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Full Length Research Paper

In vitro expression of apocarotenoid genes in *Crocus sativus* L.

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Calli were successfully induced from style explants of *Crocus sativus* L. on Murashige and Skoog's medium supplemented with α-naphthalene acetic acid and 6-benzylaminopurine. Then they were divided into three different types based on developmental stages and pigmentation progress in induced stigma-like structures. RT-PCR method was set up using calli in different developmental stages to detect expression levels of *CsLYC, CsBCH1, CsZCD* and *CsUGT2* genes for apocarotenoids biosynthesis via mevalonic acid pathway in *C. sativus*. The results obtained from *in vitro* investigation of *CsUGT2* expression levels in all three developmental stages were analyzed and compared with the expression levels of selected genes carried out on intact stigmas *in vivo*. Apparently, this gene was only expressed in the stage III of the three *in vitro* different SLSs developmental stages. Furthermore, the expression levels of *CsLYC, CsBCH1, CsZCD* were detected in stage III with fully developed SLSs and were comparable with those of in red intact stigmas.

Key words: *Crocus sativus* L., apocarotenoids, gene expression, mevalonic acid pathway, stigma-like structure.

INTRODUCTION

Saffron is the dried stigmas of *Crocus sativus* L. (Iridaceae) and the most precious and expensive spice. *C. sativus* is characterized by long and high apcarotenoid containing stigmas that are harvested manually and subjected to dessication.

Due to the presence of water soluble carotenoid derivatives in saffron which are known to have antioxidant, anticarcinogenic and antitumors activities, this spice is often used for medicinal purposes (Abdullaev, 2002).

Saffron has three major carotenoid derivatives crocin, picrocrocin and safranal that are responsible for its intense color, bitter taste and aroma, respectively. These components are obtained from the oxidative cleavage of the carotenoids stigmas (Bouvier et al., 2003; Moraga et al., 2004). Biosynthesis of *Crocus* apocarotenoids is regulated through development and enlargement of the stigmas (Grilli-Caiola and Canini, 2004). During saffron development, its stigma changes in color from white 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).

Carotenoids are terpenoid and ubiquitous in nature which can be synthesized *in vivo* through 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-C-methyl-D-erythritol 4-phosphate pathway) takes place in

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Abbreviations: SLSs; Stigma-like structures.

plastids that provides the precursors for carotenoids (Rohmer et al., 1993; Arigoni et al., 1997). The MVA pathway starts with synthesis of mevalonate through three molecules of acetyl CoA and then continues with production of isopentenyl diphosphate (IPP) molecules, geranyl geranyl pyrophosphate (GGPP) (Naik et al., 2003), colorless phytoene, colored lycopene, β-catrotene, (Britton et al., 1998) and zeaxanthin (Bouvier et al., 2003). This pathway possesses many enzymes which are catalyzed the reactions and coded by related key genes such as *PSY*, *LYC*, *CCD*, *BCH* and *ZCD*. β-Carotene with two rings is built up via cyclization of lycopene with lycopene-β-cyclase (*LYC*) (Britton et al., 1998). The hydroxylation of β-carotene in MVA pathway is catalyzed by β-carotenoid hydroxylase that coded by *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 *CsUGT2* gene in chromoplast of stigmas (Teale et al., 1992) and then sequestered into the central vacuole of the fully developed stigmas (Dufresne et al., 1997; Bouvier et al., 2003). *CsUGT2* gene is highly expressed in fully developed stigmas (Moraga et al., 2004) and has glucosylation activity against crocetin, crocetin β-D-glucosyl ester and crocetin β-D-gentibiosyl ester (Côté et al., 2000). The expression levels of *CsUGT2* gene are correlated with the biosynthesis and accumulation of saffron apocarotenoids. In *C. sativus* stigmas, insoluble crocetin is transformed via glucosylation into soluble and stable carotenoid of crocin, which is responsible for saffron intense color. Also Safranal which is responsible for saffron aroma is formed by deglucosylation and dehydration of picrocrocin (Teale et al., 1992).

