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Understanding Carotenoid Metabolism in Saffron Stigmas: Unravelling Aroma and Colour Formation

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ABSTRACT

Unusual in plants, the *Crocus sativus* stigma accumulates large amounts of specific glucosylated apocarotenoids that contribute to the colour, flavour and aroma of saffron spice, the processed stigma of this species. These compounds are generated from the oxidative cleavage of carotenoids followed by specific glucosylation steps. Apocarotenoid biosynthesis and its regulation during saffron stigma development is a complex process that occurs alongside the development of the stigma, changing the organoleptic properties of the spice obtained. The expression pattern of the genes involved in the production of these compounds, their precursor's changes as the stigma develops and the control of gene expression are all thought to be the main regulatory mechanisms for alterations in apocarotenoid levels. In *C. sativus* the carotenoid cleavage enzymes are especially important due to their involvement in apocarotenoid formation. Although several of these enzymes have been recently characterized, the enzyme involved in the generation of the main saffron apocarotenoids remains at the moment elusive. This brief review provides a comprehensive picture of the molecular regulation of colour and flavour biosynthesis in *C. sativus* along with what is currently known about the players involved.

Keywords: apocarotenoids, chromoplast, *Crocus sativus*, gene duplication, gene expression
Abbreviations: **ABA**, abscisic acid; **CCD**, carotenoid cleavage dioxygenase; **CHY**, carotene hydroxylase; **GGPP**, geranylgeranyl diphosphate; **GTs**, glycosyltransferases; **HMGR**, 3-hydroxy-3-methylglutaryl CoA reductase; **IPP**, isopentenyl diphosphate; **LCY**, lycopene cyclase; **MEP**, methyl-erythritol phosphate; **MVA**, mevalonate pathway; **NCED**, 9-*cis*-epoxycarotenoid dioxygenase; **PDS**, phytoene desaturase; **PSY**, phytoene synthase; **VDE**, violaxanthin de-epoxidase; **ZEP**, zeaxanthin epoxidase; **ZDS**, zeta-carotene desaturase

CONTENTS

BIOLOGICAL IMPORTANCE OF CAROTENOIDS AND APOCAROTENOIDS

Plant carotenoids are C_{40} carbohydrates with a chain of conjugated double bonds, creating a chromophore that absorbs light in the blue range of the spectrum. Flowers and fruits of many species are coloured due to the accumulation in the chromoplasts of carotenoid pigments that provide distinct colouring to the tissues (Hirschberg 2001). These colours, ranging from yellow to orange and red, visually attract pollinators and facilitate seed dispersal by animals. The carotenoids in flowers and fruits are considered sec-ondary metabolites as they contribute to plant fitness but are not necessarily essential for physiology in these tissues. On the other hand, in photosynthetic organisms, carotenoids have important roles as accessory light-harvesting pigments, effectively extending the range of light absorbed by the photosynthetic apparatus. They also perform an essential photoprotective role by quenching triplet state chlorophyll molecules and scavenging singlet oxygen and other toxic oxygen species formed within the chloroplast (Niyogi 2000). Heat and light stress tolerance is also mediated by carotenoid antioxidants that protect membranes from lipid peroxidation (Davison 2002; Havaux *et al*. 2007; Johnson *et al*. 2007). Constitutive expression of carotenoid biosynthesis genes has been observed in all green tissues examined. By contrast, accumulation of high concentrations of carotenoids in flowers and fruits is correlated with upregulation of genes that enhance the flux of the biosynthetic pathway (Botella-Pavia and Rodríguez-Concepción 2006).

Apart from these functions, carotenoids serve as precursors of several physiologically important compounds, synthesized through oxidative cleavage and generally known as apocarotenoids (Walhberg and Eklund 1998). Representative examples are the ubiquitous chromophore retinal (von Liting and Vogt 2000; Redmond *et al*. 2001), chordate morphogen retinoic acid (Campo-Paysaa *et al*. 2008), phytohormone abscisic acid (Schwartz *et al*. 1997) and fungal phero-

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Invited Review

Fig. 1 Crocetin esters are present in *Crocus sativus* stigmas. *C. sativus* is a small bulbous plant characterized by its long red stigmas.

mone trisporic acid (Burmester *et al*. 2007). In addition, a group of C_{15}-apocarotenoids, the strigolactones, are essential signalling molecules which attract both symbiotic arbuscular mycorrhizal fungi and parasitic plants (Akiyama 2007; Bouwmeester *et al*. 2007). It was recently shown that strigolactone functions as a novel plant hormone regulating shoot branching (Gomez-Roldan *et al*. 2008; Umehara *et al*. 2008). Furthermore, the development of arbuscular mycorrhiza is accompanied by accumulation of cyclohexenone (C_{13}) and mycorradicin (C_{14}) derivatives (Schliemann *et al*. 2008), apocarotenoids arising from the cleavage of xanthophylls and leading to yellow pigmentation of the roots (Walter *et al*. 2000). C_{13}-apocarotenoids, such as β-ionone, constitute an essential aroma note in tea, grapes, roses, tobacco and wine (Rodríguez-Bustamante and Sánchez 2007). These volatile compounds are also synthesized and released by cyanobacteria (Jüttner 1984), and have important ecological roles as sensory signals. Some apocarotenoids, such as bixin and crocetin, represent plant pigments of economic value. Crocetin is synthesised by *C. sativus* (**Fig. 1**) and other related species, *Buddleja* (Liao *et al*. 1999), *Jacquinia angustifolia* (Eugster *et al*. 1969), *Coleus forskolii* (Tandon *et al*. 1979), *Gardenia jasminoides* (Pfister *et al*. 1996), and by the cyanobacterium *Microcystis aeruginosa* (Jütner and Höflacher 1985), but in none of these species does crocetin accumulate at levels as high as those detected in saffron stigmas.

