Chapter 15

Molecular biology of *Crocus sativus*

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Chapter Outline

15.1 Introduction

Saffron (*Crocus sativus*) was once an orphan crop for plant molecular biologists and geneticists and only limited molecular information was available. Fortunately, the trend has changed significantly and many researchers in different parts of the world including Europe, India, Iran, Turkey, and Pakistan are now engaged in molecular studies of saffron. Here we summarize advances in saffron molecular genetics and biology that have deepened our understanding of development, secondary metabolism, interaction with microorganisms, and response to environmental stresses of this valuable crop.

15.2 Flower development

Flowers, and more specifically the stigmata, are the commercially valuable organs in saffron, and thus flower development in saffron has turned into an exciting and fast moving field of research.

Flower organs are organized in four whorls, namely sepals, petals, stamens, and carpels. A group of homeotic transcription factors called MADS-box proteins, composed of different classes A, B, C, D, and E, are the major players in flower organ identity and development (Prunet and Meyerowitz, 2016; Theißen et al., 2016). Based on the current model of flower development, known as the Quartet model, the first whorl (sepals) is determined by class A and E genes, the second whorl (petals) is specified by class B and E genes, the third whorl (stamens) is determined by the class B, C, and E genes, the fourth whorl (carpels) is determined by class C and E genes, and ovule development in the carpels is under control of the class C, D, and E genes (Theißen et al., 2016).

The genes identified in saffron that are involved in flower development are summarized in Table 15.1. Tsaftaris and coworkers cloned the first MADS-box genes from saffron. They cloned and characterized three homologous *APETALA-like* genes from *C. sativus: CsAP1-a, CsAP1-b*, and *CsAP1-c* (Tsaftaris et al., 2004). Later, two *AGAMOUS1-like* genes (*CsAG1*) were identified (Tsaftaris et al., 2005). These two genes, which are two isoforms resulting from alternative splicing, are expressed in flowers particularly in stamen and carpels (Tsaftaris et al., 2005). Five *PISTILLATA/GLOBOSA-like* MADS-box genes have been identified in saffron. Interestingly the expression pattern of the genes is different from their expression in *Arabidopsis*; they are not only expressed in the second and third whorls but also in the first whorl (Kalivas et al., 2007). Three *SEPALIATA3* (*SEP3*)-like genes that are identified in saffron are expressed in all flower organs but not in the leaves (Tsaftaris et al., 2011). The orthologues of *APETALA2* gene in saffron, *CsAP2*, is expressed in all the tested organs (Tsaftaris et al., 2012b). Ectopic expression of *CsatCEN/TFL1-like*,

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TABLE 15.1 Genes involved in flower development in *Crocus Sativus*.

Genes in *C. sativus*	Orthologues in *Arabidopsis*	MADS-box class	Organ of expression	Reference
CsAP1	*APETALA1*	*A*	Sepals and petals	Tsaftaris et al. (2004)
CsAP2	*APETALA2*	–	Corm, leaf, flower	Tsaftaris et al. (2012b)
CsAP3	*APETALA3*	*B*	Petal and stamen	Wafai et al. (2015)
CsPI	*PISTALLATA*	*B*	Petal and stamen	Kalivas et al. (2007)
CsAG	*AGAMOUS*	*C*	Stamens and carpels	Tsaftaris et al. (2005)
CsMYB1	*MYB*	–	Stigma	Gómez-Gómez et al. (2012)
CsSEP3	*SEPALIATA3*	*E*	Flower	Tsaftaris et al. (2011)

a *CENTRORADIALIS/TERMINAL FLOWER1* (*CEN/TFL1*)-like gene, in *Arabidopsis tfl1* mutant restored the wild phenotype (Tsaftaris et al., 2012a). *CsMYB1*, an R2R3 MYB factor from saffron, was identified that interestingly shows no expression in those *Crocus* species with branched stigma morphology, indicating that this transcription factor is probably involved in stigma morphology (Gómez-Gómez et al., 2012).

Flower development is not a very tuned process; on the contrary, like any other biological processes, flowering is a regulated process but with a level of stochasticity. For example, with a very low frequency, about 1.2×10^{-6}, there are saffron flowers with abnormal numbers of flower organs, like stigma numbers more than three (Estilai, 1978; Ghaffari and Bagheri, 2009). Cytological and morphological examinations have revealed that this abnormality is not controlled genetically, but it is probably because of fusion of two or more buds (Ghaffari and Bagheri, 2009). In fact, variation in floral organ is a common phenomenon in many other plant species, and is mainly the result of stochasticity in organ fate determination during flower development (Kitazawa and Fujimoto, 2014).

