PCR analysis revealed that *CsLYC* gene expression gets up-regulated by 82% from yellow to orange and by 8% from orange to scarlet stages of stigma development (Fig. 5).

Discussion

Relative quantification of apocarotenoids

Carotenoid accumulation and composition during stigma development of *C. sativus* is highly regulated by the coordinated transcriptional activation of carotenoid biosynthetic genes (Castillo et al. 2005). During the development of saffron, the stigma changes in color from white to scarlet, passing through yellow and orange stages, parallel stigma growth and apocarotenoid accumulation. Apocarotenoids increased 7% from the yellow to the orange stage and up to 88% in the scarlet stage (Himeno and Sano 1987). The accumulation of carotenoids during this process reaches their maximum levels at the time of anthesis. Beta-carotene and zeaxanthin increased by 60.5 and 85%, respectively, from the orange to the scarlet stage (Castillo et al. 2005). Total content of crocin and safranal depends upon storage of stigma and extraction method of apocarotenoids (Caballero- Ortega et al. 2007), hence the amount of these compounds reported by different workers varies. Alonso et al. 2001 reported that crocins vary from 0.85to 32.4% dry weight at the scarlet stage of stigma development. Other values reported vary between 29 (Li et al. 1999) and 45.99% dry weight (Caballero-Ortega et al. 2007) for Iranian saffron and 67.3% dry weight (Sujata et al. 1992) for Indian saffron. Safranal levels reported in some studies are around 8% dry weight (Sujata et al. 1992) at maturity. Safranal vary between a 6t and 29% dry weight (Hadizadeh et al. 2007). Lage et al. 2009 reported that crocin and safranal content vary between 17-37 and 0.1-0.48%, respectively, under Moroccan conditions at the scarlet stage of stigma development.

Expression of apocarotenoid biosynthesis genes

Expression of apocarotenoid biosynthesis genes *viz.* *CsBCH*, *CsZCD*, *CsLYC*, and *CsGT-2* was studied through relative semi-quantitative during three stages of stigma development. These stages were comparable with those

obtained from *in vivo* studies by Himeno and Sano (1987). The three developmental stages were chosen based on length, pigmentation, and apocarotenoid content. Although reverse transcription PCR analysis is the semi-quantitative method of gene expression, it is a very good method to determine a comparative level of gene expression and is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts. RT-PCR is increasingly used to detect small changes in gene expression that would otherwise be undetectable (Freeman et al. 1999). In comparison to our findings, Castillo et al. 2005 found maximum expression of *CsLYC*, *CsBCH*, and *CsGT-2* at the scarlet stage of stigma development, but they did not observe any *CsLYC* and *CsZCD* expression in the orange and yellow stages of stigma development which may be due to different clonal selection or different dates of sample collection. Ahrazem et al. 2010 studied the expression pattern of two lycopene-ß-cyclase genes, *CstLcyB1* and *CstLcyB2a*, and found that *CstLcyB2a* encodes chromoplast-specific lycopene cyclases, with an expression analysis showing strongly in flower stigmas where it activates and boosts ß-carotene accumulation while *CstLcyB1* transcripts were observed in leaves, tepals, and stigmas at lower levels. It is documented that in *C. sativus* the development of the stigmas occurs concomitantly with transition of amyloplasts to chromoplasts and parallel with biosynthesis and accumulation of apocarotenoid which relates to expression levels of these apocarotenoid biosynthetic genes (Bouvier et al. 2003; Moraga et al. 2004).

Quantitative changes in *CsZCD* and *CsLYC* genes

Quantitative RT-PCR has become a powerful tool for analysis of gene expression because of its high throughput, sensitivity, and accuracy (Bustin 2000). Since semi-quantitative expression results reveal that *CsBCH* and *CsGT2* were not expressed during the orange and yellow stage of stigma development, hence relative quantification through quantitative real-time PCR was done only for *CsZCD* and *CsLYC* genes which showed differential expression during stigma development. In our previous study on the relative expression of the *CsZCD* gene during different stages of stigma development (Mir et al. 2012), we observed that the *CsZCD* gene expression gets up-regulated by 8% from yellow to orange and then by 33% from orange to scarlet stage of stigma development in saffron (Mir et al. 2012). In the present study, the relative expression of the *CsLYC* gene was studied through real-time PCR analysis. An abrupt increase in expression of *CsLYC* from the yellow to orange stage of stigma development was observed. Here, the scarlet stage which has a fully developed stigma was treated as a positive calibrator. Higher expression of *CsLYC* gene during scarlet stage of stigma development has been reported using semi-quantitative reverse transcription PCR (Castillo et al. 2005; Rubio et al. 2004). Semi-quantitative expression of lycopene cyclase gene has been done in different tissues of saffron flower and maximum expression has been observed in saffron stigmas at the maturity stage (Ahrazem et al. 2010). This is the first report of quantitative analysis of *CsLYC* gene expression during stigma development in saffron. The prelude to this would be to validate a set of reference genes along with other important genes from the same pathway to harmonize the data from various experiments that are expected to follow suit.

Conclusions

In *Crocus sativus* during stigma development there are significant changes in the content of apocarotenoids and apocarotenoid biosynthetic genes. Two genes, *CsZCD* and *CsLYC*, showed clear changes in expression during different stages of stigma development, but *CsBCH* and *CsGT2* were expressed only during the scarlet stage of stigma development. Our study found a clear correlation between apocarotenoid content and apocarotenoid gene expression during different stages of stigma development. In order to find out the main regulatory gene responsible for apocarotenoid biosynthesis, we need to correlate apocarotenoids genes with level of apocarotenoids and substrates like zeaxanthin, beta-carotene, etc., during different stages of stigma development. The present findings will play an important role to find out the stage of flower development under *in vivo* or *in vitro* conditions and the quality of stigma can be improved by inducing overexpression of these genes in plant by genetic manipulation approaches.

Acknowledgements

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