Biosynthesis of Carotenoids in Saffron

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Abstract

Saffron, the dry stigma of *Crocus sativus*, is made of carotenoids. Very little is known about why carotenoids accumulate in the stigma tissue of *C. sativus*, when this accumulation starts and how these compounds are made in saffron. To help to answer these questions is necessary to identified and characterised the enzymes involved in the biosynthesis of these compounds and study how the genes codifying for these enzymes are expressed and regulated during the stigma development. Carotenoid biosynthetic enzymes are encoded by nuclear genes, and the corresponding precursor proteins are post-translationally imported into plastids. Therefore, translational and post-translational steps are potential targets for regulation of carotenoid biosynthesis. Partial clones for genes encoding PSD, PSY, BCH and NCDE carotenogenic enzymes have been isolated from stigma tissue and their expression analysed during stigma development and carotenoid accumulation.

INTRODUCTION

Carotenoids are a large group of compounds, often highly coloured, which are derived from isoprenoid precursors. These structurally diverse pigments have many different biological functions, such as species-specific coloration, photoprotection, and light harvesting, and they serve as precursor of many hormones. Some bacteria, fungi, and algae also produce carotenoids. Carotenoids are produced in most plants tissues, but are most apparent in flowers and fruits, where probably aid in the attraction of pollinating insects and of animals that contribute to seed dispersal. Although animals do not possess the ability to synthesize their own carotenoids, they are able to acquired carotenoids via their diet. For humans dietary carotenoids are the precursors of vitamin A and retinoids and are also essential as antioxidants in protecting against certain degenerative diseases such as macular degeneration of the eye and in the prevention of prostate, breast and other cancers (Slattery et al., 2000; Nishino et al., 2000).Research into carotenoid biosynthesis has been carried out in many organisms (bacteria, fungi, and plants). In the last decade, molecular approaches have led to the isolation of a number of genes involved in plant carotenogenesis (Bartley and Scolnik, 1995). The earlier steps of the carotenoid biosynthetic pathway are common to all plant and bacterial systems. Nevertheless, a systematic flux analysis of carotenoid biosynthesis, including the determination of individual enzyme activities and functional expression levels, has not yet been carried out. The genes from most of the major enzymes involved in the carotenoid pathway have been cloned from both plant and microbial sources using several strategies. The colour complementation strategy it has been widely used and is based on the expression in *E. coli* of the genes of the *crt* cluster of *Erwinia uredovora* (Misawa et al., 1990; Armstrong, 1994). This system has provide convenient for identifying the function of gene products (Sun et al., 1996; Cunningham et al., 1996; Bartley et al., 1999), dissection of the pathway (Hundle et al., 1993), study of transcriptional regulators of carotenogenic genes (Penfol et al., 1994) and isolation of a new genes encoding enzymes of the carotenoid biosynthetic pathway (Klein et al., 1991) or enzymes catalysing the synthesis of carotenoids precursors (Cunningham and Gant, 2000; Carretero-Paulet et al., 2002). The use of transposable elements as molecular tags for gene isolation (transposon tagging) and

characterization has been exploited extensively in plants. This strategy has allowed the identification of carotenogenic genes as the *Pink sculletum1* (*Ps1*) gene of maize predicted to encode lycopene β-cyclase (Singh et al., 2003). And also it has been possible the isolation of enzymes and proteins involved in the modulation of carotenogenic enzymes. By the use of T-DNA-tagged, *Arabidopsis* populations have been selected for analysis based on their segregation for pigment mutations (Norris et al., 1995). Reverse genetics and chemical mutagenesis have been also exploited for the isolation of plant carotenogenic genes. The fact that carotenoids are coloured made this strategy one of the easiest ways to obtained genes involved in the biosynthesis and accumulation of carotenoids, is the case of the tomato *YELLOW FLESH*, affected in the PSY gene (Fray and Grierson, 1993), the *DELTA* mutation that affects the lycopene ε-cyclase gene (Ronen et al., 2000) and the *tangerine* mutant of tomato that lacks carotenoid isomerase activity (Park et al., 2002). Also is possible to obtain genes that codified for enzymes or proteins not directly involved in carotenoids biosynthesis but that can modulate the activity of other genes. Albino, or variegated phenotypes could be the result of carotenoid deficiencies. The cloning of the gene IMMUTANS has identified a new important factor for phytoene desaturations (Carol et al., 1999; Wu et al., 1999).

