



Relative expression of *CsZCD* gene and apocarotenoid biosynthesis during stigma development in *Crocus sativus* L.

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Abstract *Crocus sativus* is a triploid sterile plant characterized by its red stigmas, which produce significant quantities of carotenoid derivatives formed from the oxidative cleavage of β -carotene and zeaxanthin. The accumulation of three major carotenoid derivatives- crocin, picrocrocin, and safranal- is responsible for the color, bitter taste, and aroma of saffron, which is obtained from the dried stigma of *Crocus*. Maximum apocarotenoid accumulation occurs during fully developed scarlet stage of stigma development. Zeaxanthin is the precursor for biosynthesis of apocarotenoids. *Crocus* zeaxanthin 7, 8 (7, 8)-cleavage dioxygenase gene (*CsZCD*) encodes a chromoplast enzyme that initiates the biogenesis of these apocarotenoids by cleaving zeaxanthin. The Reverse Transcription-PCR analysis revealed that *CsZCD* gene expression followed different patterns during stigma development. Highest levels of *CsZCD* gene expression was observed in fully developed scarlet stage of stigma. Real Time PCR analysis showed that there is a sharp increase in gene expression from yellow to orange and orange to scarlet stages of stigma development. Increase in *CsZCD* gene expression parallels with the apocarotenoid content during the development of stigma, suggesting its regulatory role for apocarotenoid biosynthesis and stigma development in saffron.

Keywords Saffron · *CsZCD* · Real time PCR · Gene expression · Apocarotenoid

Introduction

Crocus sativus (Saffron) belong to family Iridaceae and is most expensive spice of the world. It is characterized by its long red stigmas, which produce and store significant quantities of carotenoid derivatives, formed from the oxidative cleavage of β -carotene and zeaxanthin (Rubio et al. 2009; Bouvier et al. 2003; Moraga et al. 2004). Carotenoids and their derivatives play an important role in plants and other living organisms (Cazzonelli 2011). Biosynthesis of *Crocus* apocarotenoids is developmentally regulated (Grilli-Caiola and Canini 2004). During saffron development, its stigma changes in color to scarlet, passing through yellow and orange colors (Himeno and Sano 1987). This stigma growth and accumulation of apocarotenoid simultaneously occur with conversion of amyloplasts to chromoplasts (Bouvier et al. 2003). The quality of saffron is certified in the international trade market following the ISO 3632 Normative since 1993. The most important parameter is colouring strength, calculated from UV–vis measurements at 440 nm in aqueous extracts of this spice. Such measurements are related with the total crocins content. Other important components are picrocrocin and safranal, responsible for flavor and aroma respectively (Fernandez 2004). The amounts of these main compounds are used to express the quality of saffron.

Crocus zeaxanthin cleavage dioxygenase gene (*CsZCD*) codes for a chromoplast enzyme that initiates the biogenesis of crocetin glycosides and picrocrocin (Bouvier et al. 2003). Previous studies and literature showed the biogenesis of crocetin from β -carotene and zeaxanthin (Rubio et al. 2009; Gomez-Gomez et al. 2010). *CsZCD* specifically catalyzes

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the synthesis of crocetin dialdehyde and hydroxy-cyclocitral from zeaxanthin. Thus, *CsZCD* is the Crocus 7,8 (7,8)-zeaxanthin cleavage dioxygenase that initiates the synthesis of saffron pigment and aroma and is expressed specifically in the chromoplasts during the active period of zeaxanthin cleavage (Bouvier et al. 2003). The quantitative and qualitative changes in the carotenoid and the apocarotenoid profile in *C. sativus* stigmas have been studied previously (Castillo et al. 2005; Rubio et al. 2009; Ahrazem et al. 2010), and it has been shown that transcriptional regulation of a β -carotene hydroxylase and lycopene cyclase genes is involved in the observed changes. In this study, the relative quantification of *CsZCD* gene at three different stages of stigma development in *C. sativus* is described in order to study the involvement of this gene 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). Three stages of stigma development were taken in present study, stage II corresponds to a yellow undeveloped stigma, stage VIII represents scarlet fully developed stigma and stage IV represents an orange undeveloped stigma. Most likely with this paper these will be treated as yellow, orange and scarlet stages of stigma development. The apocarotenoid accumulation and expression patterns of *CsZCD* gene were studied during these three stages of stigma development.