Carotenoids are important not only for plants, but also for animals and humans since they have long been recognized as essential nutrients and beneficial health compounds (Fraser and Bramley 2004). As animals and humans are unable to synthesize carotenoids *de novo*, they have to depend on diet for these essential products. Xanthophylls, such as lutein and zeaxanthin, are essential components of the macular pigments in eyes and offer protection against macular degeneration, the leading cause of age-related blindness (Krinsky *et al*. 2003). "Pro-vitamin A" carotenoids, such as β-carotene and α-carotene, provide the primary dietary sources of vitamin A. Some carotenoids such as lycopene, rich in the tomato, are strong antioxidants and have a protective function in reducing the risk of cancer and cardiovascular diseases (Chan *et al*. 2009). All these important health benefits to animals and humans make the research on carotenoid metabolism exceptionally important.

CAROTENOID BIOSYNTHESIS IN FLOWERING PLANTS

In higher plants, carotenoids are derived from isopentenyl diphosphate (IPP) and are produced in plastids by the methyl-erythritol phosphate (MEP) pathway (**Fig. 2**) (Lichtenthaler 1999; Hunter 2007). Genetic and molecular studies have established that nuclear genes encode all the enzymes of the pathway (reviewed in Cunningham and Gantt 1998; Botella-Pavia and Rodriguez-Concepcion 2006; Giuliano *et al*. 2008; Lu and Li 2008). Four molecules of IPP are converted to geranylgeranyl diphosphate (GGPP) (C_{20}) by the action of IPP isomerase (IPI) and GGPP synthase (GGPS). The condensation of two molecules of GGPP by phytoene synthase (PSY) gives rise to 15-*cis*-phytoene (C_{40}), the first specific compound in the carotenoid pathway (**Fig. 3**). Phytoene is converted into lycopene by the action of two desaturases: phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS). This pathway gives rise to poly-*cis* compounds that are converted to their all-*trans* forms through the action of the carotenoid isomerases CrtISO (Isaacson *et al*. 2002; Park *et al*. 2002) and ZISO (for 15-*cis* zeta-carotene isomerase) (Li *et al*. 2007). Lycopene is the substrate of two competing cyclases: epsilon-cyclase (LCY-ε) and beta-cyclase (LCY-β), acting together on the two ends of the molecule and leading to the formation of α-carotene, whereas the action of LCY-β alone forms β-carotene. Beta- and α-carotene are hydro-xylated by non-heme (CHY1, CHY2) as well as cyto-chrome P450 (CYP97A and CYP97C) hydroxylases. CYP97C hydroxylates the epsilon-ring of lutein (Tian *et al*. 2004). Beta-xanthophylls are epoxidated–de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), giving rise to the xanthophyll cycle. The subsequent opening of the cyclohexenyl 5-6-epoxide ring in violaxanthin gives rise to neoxanthin. A hypothetical enzyme complex containing enzymes from the isoprenoid pathway, IPI and GGPS, membrane-associated enzymes of the carotenoid pathway, PDS, PSY, ZDS, CrtISO, ZISO, and LCY, have long been hypothesized (Cunningham and Gantt 1998).