The first committed step in carotenoid biosynthesis is the condensation of two geranylgeranyl diphosphate (GGPP) moieties to render phytoene, a colourless carotenoid (Figure 1). The gene responsible for this reaction, phytoene synthase, has been cloned from microbes and plants. In addition GGPP is the precursor of tocopherols, and chlorophyll. As the first committed step in carotenoid biosynthesis PSY has been considerate to be a regulatory point in the pathway (Bramley et al., 1992; Fraser et al., 2000). Phytoene then undergoes four sequential desaturation steps, via intermediates, neurosporene and zetacarotene, to lycopene. As double bonds are added and the number of conjugated double bonds increases, and the carotenoids gain colour. In plants and in cyanobacteria these steps are catalysed by two enzymes, phytoene desaturase (PDS) and zetacarotene desaturase (ZDS), each mediating two symmetrically positioned desaturations, whereas this is achieved in fungi and non-photosynthetic bacteria by only one gene product CrtI. The cyclation of the linear carotenoid lycopene is catalysed by two types of cyclases (Cunningham and Gantt, 2001; Ronen et al., 2000). The lycopene β-cyclase forms the bicyclic β-carotene. The lycopene ε-cyclase adds one ε-ring to form δ-carotene rather than the bicyclic ε-carotene. Only in lettuce ε-carotene have been detected. Lutein and zeaxanthin are dihydroxy xanthophylls that are produced from their corresponding carotene precursors by the action of ε- and β-ring carotenoid hydroxylases. The formation of zeaxanthine involved the β-carotene hydroxylase enzyme, and the corresponding gene has been cloned in the three phyla. The formation of lutein requires the action of a second hydroxylase, the ε-carotene hydroxylase in addition to the β-carotene hydroxylase. The ε-carotene hydroxylase has not been cloned yet from any organism, but it has been identified genetically in *Arabidopsis* (Pogson et al., 1996). Lutein and zeaxanthin have identical chemical formulas and similar structures, but small differences in their ring structures, conjugated double bound systems, and hydroxylation stereochemistry allow for distinct and specialized roles in photosystem structure, light harvesting, and photoprotection.

Apocarotenoids are metabolically derived from the oxidative cleavage of C_{40} carotenoids. The enzymatic oxidative cleavage of carotenoids is found in animals, bacteria and plants. Because the important role and accumulation of some of these compounds, apocarotenoids are considerate as real secondary metabolites. It is the case of vitamin A (retinal), and the phytohormone abcisic acid. In addition, many apocarotenoids have economic significance. Bixin (annatto) is a food colorant frequently used to give dairy products a creamy/yellow colour. The pigment in saffron that gives it a characteristic orange/red colour is the apocarotenoid, crocin. Cleavage products derived from the ends of the zeaxanthin precursor are converted to picrocrocin and safranal (C_{10}), which are important components of saffron's flavour and aroma (Figure 2).

Recently, several plant dioxygenases that cleave carotenoid chromophore at

defined sites have been identified. Maize VP14, the first characterized of this series, catalyses the cleavage of 9-cis-violaxanthin and 9-cis-neoxanthin into xanthoxin, the precursor of abscisic acid (Schwartz et al., 1997). Since the characterization of VP14, ABA biosynthetic enzymes have been identified in several plants (Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000). Based upon sequence similarity, an enzyme necessary for vitamin A biosynthesis has been identified in *Drosophila* (von Lintig and Vogt, 2000) and vertebrates (Kiefer et al., 2001). More recently, enzymes with broad substrate specificity have been identified in *Arabidopsis* (Schwartz et al., 2001).

MATERIAL AND METHODS

Stigmas and tissues from *Crocus sativus* L. plants were collected at two developmental stages defined according to Himeno and Sano (1987): stage II, closed with the first signs of yellow pigmentation; stage IV, closed with orange colour and stage VIII dark red pigmented. All the tissues used were collected and immediately frozen in liquid nitrogen and stored at -80°C until required.