Material and methods

Plant material

Standards of crocin, safranal and picrocrocin were obtained from Sigma Aldrich. Stigmas were collected at three different stages (Yellow, orange and scarlet color). Samples were dried in saffron drier (Developed by SKUAST-K, Srinagar) at 60 °C. Dried samples were ground in pestle and mortar and were passed through a 0.5 mm mesh and stored in the dark at 4 °C until they were used for extraction and apocarotenoid estimation. For RNA isolation, stigmas were collected at Central Institute of Temperate Horticulture, Rangreth, Srinagar and quickly immersed in liquid nitrogen and then stored at -80 °C for further use.

Extraction procedure

A 100 mg mass of dried stigma was extracted with 5 ml of cold 50 % (v/v) ethanol in a pestle and mortar, it was then transferred to a screw-capped 50 ml tubes and a total volume of 20 ml 50 % (v/v) ethanol was added to it. Tubes were sonicated for 20 min on ice and then centrifuged at 4,000 rpm for 15 min and washed twice with 5 ml 50 %

(v/v) ethanol. The supernatant was used for analysis by spectrophotometric procedures.

Spectrophotometry

Saffron samples were analyzed according to the ISO 3632 trade standard (ISO/TS 3632-1/2. 2003). According to ISO, picrocrocin, safranal and crocins are expressed as direct reading of the absorbance of 1 % aqueous solution of dried saffron at 257, 330 and 440 respectively. The supernatant (1 ml) was diluted to 5 ml with 50 % (v/v) ethanol for analysis using Nanodrop (Thermo scientific). A standard curve was prepared by measuring the absorption of crocin, safranal and picrocrocin at 440 nm, 330 nm and 257 nm respectively. Sample supernatants were diluted 100 times and readings were taken at 440 nm, 330 nm and 257 nm for crocin, safranal and picrocrocin respectively. Measurements of $E^{1\%}$ of an aqueous saffron extract at 440, 330, and 257 nm, respectively, were done using a 1 cm pathway quartz cell. Results are obtained by direct reading of the absorbance, D , at three wavelengths, as follows:

$$E^{1\%1\text{cm}} = (D \times 10,000) / (M \times (100 - H)).$$

Where D is the specific absorbance; m is the mass of the saffron sample, in grams; H is the moisture and volatile content of the sample, expressed as a mass fraction. Moisture and volatile contents were identified by using powdered saffron stigmas. The sample were ground with a pestle and mortar and passed through a 0.5 mm mesh. After weighing, the powdered samples were kept uncovered in an oven set at 103 °C for 16 h. The moisture and volatile matter content are expressed as a percentage of the initial sample using the following relation: (initial mass - constant mass)/initial mass \times 100.

RNA extraction

Frozen stigmas from three stages of stigma development (yellow, orange and scarlet) were ground in cold and sterilized mortar and pestle into fine powder and total RNA was extracted using RNA isolation kit (Roche Applied Science, Penzberg, Germany) following the manufacturer's protocol. Quality of the extracted RNAs was checked by measuring the absorbance at 260 and 280 nm and RNAs with ratio of OD 260/280 ranging from 1.2 to 1.5 were used for cDNA synthesis.

cDNA preparation

For each sample, 5 μ g of total RNA as template and 18-bp oligo dT primer and first strand cDNA synthesis kit (Roche Applied Science, Penzberg, Germany) were used

for first-strand cDNA synthesis as per manufacturer's instructions. The synthesized cDNA was stored at -20°C for gene expression study.