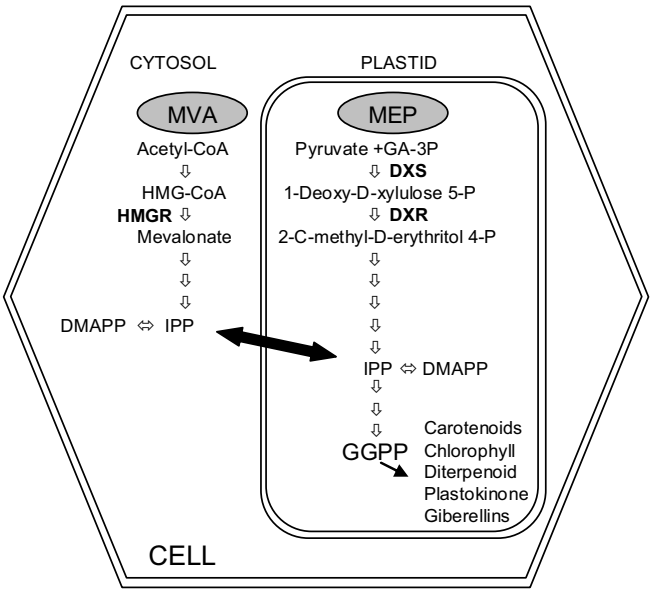

Fig. 2 Location and expression of the selected candidate genes and products in the isoprenoid pathway. A mevalonic acid (MVA) pathway is localized in the cytosol and endoplasmic reticulum to supply IPP for the synthesis of cytosolic and mitochondrial isoprenoids. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is localized in plastids. In all three compartments, IPP (C5) is utilized by prenyltransferases to produce a variety of linear allylic prenyl diphosphates of increasing size. Geranyl diphosphate (C10), farnesyl diphosphate (C15) and geranylgeranyl diphosphate (C20) are key intermediates for the synthesis of the wide range of end products derived from the isoprenoid pathway.

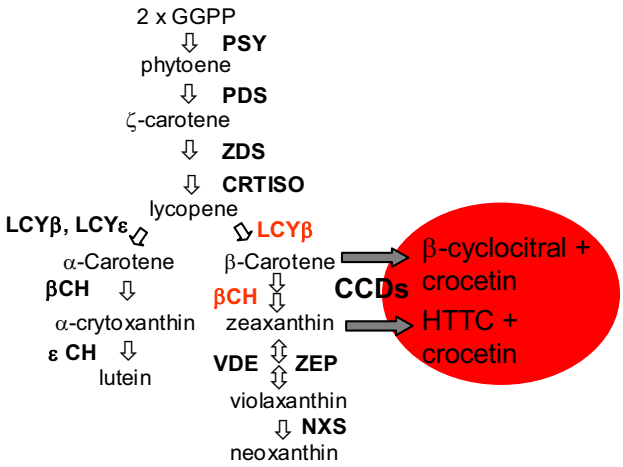

Fig. 3 Schematic carotenoid biosynthetic pathway in *C. sativus* stigma and main apocarotenoids generated. Enzymatic reactions are represented by arrows. GGPP, geranyl geranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, carotene desaturase; CRTISO, carotene isomerase; LCYB, lycopene B-cyclase; LCYε, lycopene E-cyclase; BCH, B-carotene hydroxylase; ECH, E-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCD, carotenoid cleavage dioxygenase; HTTC, 2,6,6-trimethyl-4-hydroxy-1-carboxaldehyde-1-cyclohexene. In red text are the key enzymes that control carotenoid biosynthesis in saffron stigma.

CHROMOPLAST-SPECIFIC CAROTENOID BIOSYNTHESIS PATHWAY

The amounts and identities of the various carotenoids in the photosynthetic membranes of green plants are relatively well conserved. In contrast, carotenoid pigmentation in non-green plant plastids varies broadly both in quantity and composition. In flowers, fruits and roots, carotenoids are synthesized and also located in chromoplasts, with organelles being related to and often derived from these chloroplasts. There is also great diversity in the morphology of chromoplasts (Sitte *et al*. 1980). Simultaneous with chromoplast formation, an active synthesis of carotenoids begins and special carotenoid-bearing structures start to develop. These are structurally diverse, and several types are often present in the same organelle. Some carotenoid-containing structures may be transient and disappear again in senescent chromoplasts. The most common carotenoid-containing structures present in senescent chromoplasts are plastoglobules, spherical lipid droplets that lie singly or in groups in the chromoplast stroma (Brehelin and Kessler 2008). Furthermore, in plastoglobules from red pepper chromoplasts, Ytterberg *et al*. (2006) identified the carotenogenic enzymes ZDS, LYC-β or CYC-β and two β-carotene hydroxylases.

Recent studies suggest the presence of a specific chromoplast pathway for carotenoid biosynthesis in order to enhance carotenogenesis in flowers and to increase pigmentation in fruit chromoplasts. This pathway is generated by gene duplication of specific carotenogenic genes (Gallagher *et al*. 2004). Genes coding for GGPS, PSY, LYC, and CHY have been isolated from different plants, and each is encoded by at least two genes. In each of these enzymes, one isoform is constitutively expressed in leaves, whereas the other is specific for chromoplasts in flowers and/or fruits (Galpaz *et al*. 2006). In *C. sativus* two genes for PSY (Rubio *et al*. 2003), CHY (Castillo *et al*. 2005) and LCY (Ahrazem *et al*. 2009) have been identified. The presence of a specific chromoplast pathway of carotenoid biosynthesis in *C. sativus* might play an important role in the high apocarotenoid accumulation in stigma tissue.

Transcriptional regulation of the chromoplast-specific carotenoid gene expression appears to be a major mechanism regulating the biosynthesis and accumulation of specific carotenoids. Examples are found in tomato and pepper fruits and flowers, where the accumulation of specific carotenoids coincides with increased expression of upstream carotenogenic genes and reduced expression of genes downstream from the accumulating carotenoids (Hirschberg 2001). Post-transcriptional regulation at enzymatic levels also plays a role in controlling carotenoid biosynthesis and accumulation. Metabolic turnover of carotenoids by carotenoid cleavage dioxygenases helps to maintain the steady levels of carotenoids, and also produces important apocarotenoid molecules.