Our aim is to investigate the expression levels of four selected key genes (*CsLYCb, CsBCH1, CsZCD* and *CsUGT2*) for apocarotenoid biosynthesis in *Crocus sativus* via MVA pathway during developmental stages of *in vitro* formed stigma-like structures.

MATERIALS AND METHODS

Plant materials

Flowering corms of *C. sativus* were collected from saffron field of the University of Tehran (Karaj, Iran) in November to December 2008. Flowering buds with an average length of 7 - 12 cm were excised from corms and used for culture.

Sterilization and culture

Floral buds thoroughly washed in running tap water (30 min) and then sterilized with disinfection fluid containing 0.5% benzylkonium chloride (Tolidaru Co., Iran) solution (15 min), 70% ethanol (2 min), sodium hypochlorite 1% supplemented with a few drops of Tween 80 (20 min); and finally rinsed 3 times with sterile distilled water.

Style organs were aseptically separated from the sterilized buds and cultured on Petri dishes containing MS medium (Murashige and Skoog, 1962) supplemented with 10 mg/l NAA and 10 mg/l BAP for induction of SLSs. All cultures were kept under 22 ± 2°C temperatures in darkness. Calli were subcultured every 28 days and collected from three developmental stages, freeze-dried and then stored at - 80°C for further uses.

RNA extraction

Freeze dried calli from stages I-III were ground in cold and sterilized mortar and pestle into fine powder and total RNA was extracted using RNeasy Plant Mini kit (Qiagen, USA) following the manufacturer's protocol. Quality of the extracted RNAs was checked by measuring the absorbance at 260 and 280 nm by a spectrophotometer (Milton Roy-Spectronic 601-Co USA) and RNAs with ratio of OD_{260}/OD_{280} ranging from 1.2 to 1.5 were used for cDNA synthesis and ribosomal RNA profile were visualized with ethidium bromide staining following agarose gel electrophoresis.

cDNA Preparation

For each sample, 5-10µg of total RNA as template and 18-bp oligo dT primer and a first-strand cDNA synthesis kit RTpreMix (Bioneer Korea) were used for first-strand cDNA synthesis as described by the manufacturer. The synthesized cDNA was stored at -20°C for gene expression study.

RT- PCR

Amplification was carried out using gene-specific forward and reverse primers, cDNA obtained as templates and PCRpreMix (Bioneer Korea) for amplification of Cs*LYC, CsBCH1, CsZCD, CsUGT2* and as well as Cs*TUB* as internal control, according to the manufacturer's instructions. Gene-specific primers were designed (Bouvier et al., 2003; Castillo et al., 2005) to flank introns.

Designed forward and reverse primers were 5'-AGATGGTCTT CATGGATTGGAG-3' and 5'-ATCACACACCTCTCATCCTCTTC-3' for *CsLYC* gene (GenBank access No. AJ888515), 5'-TCGA GCTTCGGCATCACATC-3' and 5'-GCAATACCAAACAG CGTGATC-3', for *CsBCH1* gene (GenBank access No. AJ416711), 5'-GTCTTCCCCGACATCCAGATC-3' and 5'-TCTCTATCGGGC TCACGTTGG-3' for *CsZCD* gene (GenBank access No. AJ489276), 5'-GATCTGCCGTGCGTTCGTAAC-3' and 5'-GATGA CAGAGTTCGGGGCCTTG-3' for *CsGT2* gene (GenBank access No. AY262037) and finally 5'-ATGATTTCCAACTCGACCAGTGTC-3' and 5'-ATACTCATCACCCTCGTCACC ATC-3' for *CsTUB* gene (GenBank access No. AJ489275) (Bouvier et al., 2003; Castilla et al., 2005). The lengths of the products for five genes of *CsLYC*, *CsBCH1*, *CsZCD*, *CsGT2* and *CsTUB* were 247, 495, 241, 400 and 225 bp, respectively.