15.3 Gametogenesis and interspecific hybridization

In angiosperms, male and female gametes are produced during microsporogenesis and megasporogenesis, respectively. In anthers, microsporocytes go through meiotic divisions to produce microspores, which will form male gametophyte (pollen grain) by subsequent mitotic divisions. In an analogous scenario in ovules, megasporocytes produce magaspores by meiosis, which eventually form the female gametophyte (embryo sac).

The key to successful gametogenesis is to have normal meiosis divisions. In diploid species, meiosis is a straightforward phenomenon and often results in haploid gametes. But for triploid cells the chance to get haploid gametes with a whole set of chromosomes is very low, and therefore triploids are sterile. Although pollen grains of saffron are mostly alive (65% based on nitroblue tetrazolium test), they have a very low germination rate that can be justified by the cytological abnormalities (Caiola, 2005; Chichiricco and Caiola, 1986). The pollen tube cannot penetrate the ovule in *C. sativus*, suggesting that *C. sativus* probably originated from a self-incompatible species (Chichiricco and Caiola, 1986). This self-incompatibility lessens the likelihood of seed production in saffron close to impossible.

Saffron seeds can be produced by interspecific hybridization with diploid *Crocus* species (Caiola and Canini, 2010). Hand pollination of *C. sativus* flowers with pollens from *Crocus cartwrightianus* resulted in formation of seeds with different color and larger dimension compared with the diploid seeds. The seeds germinated with high percentage similar to diploid seeds, and resulted in seedlings, which subsequently produced corms without tonics. Although the corms produced cormlets after 3 years, the flowering stage of the corms was not observed eventually (Maria Grili Caiola, personal communication, September 29, 2017).

15.4 Secondary metabolites

Plant secondary metabolites are those compounds that, unlike primary metabolites (e.g., carbohydrates, lipids, and proteins), are not necessarily required for normal growth and development, but they are mainly important for plant interactions with the environment. Based on their biosynthetic origin, secondary metabolites are classified into five major groups: terpenoids, alkaloids, cyanogenic glucosides, glucosinolates, and phenolic compounds (Buchanan and Jones, 2007).

Terpenoids, also known as isoprenoids or terpenes, are all composed of basic five-carbon isopentate (isoprene) units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are isomers synthesized in the mevalonate pathway (MEV) in cytosol, or the nonmevalonate methylerythritol 4-phosphate pathway (MEP/DOXP) in plastids (Kirby and Keasling, 2008). Alkaloids are usually synthesized from common amino acids and function in chemical defense against herbivores (Croteau et al., 2000). Cyanogenic glucosides are characterized by their ability to release hydrogen cyanide (cyanogenesis) upon hydrolysis by beta-glycosidases. This makes them essential defense compounds against herbivores. Glucosinolates, which are mainly restricted to Brassicales, are sulfur-rich anionic beta-thioglycosides derived from amino acids. Phenolic compounds, which possess one or more hydroxyl groups attached to an aromatic arene (phenyl) ring, are primarily synthesized in phenylpropanoid and phenylpropanoid acetate pathways, with phenylalanine as the precursor (Buchanan and Jones, 2007). Their structures may range from simple molecules (phenolic acids) to polyphenols and polymeric compounds. Anthocyanins, which are responsible for most of the red, purple, and blue colors in flowers and fruits, belong to a large family of polyphenolic plant compounds known as flavonoids. They have important roles in attraction of pollinators and seed dispensers (Cheynier, 2012; Croteau et al., 2000).

15.4.1 Carotenoid biosynthesis in plants

Carotenoids are tetraterpenoids (C_{40}) that are derived from two isoprene isomers, IPP and DMAPP. Although there are two biosynthetic pathways for building these C_5 units, the cytosolic MEV and the plastidic MEP/DOXP pathway, only the latter is mainly involved in carotenoid biosynthesis in plants (Eisenreich et al., 2004; Rodrıguez-Concepción and Boronat, 2002). In MEP pathway, deoxyxylulose-5-phosphate (DXP) is formed from pyruvate and glyceraldehyde-3-phosphate by the action of DXP synthase (Fig. 15.1). The next step is carried out by 1-deoxyxylulose-5-phosphate