PLANT CAROTENOID CLEAVAGE OXYGENASES AND THEIR APOCAROTENOID PRODUCTS

The first gene identified as encoding a carotenoid cleavage dioxygenase (CCD) was the maize *VIVIPAROUS14* (*Vp14*) gene which is required for the formation of abscisic acid (ABA), an important hormone that mediates responses to drought stress and aspects of plant development such as seed and bud dormancy (Zeevaart and Creelman 1988). The VP14 enzyme cleaves at the 11,12 position of the epoxycarotenoids 9′-*cis*-neoxanthin and/or 9-*cis*-violaxanthin and is now classified as a 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Schwartz *et al*. 1997), a subclass of the larger CCD family. Since the discovery of Vp14, many other CCDs have been shown to be involved in the production of a variety of apocarotenoids (Auldridge *et al*. 2006a). In insects, the visual pigment retinal is formed by oxidative cleavage of β-carotene by β-carotene-15,15′-dioxygenase (von Lintig and Vogt 2000). Retinal is produced by an orthologous enzyme in vertebrates, where it is also converted to retinoic acid, a regulator of differentiation during embryogenesis (Wyss *et al*. 2000). A distinct mammalian CCD is believed to cleave carotenoids asymmetrically at the 9,10 position (Kiefer *et al*. 2001) and, although its function is unclear, recent evidence suggests a role in the metabolism of dietary lycopene (Hu *et al*. 2007). By contrast, in flowering plants no 15,15′ cleave activity has been detected. In addition to NCED, the CCDs from plants are distributed into four classes: CCD1, CCD4, CCD7 and CCD8. CCD1 and CCD4 seem to be involved in the production of aroma volatiles. The first member of the CCD1 subfamily was identified from *Arabidopsis thaliana* (Schwartz *et al*. 2001). Sequence homology then allowed the identification and characterization of orthologs from several plant species, such as *Crocus sativus* (Bouvier *et al*. 2003; Rubio *et al*. 2008), *Lycopersicum sculentum* (Simkin *et al*. 2004a), *Vitis vinifera* (Mathieu *et al*. 2005), *Cucumis melo* (Ibdah *et al*. 2006), petunia (Simkin *et al*. 2004b), *Zea maize* (Vogel *et al*. 2008), *Fragaria* × *ananassa* (García-Limones *et al*. 2008), *Medicago truncatula* (Floss *et al*. 2009), *Oryza sativa* (Ilg *et al*. 2009) and *Rosa damascena* (Fong-Ching *et al*. 2009). CCD1 is a non-heme enzyme which uses oxygen to cleave a variety of carotenoid substrates symmetrically at the 9,10 and 9′,10′ positions of cyclic and acyclic carotenes. These generate C_{14} dialdehydes, which are common to all carotenoid substrates, and two variable end-group-derived C_{13} ketones. The wide substrate specificity of plant CCD1s allows the production of divergent volatile C_{13} compounds, including β-ionophores, α-ionones, pseudoionone and geranylacetone (Schwartz *et al*. 2001). The CCD1 enzymes also recognize the 5,6 or 5′,6′ bond positions of linear carotenes leading to the volatile C_8 ketone 6-methyl-5-hepten-2-one (Vogel *et al*. 2008), and the 7,8 and 7′,8′ double bonds of acyclic carotenoid ends leading to geranial (Ilg *et al*. 2009).

We have shown that CCD4 enzymes cleave double bonds at 9,10(9′,10′) positions, and seem to be more active than the CCD1 enzymes, at least for the β-carotene substrate (Rubio *et al*. 2008). Recently, the activity of other CCD4 enzymes have been characterized from apple (*Malus x domestica*, MdCCD4), chrysanthemum (*Chrysanthemum x morifolium*, CmCCD4a), rose (*Rosa x damascena*, RdCCD4), osmanthus (*Osmanthus fragrans*, OfCCD4), and *Arabidopsis*, AtCCD4 (Huang *et al*. 2009). CmCCD4a and MdCCD4 cleaved β-carotene to yield β-ionone, while

RdCCD4, and AtCCD4 cleaved 8'-apo-β-caroten-8'-al to yield β-ionone, which demonstrates that all the CCD4 enzymes cleave their substrates at 9,10 and 9',10' positions. Although CCD1 and CCD4 enzymes cleave carotenoids at the same positions (9,10 and 9',10′), CCD4 enzymes seem to be more substrate specific than CCD1. CCD4s could not cleave linear carotenoids such as lycopene and ζ-carotene, or carotenoids containing a hydroxyl group such as zeaxanthin and lutein. It seems that CCD4s only cleave cyclic non-polar carotenoids such as β-carotene. In addition, CCD4s are targeted at the plastids (Ytterberg *et al*. 2006; Rubio *et al*. 2008), whereas CCD1 enzymes are cytosolic and lack a chloroplast transient peptide in their sequences (Bouvier *et al*. 2003; Tan *et al*. 2003; Simkin *et al*. 2004a; Auldridge *et al*. 2006b). The plastid, or more exactly, the plastoglobule location of the CCD4 enzymes, allows these enzymes to obtain access to plastid carotenoids, while the CCD1 activity is limited to carotenoids outside these organelles or once these organelles have lost homeostasis or are targeted for degradation.