RT-PCR

Reverse transcription was carried for amplification of *CsZCD* gene and *CsTUB* as internal control, using AMVRT cDNA kit (Roche Applied Science, Penzberg, Germany) according to user manual. Forward and reverse primers were 5'-GTCTTCCCCGACATCCAGATC-3' & 5'-CTCTATCGGGCTCACGTTGG-3' for *CsZCD* and 5'-TGATTTCCAACCTCGACCAGTGTC-3' and 5'-ATACTCATCACCTCGTACCATC-3' for *CsTUB* gene (Bouvier et al. 2003; Castillo et al. 2005). The lengths of the products for two genes of *CsZCD* and *CsTUB* were 241, and 225 bp, respectively. PCR was performed (Takara, Japan) with 2–5 μg of cDNA with initial denaturation at 95°C for 5 min followed by 35 cycles of amplification according to the subsequent scheme; denaturation 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

The real time PCR were 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 and ZCD gene expression at scarlet stage 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. Amplification of a single product of expected size was verified by gel electrophoresis on a 2 % agarose gel (Sigma-Aldrich, St Louis, MO, USA) and ethidium bromide staining. LightCycler 480 software (version 1.5; Roche Diagnostics) was used to collect the fluorescence data. Advanced relative quantification between three stages of stigma development was done through $2^{-\Delta\Delta\text{cp}}$ method (Livak and Schmittgen 2001).

Results and discussion

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 *CsZCD* gene (Bouvier et al. 2003; Moraga et al. 2004). The pattern of accumulation of the apocarotenoid crocetin, picrocrocin and the different crocins in developing saffron stigmas was investigated by extracting stigmas corresponding to three different developmental stages as previously described (Castillo et al. 2005). In the present study apocarotenoid accumulation was correlated with the expression of *CsZCD* gene during three developmental stages of saffron stigma. It was observed that apocarotenoid synthesis at three different stages of stigma development varied significantly. An apocarotenoid accumulation start from yellow stage but maximum accumulation was observed in scarlet stage of stigma development (Table 1). 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). The expression levels of *CsZCD* gene parallels the accumulation of the apocarotenoid compounds, suggesting that the formation of these compounds is controlled at a different level, by *CsZCD* gene expression (Rubio et al. 2008). Biogenesis of crocetin glucosides and picrocrocin are initiated by zeaxanthin cleavage by dioxygenase, which is coded by *CsZCD* has been reported (Bouvier et al. 2003). The gene *CsZCD*, which codes for a chromoplast enzyme that initiates the biogenesis of crocetin glycosides and picrocrocin, was highly expressed in the scarlet stigma when the highest levels of zeaxanthin and apocarotenoids have been detected (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

Table 1 Apocarotenoid contents in saffron stigma during different stages of stigma development

Apocarotenoid	Stage of flower development	ISO/TS 3632 value
Picrocrocin	Scarlet	79±0.67
	Orange	20.0±1.11
	Yellow	8±0.30
Crocine	Scarlet	188±1.16
	Orange	60±1.12
	Yellow	6±0.10
Safranin	Scarlet	23.2±1.16
	Orange	8±0.04
	Yellow	1.02±0.02

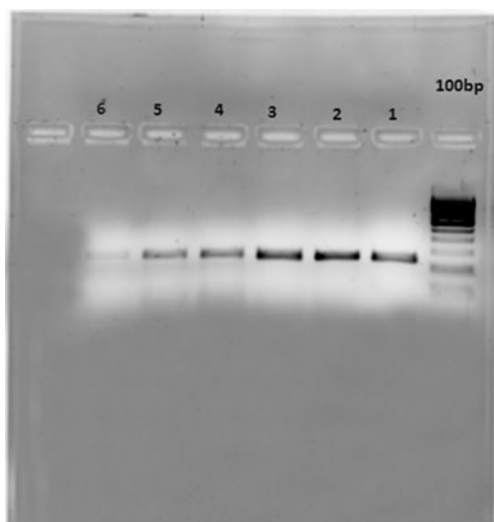


Fig. 1 *CsZCD* (Lane 4–6) and reference *Tubulin* (Lane 1–3) gene expression at three developmental stages of saffron stigma (Lane 1 & 4 for *Scarlet*, 2 & 5 for *orange* and 3 & 6 for *yellow*)