PCRs were performed (Techne-Touchgene Gradient- FTG RAD 2D-LTD-UK) according to the following conditions: 2 - 5 µg of cDNA was used. Initial denaturizing at 95°C for 5min 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 10 min. The experiments were repeated twice. Subsequently 7 µl of the PCR products were used on 1% (w/v) agarose gels electrophoresis. The images of stained gels with ethidium bromide were scanned and captured by a gel-documentation System (BioDoc-It ™ System USA).

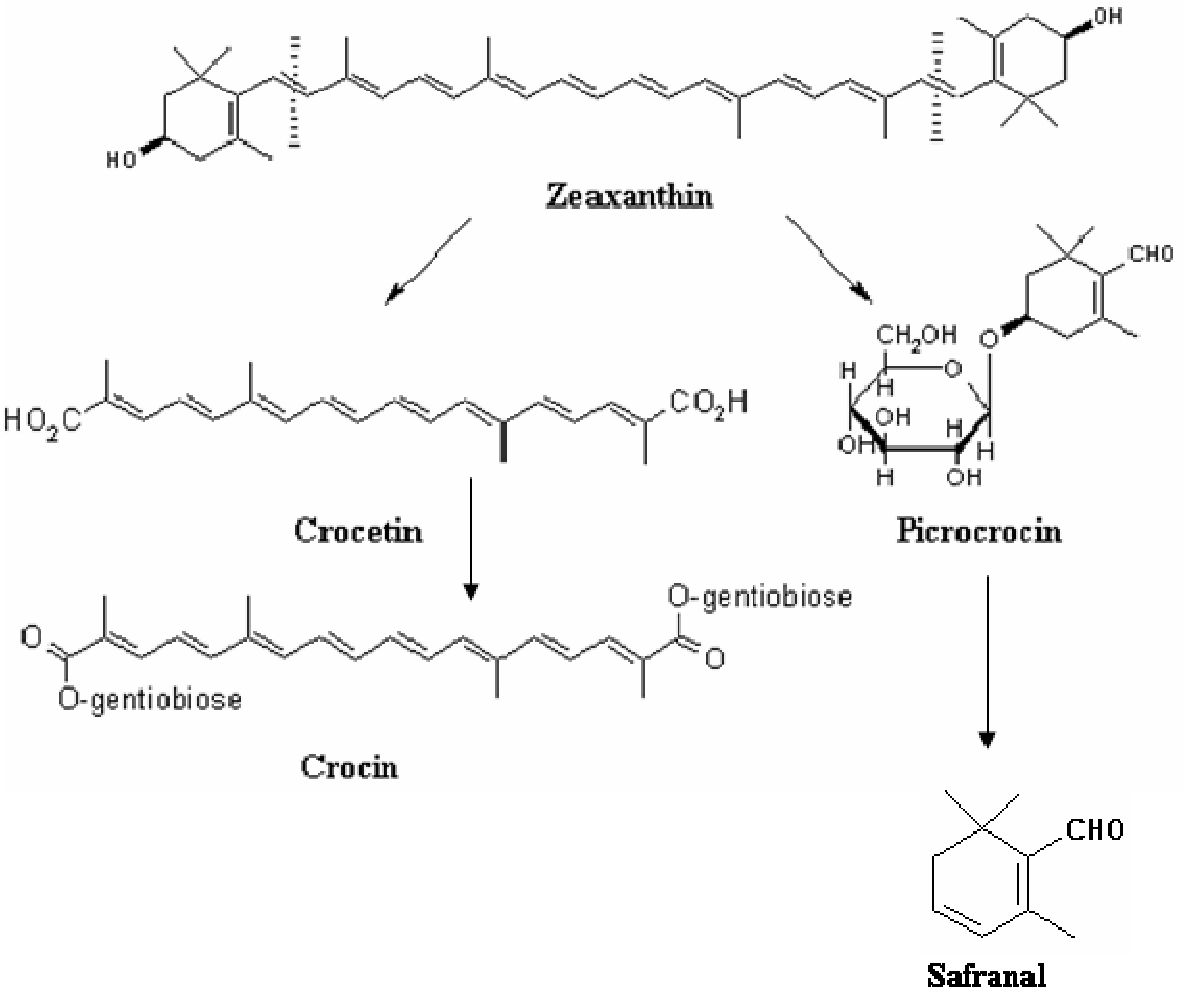

Figure 1. The biogenesis of the crocin and picrocrocin which are derived from the bio-oxidative cleavage of Zeaxanthin at points 7, 8-(7', 8'). Safranal is produced by de-glucosylation of picrocrocin (Naik et al., 2003).

RESULTS AND DISCUSSION

Calli induced from style explants were divided into three types according to their developmental stages as follows: a) Stage I (SI): colorless calli without SLSs; b) Stage II (SII): calli on which pale yellow SLSs were initiated and c) Stage III (SIII): calli with fully developed red SLSs (Figures 2A-C). These stages were comparable with those obtained from *in vivo* studies by Himeno and Sano (1987). They considered three developmental stages in *C. sativus* stigmas based on length, pigmentation and apocarotenoid content.

We have evaluated the expression levels of *CsUGT2* that lead to glucosylation of crocetin in SLSs (Figure 1), on the calli in three different developmental stages. The results obtained from RT-PCR were revealed that *CsUGT2* was highly expressed at stage III (Figures 2E and 3A). In this trial, the expression of *Tubulin* gene was used as an internal control at three stages (Figure 2D). Comparison expression levels of *CsUGT2* in SLSs confirmed the high apocarotenoid biosynthesis and its accumulation in fully developed red SLSs as compared to stages I and II, which had no sign of the gene expression. This result might be due to the low apocarotenoid content in immature SLSs. 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 *CsUGT2* gene (Bouvier et al., 2003; Moraga et al., 2004).

We set up another experiment using calli with mature and red SLSs at stage III to evaluate the *in vitro* expression levels of three genes namely *CsBCH1, CsLYC* and *CsZCD* which are involved in apocarotenogenesis of stigmas via MVA pathway (Castillo et al., 2005). Results showed that all three genes as well as *CsUTG2* are highly expressed in fully developed red SLSs at stage III (Figure 3). Bouvier et al. (2003) reported that biogenesis of crocetin glucosides and picrocrocin are initiated by zeaxanthin cleavage by dioxygnase, which is coded by