For gene isolation and cloning total RNA and mRNA were isolated from developed saffron stigma by using Ambion PolyAtrack and following manufacturer's protocols (Ambion). The first-strand cDNAs were synthesized by RT from 2 µg of total RNA using a first-strand cDNA synthesis kit (Pharmacia) and an oligo dT primer. These cDNAs were used as templates for the PCR reactions. A Touch Down-PCR program was run using the following conditions: 94°C for 3 min, 10 cycles at 94°C for 30 s, 58°C – 0.2°C/cycle for 30 s, and 72°C for 1 min, and 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The primers used were: BCH-f, 5'-GACCCAAGGAATTGGAAGAAGT-3; BCH-r, 5'-ACCACTTCCTCCAATTCCTTGG-3'; PDS-f, 5'-ARTAYCTGGCNGAYGCNGGNCA-3'; PDS-r, 5'-TRGTKCANTADCGNGTKCGNAC-3' PSY-f, 5'-ARGTNTGYAARGARTAYGCNAARAC-3'; PSY-r, 5'-GNTGNCGNGTYAGNCTKGGNCG-3'; NCDE-f, 5'-TYGAYGGNGAYGGNATGG-3; and NCDE-r, 5'-CYTTCCANAGRTCRAARCA-3'. The amplification products were purified by agarose-gel electrophoresis with the BioTools Extraction Kit (BioTools, Spain), cloned in the pGEM-T easy vector (Promega), and sequenced in both directions using primers from the vector. Computer-aided sequence similarity searches were made using BLAST (NCBI; http://www.ncbi.nlm.nih.gov).

For RT-PCR experiments. Total RNA was isolated from saffron tissues by using the TRIzol reagent (Invitrogen). From total RNA the cDNA was synthesized using the Ready-To-Go You-Prime First-strand Beads (Amersham Biosciences). The amplification procedure by PCR consisted of an initial denaturing step at 94°C for 3 min., followed by 35 cycles of 20 sec at 94°C, 20 sec at 55°C and 30 sec at 72°C. Various initial concentrations of RNA, ranging over 10 fold difference, were used to demonstrate the differential accumulation of the mRNA in the analysed tissues. Gene-pecific oligonucleotides designed from the sequenced clones were used. PCR products were separated by electrophoresis in 3% agarose gels stained with ethidium bromide and were photographed with the IP-010-Sd photo-documentation system (Vilber Lourmat). The program PhotoCaptMw was used to quantify the intensity of the ethidium bromide stained DNA bands from the positive images of the gel.

For carotenoid analysis from stigma tissue, extracts and reverse-phase HPLC was performed as described by Alonso et al (1998).

RESULTS AND DISCUSSION

To the date, there are more than 150 gene sequences encoding for the carotenogenic enzymes that catalysed the biosynthetic steps for dihydroxy xanthophylls. All the amino acid sequences of the carotenogenic enzymes present defined structural or functionally important domains. The marked conservation of the amino acid sequences between monocot and dicot carotenogenic enzymes suggest that subtle perturbations on these functional conserved domains could be detrimental. In addition, sequence

comparison revealed a number of conserved regions in the genes encoding for carotenoid dioxygenases enzymes.

Cloning Saffron Carotenogenic Enzymes by a PCR Strategy

Based upon the degree of sequence similarity of these genes, we followed a PCR strategy for the isolation of the saffron carotenogenic genes. cDNA was prepared by reverse transcription of poly(A)$^+$ from total RNA isolated from saffron stigma. The BCH primers were used to amplified an approximate 480 bp fragment (CsBCH, AJ416711). The sequence of this fragment showed high similarity with carotene hydroxylases from various plants. Degenerated primers for PSY allowed the isolation of two partial clones of y and x bp that showed similarity with several phytoene synthase genes. The PDS primers were used to amplified a fragment of 400 pb. Using the degenerated primers NCDE-F and NCDE-R, 600-bp and 700-bp fragments were amplified. The putative protein sequence of the obtained fragments CsCCD1, CsCCD2 and CsCCD3 (gene bank accession nos:AJ416712, AJ416713, AJ416714) showed high similarity with other NCDE enzymes. Based upon the degree of sequence similarity of these genes and genes present in the database that codify for carotenoid cleavage enzymes, it can be inferred that the encoded proteins catalyse reactions in which a double bond is oxidatively cleaved, yielding two products with aldehyde groups at the site of cleavage. The isolation of full cDNA clones is necessary to determine which, if any, of the identified clones is responsible for the synthesis of saffron's apocarotenoids.