scarlet stage (Himeno and Sano 1987). In the present study picocrocins increased 2.5 folds from the yellow to the orange

stage and further 4 folds from orange to scarlet stage. Crocin content showed 10 and 3 folds increase from yellow to orange and orange to scarlet stages respectively and safranal showed 8 and 4 folds increase from yellow to orange and orange to scarlet stages respectively. Accumulation of carotenoids during this process, which reach their maximum levels at the time of anthesis. β -Carotene and zeaxanthin increased by 60.5 % and 85 %, respectively, from the orange to the scarlet stage (Castillo et al. 2005).

Reverse transcription and real time PCR was performed with RNA purified from stigmas in stages. The Reverse Transcription-PCR analysis revealed that accumulation of the mRNAs corresponding to the *CsZCD* gene studied during stigma development followed different patterns during stigma development (Fig. 1). Highest levels of *CsZCD* gene expression was observed in the scarlet stage of stigma development. These results are in accordance with the results observed by Castillo et al. 2005. In addition, the expression levels of *CsZCD* were analyzed in each step (Fig. 1). The expression levels were correlated with the accumulation of apocarotenoids during these stages of stigma development. Therefore, both *CsZCD* gene expression

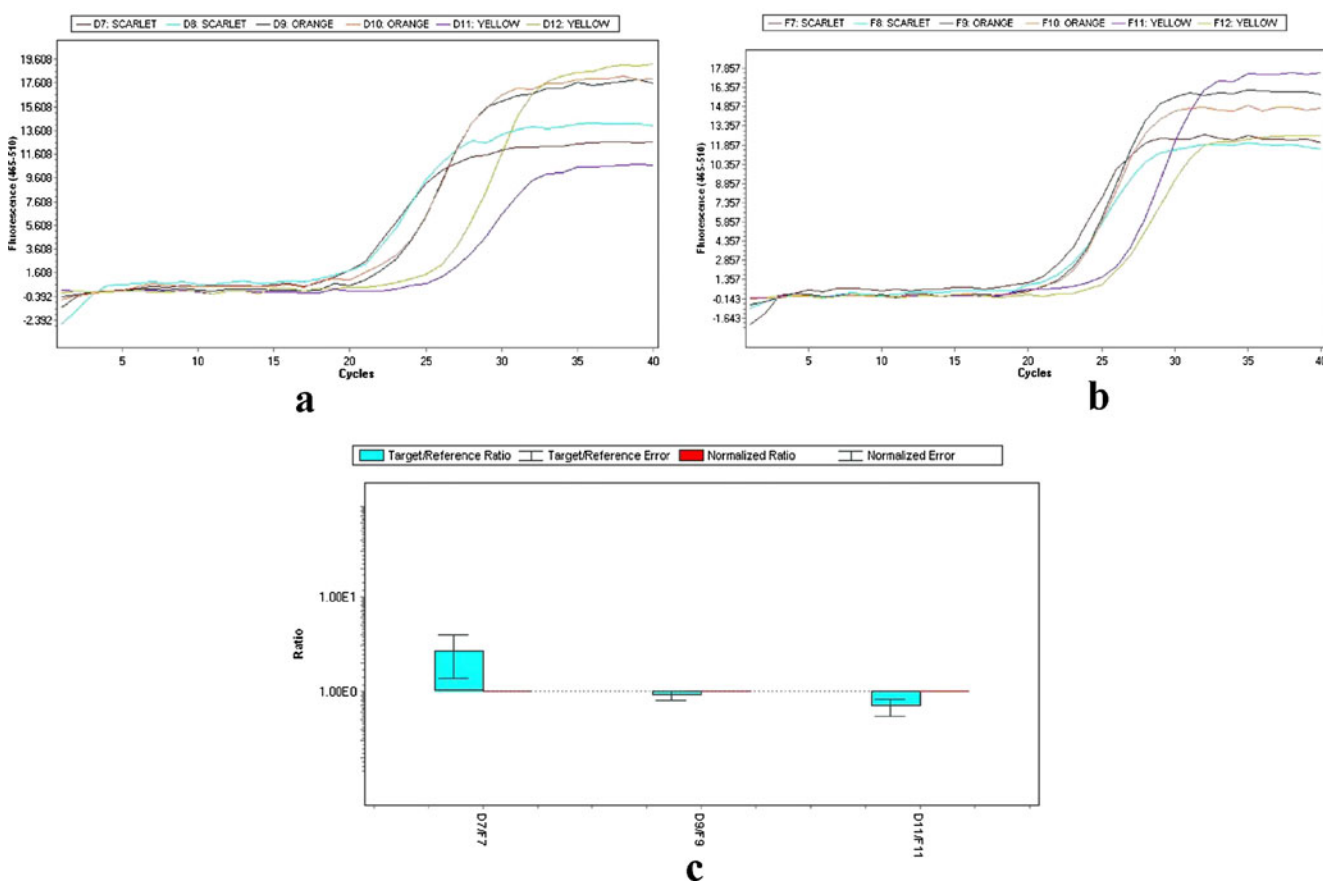


Fig. 2 Amplification curves of *CsZCD* (a) and *Tubulin* (b) genes at *scarlet*, *orange* and *yellow* stages of stigma development. Bar graph (c) representing the relative expression ($2^{-\Delta\Delta C_t}$) of *CsZCD* over

tubulin gene, bars represent means for three replications and error bars represent standard error of the mean

and accumulation of apocarotenoids occur in parallel (Bustin 2000). Real Time PCR amplification of *CsZCD* and tubulin genes at yellow, orange and scarlet stages of stigma development is shown in Fig. 2. There was 2.69 folds relative increase in *CsZCD* gene expression over tubulin gene expression in scarlet stage and 0.90 and 0.69 folds decrease in *CsZCD* gene expression over tubulin gene expression in