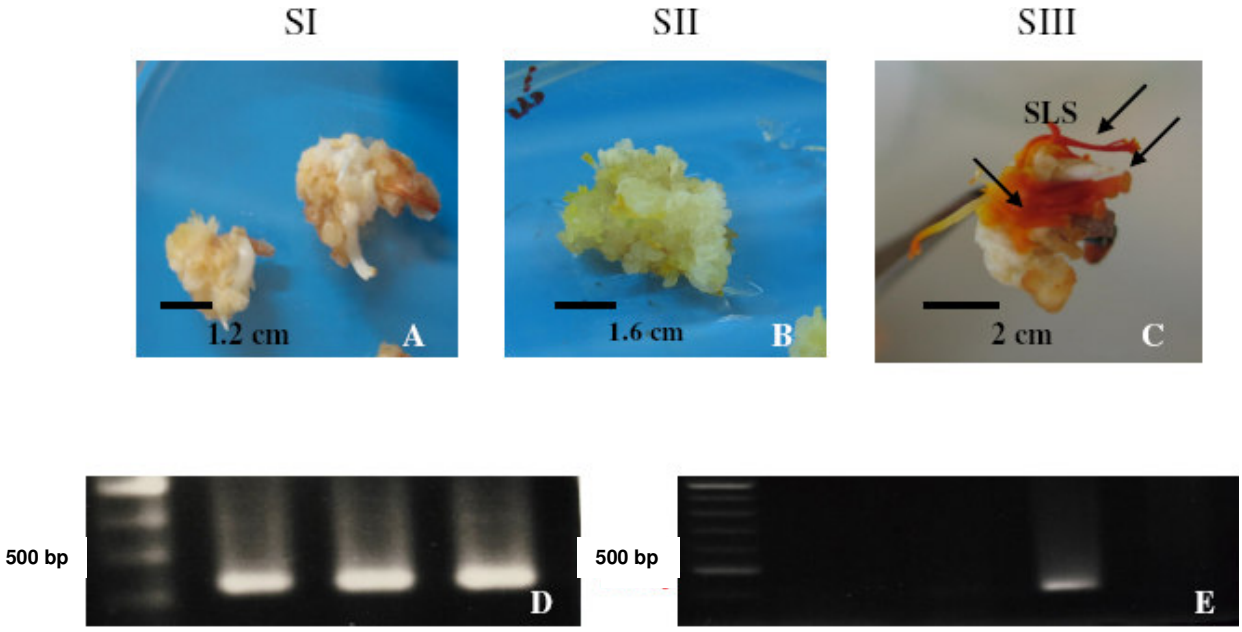

Figure 2. (A) Colorless calli at stage I (SI) without SLSs. (B) Pale yellow calli at stage II (SII) on which SLSs were initiated. (C) Fully developed red SLSs on calli at stage III (S III). (D) Expression levels of *Tubulin* gene (*CsTUB*) in *C. sativus* as internal control at stage I-III (TSI, TSII and TSIII), respectively with 2000 bp-marker (M). (E) Expression levels of glucosyltransferase (*CsGT2*) in *C. sativus* at three stages I, II, III (GSI, GSII and GSIII).

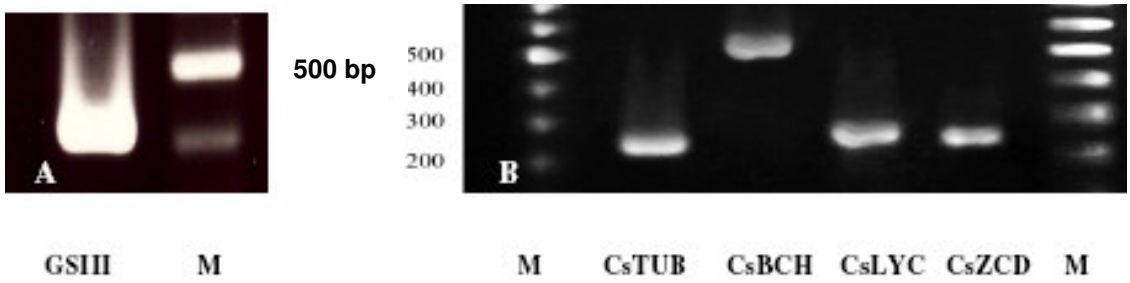

Figure 3. (A). Expression levels of glucosyltransferase (*CsGT2*) gene in fully developed red SLSs of *C. sativus* at stage III (GSIII). (B) Expression levels of three apocarotenogenic genes of: *β*-carotene hydroxylase (*CsBCH*), lycopene-β-cyclase (*CsLYC*), zeaxanthin cleavage dioxygnase (*CsZCD*) at stage III in *C. sativus* with expression of *Tubulin* gene (*CsTUB*) as internal control, 2000 bp-marker (M)

CsZCD. This gene is highly expressed in the fully developed red SLSs. According to Bouvier et al. (2003), our results based on *CsZCD* gene expression showed that this gene to be identical to fully developed natural stigmas.

The next gene studied here was *CsBCH1*, which its expression levels were accompanied with the highest levels of carotenoid accumulation in developed stigmas. Due to the importance of zeaxanthin as a precursor, *CsBCH1* is considered as a key gene for apocarotenoid biosynthesis in saffron stigmas (Bouvier et al., 2003). Castillo et al., (2005) have reported that *CsBCH1* gene has a higher expression level in developed stigmas which is comparable with our *in vitro* results at stage III (Figure 3B).