The CCD7 and CCD8 enzymes are implicated in the generation of the apocarotenoid hormone strigolactone involved in shoot branching. CCD7 and CCD8 are conserved across angiosperm species including monocotyledons: MAX3 (more axillary shoots), RMS5 (ramosus) and HTD1 (high tillering dwarf)/D17 encode (CCD7) (Sorefan *et al*. 2003; Booker *et al*. 2004, 2005; Johnson *et al*. 2006; Zou *et al*. 2006). Recombinant AtCCD7 exhibits regioselectivity for the 9,10 position similar to CCD1, yet it cleaves only once asymmetrically, resulting in C_{13} and C_{27} products (Schwartz *et al*. 2004). MAX4, RMS1, D10 and DAD1 (decreased apical dominance) encode another sub-class of CCDs designated as CCD8 (Snowden *et al*. 2005; Sorefan *et al*. 2006; Arite *et al*. 2007; Simons *et al*. 2007), and cleave the product generated by CCD7 (Schwartz *et al*. 2004; Alder *et al*. 2008), but are also able to act directly on carotenoid substrates (Auldridge *et al*. 2006b). Therefore, CCD7 and CCD8 might catalyse sequential carotenoid cleavage reactions, although further studies should be carried out to ascertain their role in plants.

REGULATION OF CAROTENOID BIOSYNTHESIS DURING STIGMA DEVELOPMENT IN SAFFRON

Since carotenoids are just one class of isoprenoids, the regulation of their formation must involve the co-ordinated flux of isoprenoid units (IPP) into the C_{40} carotenoids as well as the other branches of the isoprenoid pathway. In higher plants, the five-carbon building blocks of all terpenoids, IPP and dimethylallyl diphosphate, are derived from two independent pathways localized in different cellular compartments: the MEP or nonmevalonate pathway, localized in the plastids, and the cytosol-localized mevalonate pathway (MVA) (**Fig. 2**). The MEP pathway furnishes the formation of monoterpene-, diterpene- and carotenoids (Lichtenthaler *et al*. 1997; Rhomer 1999). In snapdragon flowers it provides both monoterpene and sesquiterpene formation (Dudareva *et al*. 2005). During the development of *C. sativus* stigmas, the 1-deoxylulose-5-phosphate synthase, DXS, which is the first enzyme specific to the MEP pathway, is highly expressed in all developmental stages, whereas the 3-hydroxy-3-methylglutaryl CoA reductase, HMGR, the enzyme which catalysed the third step of the MVA pathway, is expressed at low levels (Rubio *et al*. 2009), suggesting that DXS plays an important role in the control of isoprenoid biosynthesis in the stigma tissue, characterized by high levels of carotenoid derivatives.

Carotenogenesis in ripening fruit and petals has been studied extensively (Hugueney *et al*. 1996; Hirschberg 2001; Moehs *et al*. 2001; Zhu *et al*. 2002, 2003; Kato *et al*. 2004). In these tissues development and carotenoid accumulation parallels chloroplast to chromoplast transition. In *C. sativus*, however, the development of the stigma occurs concomitantly with the amyloplast to chromoplast transition and the stigma never turns green during this process. Chromoplasts in *C. sativus* have a tubular structure and show numerous plastoglobules and vesicles (Grilli-Caiola and Canini 2004). Stigma development also parallels carotenoid accumulation (Castillo *et al*. 2005), making this tissue a good model system to study carotenoid formation and accumulation during this transition process. Carotenoid accumulation that occurs in the transition of green to red in tomato fruit chromoplasts is mediated by transcriptional regulation of *PSY* and *PDS*. In *C. sativus* the transcript levels of these carotenogenic genes, *CsPSY* and *CsPDS,* are relatively low and modulated during development, displaying their lowest levels at early developmental stages and the highest in the red and preanthesis stages. In contrast, the transcript levels of the chromoplast-specific lycopene cyclase, *CstLcyB2a*, and the chromoplast specific carotene hydroxylase, together with the *CsCHY1* genes, are much higher and accumulate in the red and scarlet stages of saffron stigmas, which is consistent with the production and accumulation in this tissue of β-carotene and zeaxanthin, the main carotenoids present in stigma extract and the precursors of saffron apocarotenoids (Castillo *et al*. 2005; Ahrazem *et al*. 2009). Moreover, the analysis of the stigmas of several *Crocus* species showed that quantitative and qualitative changes in the carotenoid pigments were related to the expression levels of *CstLcyB2a* and *CsCHY1*, thus supporting the hypothesis that the major mechanism controlling carotenoid formation in *C. sativus* is transcriptionally regulated at the level of both genes. All these data suggest that the reactions catalysed by CstLcyB2a and CHY1Cs enzymes could be the limiting steps in the formation of saffron apocarotenoids in the stigma tissue, due to the accumulation of the respective substrates and products. However, the levels of carotenoids in the developed stigmas are much lower compared with the massive accumulation of apocarotenoids, suggesting a high flux rate in the carotenoid pathway and an important role for the carotenoid cleavage dioxygenases.