Monitoring Apocarotenoid Accumulation during Stigma Development

A total of eight developing stages have been defined for saffron stigmas, based on the changes in size, pigmentation and accumulation of crocin and picrocrocin (Himeno and Sano, 1987). The carotenoid content in stigma tissue during stage II and stage VIII (anthesis) was determined by HPLC analysis. Stage II is characterized by a low content in crocin and a relatively high level of crocetin and picrocrocin. By contrast stage VIII was characterized by the accumulation of crocin and other crocetin glucosides (Figure 3). Therefore, the activities responsible for the formation of crocetin glucosides are present from stage II to stage VII.

Expression of the Carotenogenic Genes in Saffron

The expression patterns of *bch, psy, pds, Csccd1, Csccd2* and *Csccd3* genes were examined in three stages of stigma development. Expression levels of these genes were monitored by RT-PCR because northern analysis was hampered by low abundance of the transcripts and possible cross-hybridisation between closely related gene family members. Therefore, gene-specific primers were designated for the RT-PCR experiments. The expression levels of *psy* and *pds* reach their maximum preceding the increase in stigma pigmentation. The kinetics of transcript accumulation is very similar for both genes (Figure 4). In contrast, the level of the transcript of *bch* , stayed similar from stage II to stage IV and reach the highest levels at the time before anthesis, coincident with the highest carotenoid accumulation in the tissue (Figure 4). The *Csccd1* mRNA was mainly detected in immature stigmas and less in fully developed stigmas. Similar expression levels were detected in other tissues (Figure 5). C*sccd2* expression levels were quite low in all the cases. The mRNA was mainly detected in fully developed stigmas, and much less in young undeveloped stigmas. No signal was detected for *Csccd2* in the other tissues tested (data non shown). *Csccd3* was mainly detected in leaves and full developed stigma and the expression was reduced in the other stigma developmental stages (Figure 5).

CONCLUSIONS

During the development of the stigma tissue clear changes in levels of total apocarotenoids were observed. A dramatic increase of the apocarotenoid content was observed in the fully developed stigma. In order to investigate whether this increase during the stigma development was accompanied by an changes in the steady-state levels

of carotenogenic genes transcripts, we isolated saffron cDNA fragments for *psy*, *pds*, *bch* and *NCDE*. The individual clones are all homologous to other plant carotenogenic cDNAs.

The steady-state levels of *psy* and *pds* transcripts increased co-ordinately in stage IV, coincident with an increase in stigma coloration and carotenoid biosynthesis. The increases in the expression of these genes preceded the expression of *bch*, which protein products acts downstream in the carotenogenic pathway. The strongest increase of transcripts level was observed for the *bch* transcripts, which is consistent with the highest growth, production and accumulation of zeaxanthin-derivate apocarotenoids in the stigma.

Based on the conserved regions of the plant carotenoid dioxygenase enzymes, we have isolated three clones: *Csccd1*, *Csccd2* and Csccd3 with similarity with plant carotenoid dioxygenases. The high expression levels observed for *Csccd3* suggest that the gene product could be involved in saffron apocarotenoid biosynthesis.