The fourth gene studied in our experiment was *CsLYC*. Lycopene *β*-cyclase, the product of *CsLYC* gene, catalyzes the cyclization of lycopene to *β*-catrotene. *β*-catrotene and zeaxanthin are the important precursors of saffron apocarotenoid in MVA pathway *in vivo* and *in vitro*. Calli with SLSs at stage III showed high expression of *CsLYC* gene. Himeno and Sano (1987) have reported a comparison between the secondary metabolites, crocin, picrocrocin and safranal in SLSs and intact stigmas using HPLC. They have indicated that the SLSs show similarity to intact stigmas in morphology as well as biochemistry.

In conclusion based on our results, four studied genes are involved in MVA pathway that regulates biosynthesis of apocarotenoids in SLSs as well as natural stigmas in *C. sativus*. To the best of our knowledge, this is the first

report on the expression levels of apocarotenoid genes in saffron *in vitro*.

REFERENCES

Abdullaev FI (2002). Cancer chemopreventive and tumoricidal properties of saffron (*Crocus sativus*). Exp. Biol. Med. 227: 20–25.

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH (1997). Terpenoid biosynthesis from 1-deoxy-d-xylulose in higher plants by intramolecular skeletal rearrangement. Proc. Natl. Acad. Sci. USA, 94: 10600-10605.

Bouvier F, Suire C, Mutterer J, Camara B (2003). "Oxidative remodeling of chromoplast carotenoids: identification of the carotenoid dioxygenase *CsCCD* and *CsZCD* genes involved in *Crocus* secondary metabolite biogenesis". Plant Cell, 15(1): 47-62.

Britton G, Liaaen-Jemsen S, Pfander H (1998). Carotenoids, Biosynthesis and metabolism, Verlag, Basel, Switzerland, Vol. 3.

Castillo R, Fernandes JA, Gômez-Gômez L (2005). Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and its closer relatives. Plant Physiol. 139: 674-689.

Côté F, Cormier F, Dufresne C, Willemot C (2000). Properties of a glucosyltransferase involved in crocin synthesis. Plant Sci. 153: 55-63.

Dufresne C, Cormier F, Dorion S (1997). *In vitro* formation of crocetin glucosyl esters by *Crocus sativus* callus extract. Planta Med. 63: 150-153.

Grilli-Caiola M, Canini A (2004). 'Ultrastructure of chromoplasts and other plastids in *Crocus sativus* L. Plant Biosys. 38(1): 43-52.

Himeno H, Sano K. (1987). Synthesis of crocin, picrocrocin and safranal by saffron stigma-like structures proliferated in vitro. Agric. Biol. Chem. 51: 2395-2400.

Moraga AR, Nohales PF, Perez JA, Gômez-Gômez L (2004). "Glucosylation of the saffron apocarotenoid crocetin by a glucosyltransferase isolated from *Crocus sativus* stigmas." Planta, 219(6): 955-966.

Murashige T, Skoog MF (1962). A revised medium for rapid growth and bioassay with Tobacco tissue cultures. Physiol. Plant 15: 473-497.

Naik PS, Chanemougasoundharam A, Paul Khrana SM, Kalloo G (2003). Genetic manipulation of carotenoid pathway in higher plants. Curr. Sci. 85(10): 1423-1430.

Pfander H, Schurtenberger H (1982). Biosynthesis of C20-Carotenoids in *Crocus sativus*. Rhytochemistry., 21: 1039-1042.

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993). Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem. J. 295: 517-524.

Teale SA, Webster FX, Zhang A (1992). New aggregation pheromone for the bark beetle Ips pini and uses thereof. U.S. Patent 5: 167-955.

Wang W, Chen M, Yang C, Liu W, Lan X, Liao Z (2009). The geranylgeranyl pyrophosphate synthase gene from *Ginkgo biloba*: clonig, characterization and functional identification. Afr. J. Biotechnol. 8(7): 1203-1210.