REGULATION OF APOCAROTENOID BIOSYNTHESIS DURING STIGMA DEVELOPMENT IN SAFFRON

The flavour of a particular food or ingredient can be thought of as the sum of a complex interaction between taste receptors, the ortho- and retronasal olfactory systems, mouth texture, and visual appearance (Shepherd 2006). Saffron constitutes a complex mixture of volatile and non-volatile compounds that contribute to the overall aroma and flavour of this condiment (Tarantilis and Polissiou 1997). The major components of saffron are the apocarotenoids *cis*- and *trans*-crocins, picrocrocin (β-D-glucopyranoside of

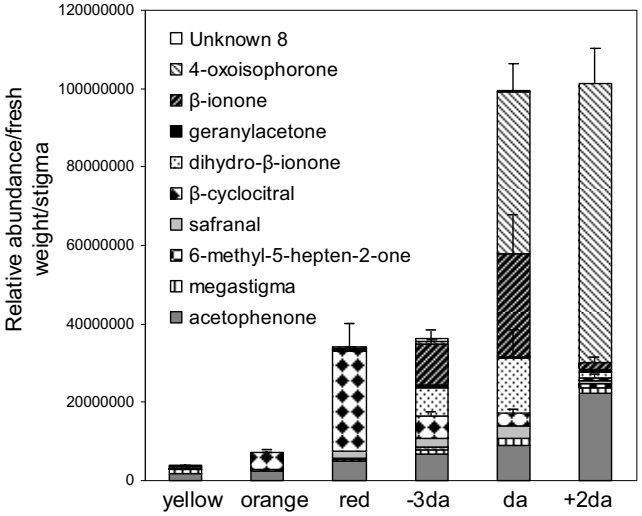

Fig. 4 Relative levels of apocarotenoid volatiles emitted during different stages of stigma development. Three days before anthesis (-3da), day of anthesis (da), and two days after anthesis (+2da).

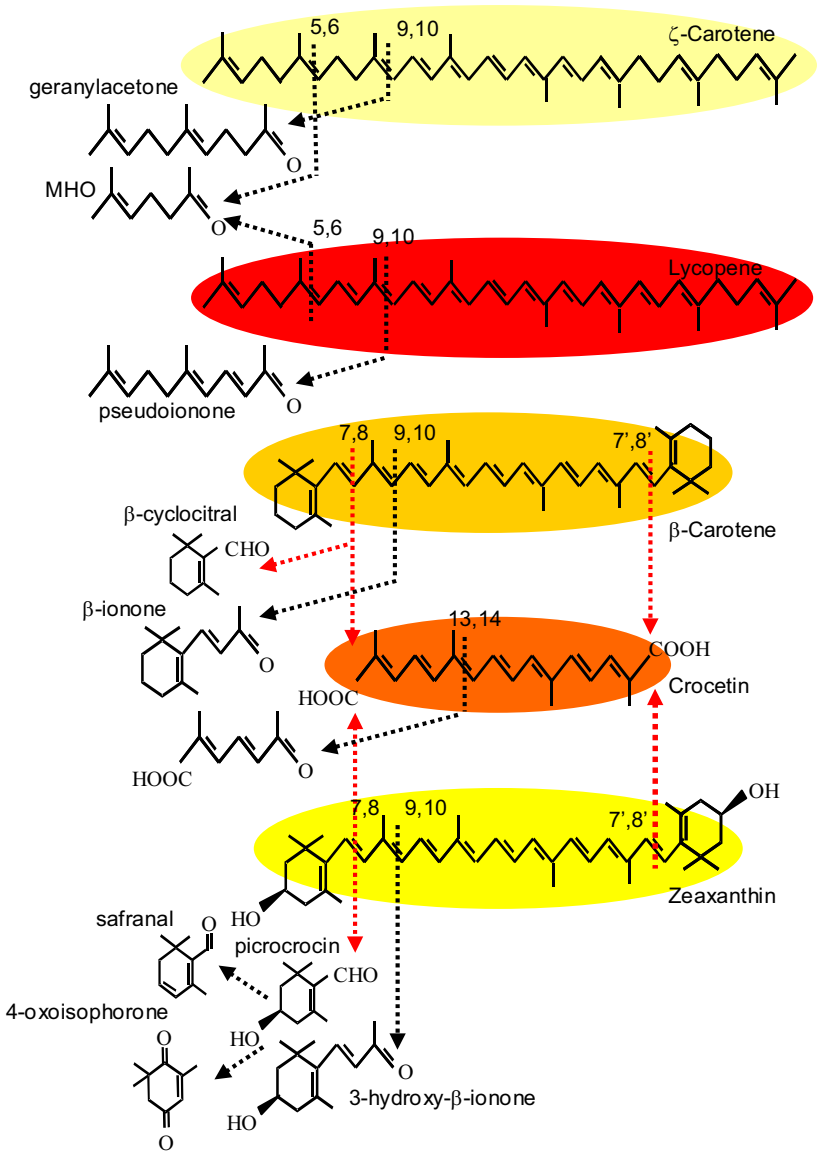

Fig. 5 Scheme for the reactions potentially catalyzed by CCD proteins in *C. sativus* stigmas. The carotenoid substrates (right) when cleaved would yield two monoaldehydes and a central dialdehyde product (left). MHO, 6-methyl-5-hepten-2-one.