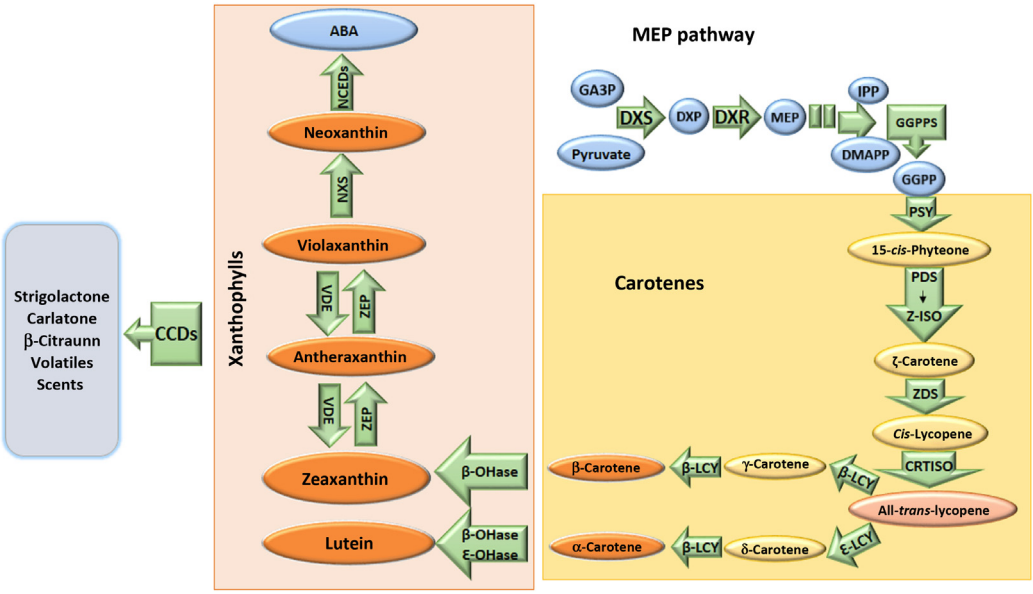

FIGURE 15.1 Schematic diagram of the carotenoid biosynthesis pathways in plants.
In *MEP* pathway, condensation of *G3P* and pyruvate catalyzed by *DXS* to produce *DXP*, which is then reduced by *DXR* to form *MEP*. After a series of catalytic reactions, *IPP* and *DMAPP* are formed, which then join together by *GGPPS* to produce *GGPP*. *PSY* catalyzes the first step in the carotenoid specific pathway that converts two *GGPP* to *cis−phytoene*. The action of desaturase and isomerase enzymes result in formation of lycopene. This is then cyclized by *β-LCY* and *ε-LCY* or *β-LCY* to form *α-carotene* and *β-carotene*. *α-Carotene* is twice hydroxylated by *ε-* and *β-OHases* to form *lutein*. Two β-ring hydroxylations of *β-carotene* by *β-OHase* give rise to *zeaxanthin*. Finally, different volatiles (e.g., *β-citraurin*) and phytohormones (*strigolactone* and *abscisic acid*) are produced by *CCDs* and *NCEDs*: *β-LCY*, β-cyclase; *β-OHase*, β-carotene hydroxylase; *CCD*, carotenoid cleavage dioxygenase; *CRTISO*, carotenoid isomerase; *DMAP*, dimethylallyl diphosphate; *DXP*, deoxy-D-xylulose 5-phosphate; *DXS*, *DXP* synthase; *DXR*, DXP reductoisomerase; *ε-LCY*, ε-cyclase; *ε-OHase*, ε-carotene hydroxylase; *G3P*, glyceraldehyde-3-phosphate, *GGPP*, geranylgeranyl diphosphate; *GGPPS*, geranylgeranyl diphosphate synthase, *IPP*, isopentenyl diphosphate; *MEP*, 2-C-methyl-D-erythritol-4-phosphate; *NCED*, 9-cis-epoxycarotenoid dioxygenase; *NXS*, neoxanthin synthase; PDS, phytoene desaturase; *PSY*, phytoene synthase; *ZDS*, ζ-carotene desaturase; *ZEP*, zeaxanthin epoxidase; and *Z-ISO*, ζ-carotene isomerase. *From Nisar, N., Li, L., Lu, S., Khin, N.C., Pogson, B.J., 2015. Carotenoid metabolism in plants. Mol. Plant 8, 68−82.*

reductoisomerase (DXR), which converts DXP to MEP. After a series of catalytic reactions, IPP and DMAPP are formed. The combination of these molecules by prenyltransferases leads to the production of geranyl diphosphate (GPP), farsenyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), which are the precursors of monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), and diterpenoids (C_{20}), respectively (Kirby and Keasling, 2008).

Carotenoid biosynthesis starts with the condensation of two GGPP molecules by phytoene synthase (PSY) giving rise to C_{40} linear 15-*cis*-phytoene. Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) catalyze desaturation reactions converting 15-*cis*-phytoene to tetra-*cis*-lycopene (Fig. 15.2). Two specific isomerase enzymes, carotenoid isomerase (CRTISO) and ζ-carotene isomerase (Z-ISO), convert poly-cis compounds to their all-trans isomers. Thus, all-trans lycopene, the first pigmented carotenoid, is formed through a series of desaturation and isomerization. Lycopene is cyclized by two competing enzymes, lycopene β-cyclase (β-LCY) and lycopene Ɛ-cyclase (Ɛ-LCY). On one hand, the addition of one and two β-rings to lycopene by β-LCY produces ϒ-carotene and orange pigment β-carotene, respectively. On the other hand, the addition of ε- and then β-rings to lycopene by Ɛ-LCY and β-LCY leads to formation of δ-carotene and α-carotene, respectively (Nisar et al., 2015). Hydroxylation of α-carotene in two sequential steps by heme-containing cytochrome P450 monooxygenases (CYP97A, CYP97C) yield to lutein, while two β-ring hydroxylations of β-carotene by β-carotene hydroxylase (BCH) give rise to zeaxanthin (Sandmann et al., 2006).