orange and yellow stages respectively (Fig. 2c). Real Time PCR analysis revealed that *CsZCD* gene expression gets up-regulated by 8 % from yellow to orange and by 33 % from orange to scarlet stage. Yellow stage of stigma development showed 25 % of gene expression with respect to scarlet stage of stigma development. So there is an abrupt increase in gene expression of *CsZCD* gene from orange to scarlet stage of stigma development. Scarlet stage which is fully developed stigma was treated as positive calibrator. Similar results using reverse transcription PCR have been observed in saffron (Rubio et al. 2004; Castillo et al. 2005). This is the first report which gives fold changes of *CsZCD* gene expression with respect to internal control during stigma development and correlates the *CsZCD* gene expression with apocarotenoid accumulation. To the best of our knowledge, no other study has attempted to analyze relative gene expression of *CsZCD* using real time in saffron. To successfully commercialize saffron spice specific for apocarotenoid values, an understanding of gene expression of key genes responsible for apocarotenoid biosynthesis during stigma development is necessary before we can harness the power of biotechnology for further utilization of these genes and mechanism for its improvement. The prelude to this would be to validate a set of reference genes along with other important genes like lycopene cyclase, β -carotene hydroxylase in order to harmonise the data from various experiments that are expected to follow suit.

Conclusion

During the development of the stigma tissue clear changes in levels apocarotenoid and *CsZCD* gene were observed. A dramatic increase of the apocarotenoid accumulation and *CsZCD* gene expression was observed in the fully developed stigma. The magnitude of expression of *CsZCD* gene need further to be correlated with other genes involved in apocarotenoid biosynthesis, so that complete information

about gene regulation in apocarotenoid biosynthetic pathway can be deciphered which can be further utilized in saffron improvement programme.

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