hydroxyl-β-cyclocitral), and its degradation product, the odour-active safranal (Kanakis *et al*. 1994). However, recent studies reveal a different volatile composition in unprocessed tissue (Rubio *et al*. 2008, 2009), suggesting the presence of a degradation process responsible for the organoleptic properties of saffron from preformed apocarotenoid compounds (Dauria *et al*. 2006), as has also been observed in other spices (Mookherjee *et al*. 1990). In addition to crocetin, picrocrocin, and its degradation product, safranal, other minor apocarotenoid volatiles have been found in the stigma tissue (Rubio *et al*. 2008, 2009). Although in minor amounts, these are sufficient to have an impact on human perception (Goff and Klee 2006). The levels of these volatiles increases as stigma develops (**Fig. 4**), suggesting an involvement with pollinator attraction (Rubio *et al*. 2008). Based on their chemical structures these compounds are likely products of oxidative carotenoid cleavage. The potential relationships between these volatiles and their carotenoid precursors following 5,6 or 5',6', 7,8 or 7',8', and 9,10 or 9',10' oxidative cleavage are shown in **Fig. 5**.

As previously mentioned, the CCD1 enzymes have broad substrate specificity, cleaving multiple linear and cyclic carotenoids at either the 5,6 or 9,10 double bond positions, and CCD4 enzymes are active over β-carotene recognizing 9,10 and 9',10' positions. Two CCD1 enzymes have been isolated from *C. sativus* (Bouvier *et al*. 2003; Rubio *et al*. 2008). *CsCCD1a* expression, however, remains practically constant throughout stigma development, with no expression in the senescent stigmas. The *CsCCD1b* gene reached maximum expression in the earlier stages and its expression dropped in the scarlet stages. By contrast, *CsCCD4a* and *b* reached the maximum expression levels in the scarlet stages (Rubio *et al*. 2009) which coincide with the highest levels of their reaction products.

The biogenesis of the main colour principles, crocins, and safranal, was proposed to be derived by bio-oxidative cleavage of zeaxanthin (Pfander and Schurtenberger 1982) by a 7,8-7′,8′ cleavage reaction. This reaction was found to be catalysed by a 7,8-7′,8′ CCD (CsZCD, zeaxanthin cleavage dioxigenase) obtained by cloning the *CsZCD* gene from *C. sativus*. CsZCD specifically catalyses the formation of crocetin dialdehyde and two molecules of 3-hydroxy-β-cyclocitral from zeaxanthin (Bouvier *et al*. 2003). Recently, two genes coding for CCD enzymes have been isolated and characterized from *C. sativus*, *CsCCD4a* and *CsCCD4b*. CsCCD4a (580 aa) and CsCCD4b (569 aa) showed 98% similarity in 369 amino acids with the previously isolated CsZCD (369 aa) (Bouvier *et al*. 2003). However, CsCCD4a and b are more than 200 amino acids longer than CsZCD, indicating that most likely the previously reported sequence represents an N-t truncated version. Furthermore, CsZCD lacks important residues and domains for the dioxygenase activity (Kloer *et al*. 2005; Kloer and Schulz 2006). CsCCD4a and CsCCD4b have a 9,10(9′,10′) cleavage acti-

vity and since they did not display the expected 7,8(7′,8′) activity for crocetin formation, these enzymes are most probably involved in the generation of β-ionone during stigma development (Rubio *et al*. 2008). Recent studies show the biogenesis of crocetin from β-carotene and zeaxanthin (Rubio *et al*. 2009). The volatile β-cyclocitral is generated by the cleavage of β-carotene at the 7,8 (7′,8′) positions, and is detected during stigma development of *C. sativus*. Therefore, the dioxygenase cleavage enzyme involved in crocetin biosynthesis should be able to recognize β-carotene and zeaxanthin as substrates.

GLUCOSYLATION OF SAFFRON APOCAROTENOIDS

The final step in the biosynthesis of the 20-carbon esterified carotenoid crocin is the transformation of the insoluble crocetin into a soluble and stable storage by glucosylation. The enzymes leading to glycoside formation, the glycosyltransferases (GTs), transfer nucleotide-diphosphate-activated sugars to low molecular weight substrates. A broad range of different carbohydrate moieties can be added, recruiting all forms of sugars independently (monoglycosides), in parallel, or in chains (di-, tri-glycosides, etc.). This gives rise to a broad spectrum of glycosidic structures for any given aglycone. The GTs are encoded by large multigene families and can be identified by a signature motif in their primary sequence (Hughes and Hughes 1994), which classifies them as a subset of Family 1 GTs. There is a need for isolating and characterizing the specific enzyme that transfers or hydrolyzes a β-glucoside whose aglycone moiety is of interest in food quality. Such biochemical data are crucial when making practical decisions as to whether or not enzymes from host plants or other sources should be added to drinks and beverages before, during or after processing in order to enhance flavour, aroma and other quality factors. Likewise, such data are essential for targeting enzymes with desirable properties for overproduction in transgenic microbial or plant hosts and improvement of their catalytic properties and stability for specific uses by genetic engineering.