ACKNOWLEDGEMENTS

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Figures

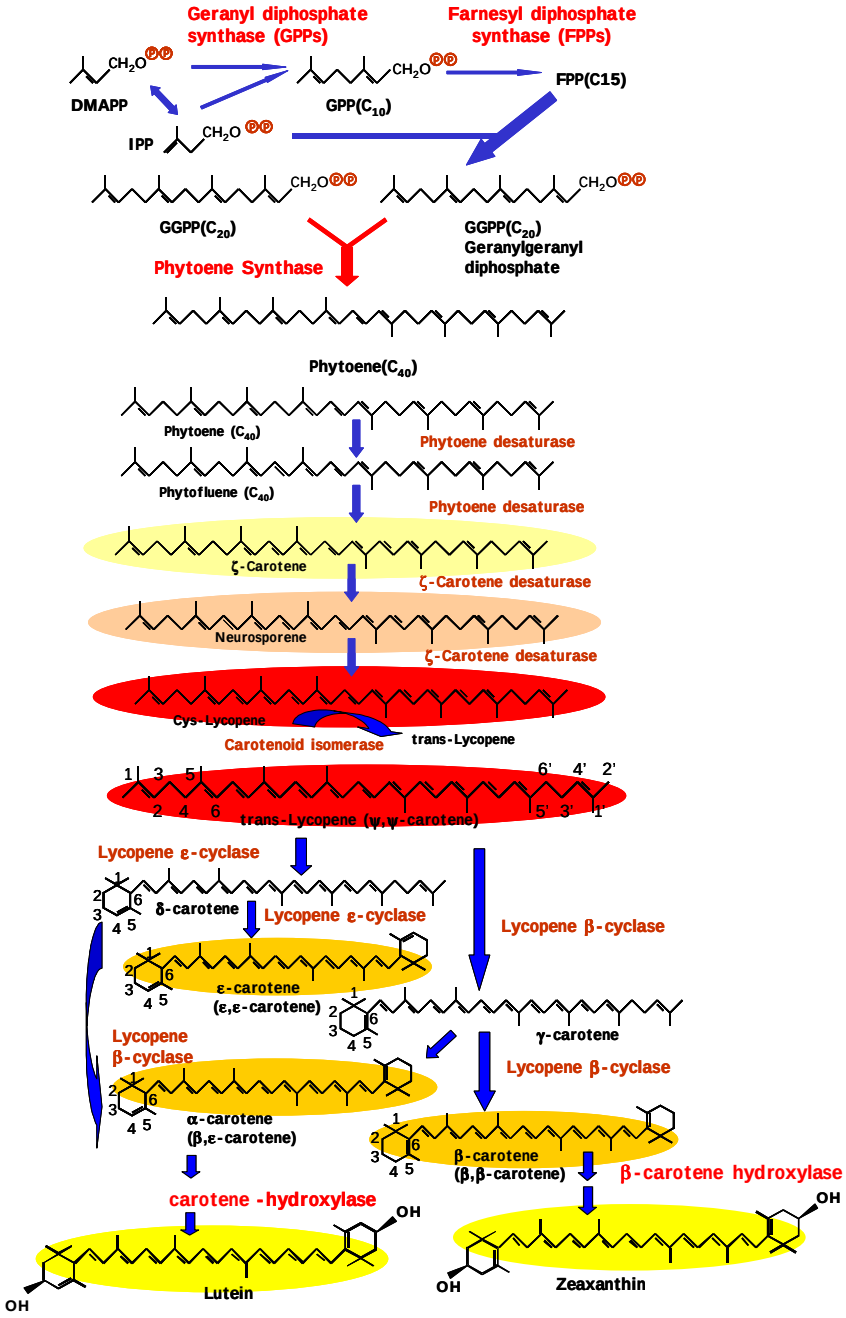

Fig. 1. Carotenoid biosynthesis in plants. The colours indicate the prevalent colour of the compounds in nature. Compounds before ξ-carotene do not absorb light in the visible region of the spectrum. The first steps of the pathway are condensation reactions that results in the formation of geranylgeranyl diphosphate (GGPP), which is considerate the carotenoid precursor.