A pathway for glucosylation of encapsulated crocetin was proposed in 1997 by Dufresne *et al*., who indicated that crocetin glucosylation into crocin is sequential and may involve two different glucosyltransferase activities. A UDP-glucose: crocetinglucosylester 6"-*O*-glucosyltransferase involved in the glucosylation of the carboxylic end of crocetin and an uridine-5'-diphosphoglucose (UDP-glucose)-crocetin 8,8'-glucosyltransferase catalysing the glucosylation of hydroxyl groups of the glucose already linked to the aglycone with formation of gentiobiosyl esters (Côte *et al*. 2000, 2001). A molecular biology approach allowed the isolation of *UGTCs2*, a glucosyltransferase enzyme involved in crocin and crocetin glucosylation in *C. sativus* stigmas (Rubio *et al*. 2004). Concomitant with stigma development, the accumulation of crocins of higher glucose content takes place (Rubio *et al*. 2009), a result which agrees with the expression patterns observed for *UGTCs2*. The *in vitro* results with the recombinant enzyme UGTCs2 showed that UGTCs2 activity promotes the formation of highly glucosylated crocetin esters using crocetin, crocetin β-D-glucosyl ester and crocetin β-D-gentibiosyl ester, thus indicating that the enzyme displays *in vitro* the two types of activities described by Dufresne *et al*. (1997). Furthermore, the enzyme was able to form a pigment more polar than crocin that was not detected in saffron stigmas. Similarly, Dufresne *et al*. (1999), using *C. sativus* cell suspensions supplemented with crocetin, detected the presence of more polar crocetin glucosides as major products that were not detected in stigma tissue.

Besides crocins and picrocrocin, other glucosylated apocarotenoids have been identified in *C. sativus*, but at low levels. The list includes a monogentiobiosyl ester, structurally related with crocin, which could be generated by the oxidative cleavage of zeaxanthin at the positions 7,8 and 13,14 (Straubinger *et al*. 1997), the β-D-glucopyranosides (4R)-4-hidroxy-3,5,5-termetilciclohex-2-enone, (4S)-4-hydroxy-3,5,5-termetilciclohex-2-enone, and the (4S)-4-hydroxymethyl-3,5,5-termethylcyclohex-2-enone, along with seven more apocarotenoids related to other compounds previously identified in various plant species (Straubinger *et al*. 1998). Thus, different glucosyltransferase activities should be responsible for the glucosylation of these compounds. In fact, several EST clones from the Saffron Gene Database (D'Agostino *et al*. 2007) encode putative glucosyltransferase enzymes, which will be of interest in determining the function and substrate specificity of these enzymes.

OTHER PLAYERS INVOLVED IN CAROTENOID ACCUMULATION

Despite significant progress concerning carotenogenesis in plants, the control mechanisms regulating overall carotenoid biosynthesis and accumulation remain an enigma. Although news strategies such as combinatorial genetic transformation, allowed to gain insight into the bottlenecks in the carotenoid pathway (Zhu *et al*. 2008). However, the regulatory genes that positively or negatively modulate carotenogenesis and the regulatory factors that govern carotenogenic gene expression in plants remain unknown. Mutant analysis allowed the isolation of the *Or* gene (Lu *et al*. 2006), which encodes a plastid-associated protein containing a Cys-rich domain found in DnaJ-like molecular chaperones and is expressed highly in tissues normally rich in proplastids or noncoloured plastids. Rather than directly regulating carotenoid biosynthesis, the *Or* gene controls carotenoid accumulation by inducing the formation of chromoplasts, which provide a metabolic sink to sequester and deposit carotenoids (Li and Van Eck 2007). Highly conserved *Or*-like open reading frames are found in several higher plant lineages, including *C. sativus* (D'Agostino *et al*. 2007) where they could be involved in apocarotenoid accumulation in the stigma tissue.

CONCLUSIONS

Since the elucidation of the carotenogenic pathway in plants, there has been a steady increase in understanding the complexities which regulate this pathway. Much of this work has been done in plants characterized by the massive accumulation of carotenoids in their fruits, and much less is known about other interesting plants such as saffron, where apocarotenoid metabolism is responsible for the economic value of this plant. Thus, the isolation and biochemical analysis of genes involved in the carotenoid metabolism should be of major interest, in addition to the measurements of flux coefficients, metabolite channelling and the interactions between carotenogenic enzymes and other protein partners. Far more information is needed before the control mechanisms of apocarotenoid accumulation in stigma tissue can be fully understood.

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