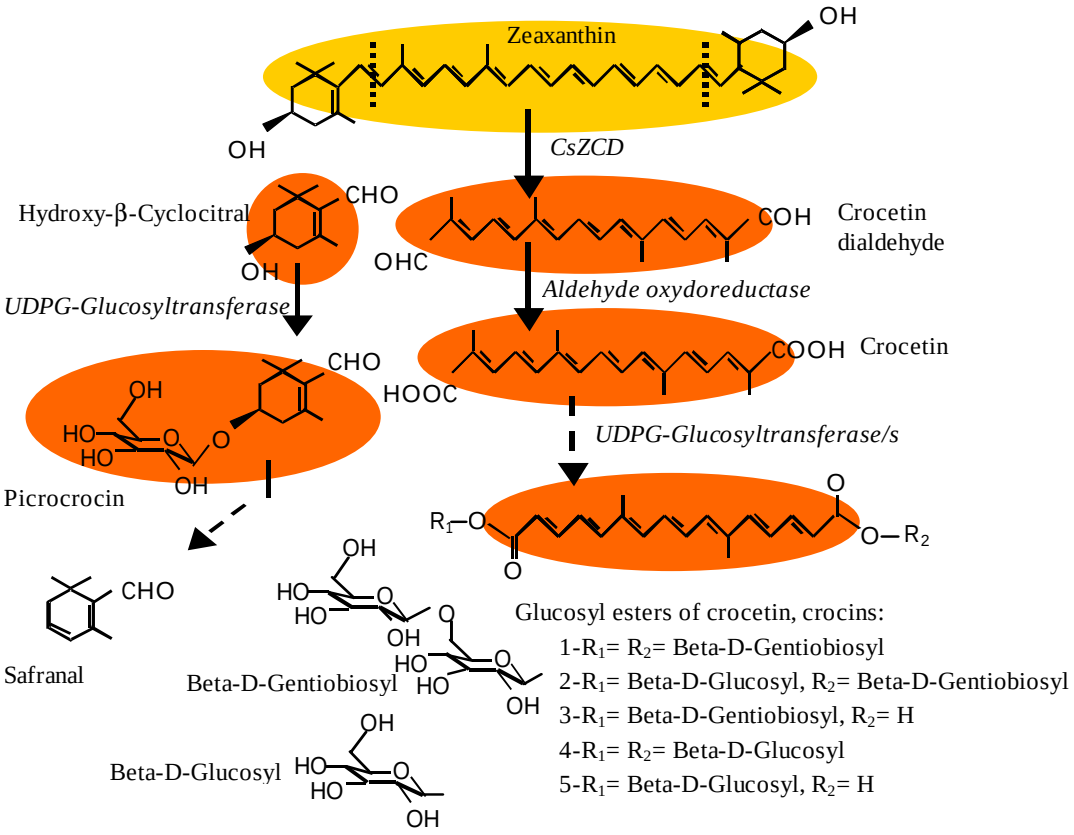

Fig. 2. Possible pathway for the biosynthesis of carotenoid metabolites in *Crocus sativus* stigma. Zeaxanthin, the postulated precursor, is cleaved and the resulted products are modified by different enzymes to render crocetin glucosides and picrocrocin.

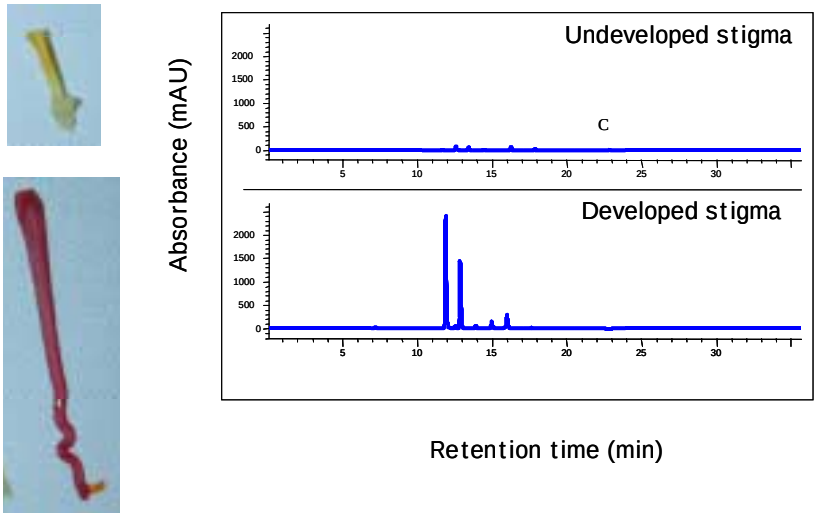

Fig. 3. Developmental stages SII and SVII of *Crocus sativus* stigmas and HPLC analysis of carotenoids and carotenoid-derived metabolites from *Crocus sativus* stigmas in both developmental stages. Compounds were detected by visible light absorbance at 440 nm.

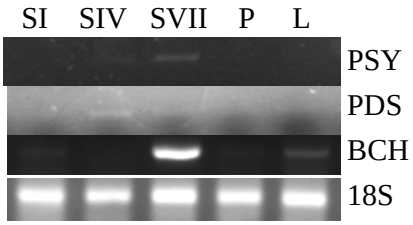

Fig. 4. RT-PCR analysis of different stigma developmental steps and plants tissues. The transcript levels of PSY, PDS and BCH are regulated during stigma development. P, petal, L, leaf. The 18S rRNA was used as endogenous RT-PCR standard. Representative experimental replicates are shown.

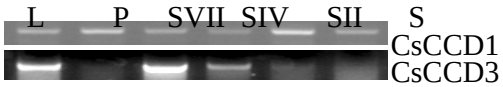

Fig. 5. RT-PCR analysis of different stigma developmental steps and plants tissues. The transcript levels of CsCCD1 and CsCCD3 are differentially regulated during stigma development. CsCCD1 is constitutively expressed in all the tissues.