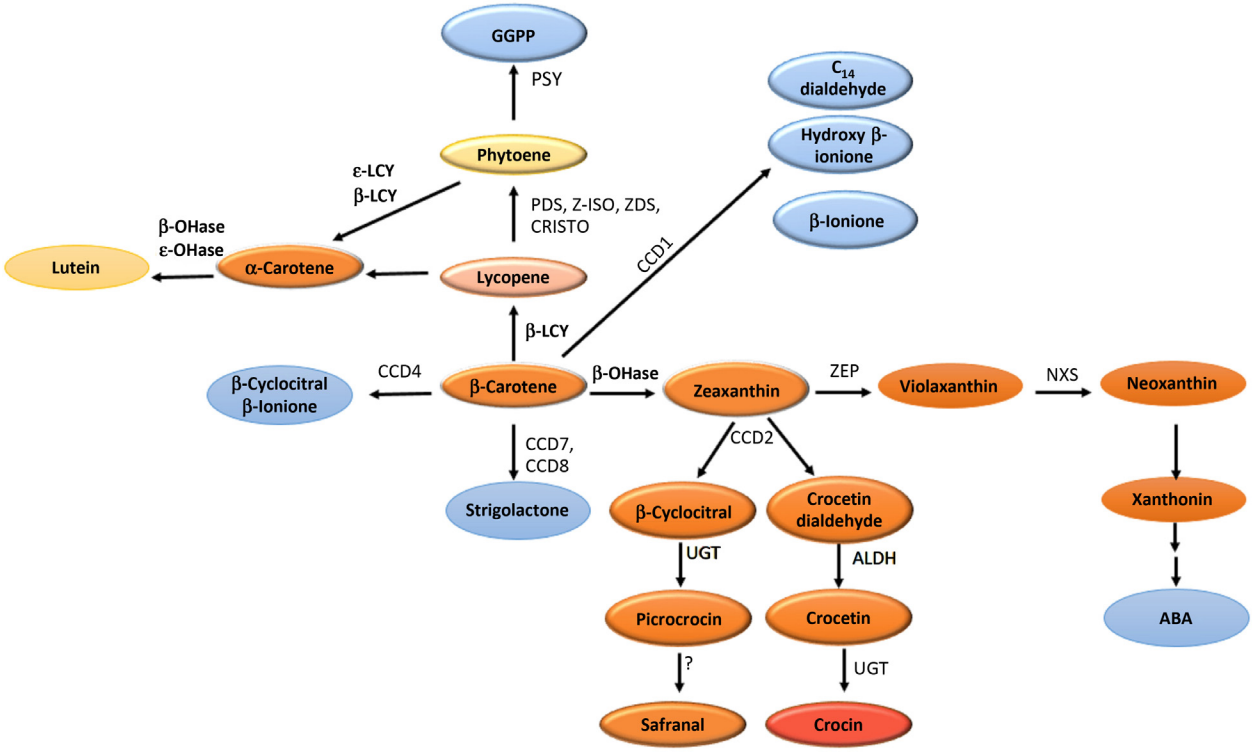

FIGURE 15.2 Schematic representation of carotenoid and apocarotenoid biosynthesis pathways in *Crocus sativus*.
Carotenoid biosynthesis starts with the condensation of two *GGPP* molecules by *PSY* giving rise to phytoene. This is converted into lycopene in a series of desaturation and isomerization reactions, which are catalyzed by PDS and ZCD (desaturases), Z-ISO and CRTISO (isomerases). Lycopene is cyclized by *β-LCY* and *ε-LCY* or *β-LCY* to form α-carotene and β-carotene. The cleavage of β-carotene furcated by *CCD1*, *CCD4*, *CCD7*, and *CCD8* yield to *β-ionone*, *C_{14} dialdehyde*, *hydroxy-β-ionone*, *β-cyclocitral*, and *strigolactone*. Moreover, hydroxylation of *β-carotene* and *α-carotene* give rise to *zeaxanthin* and *lutein*, respectively. *Zeaxanthin* is cleaved by *CCD2* to produce *crocetin dialdehyde* and *hydroxy-β-cyclocitral*. *Crocetin dialdehyde* is further dehydrogenated and glycosylated to *crocetin* and *crocin* by *ALDH* and *UGT*, respectively. *Hydroxy-β-cyclocitral* is converted to picrocrocin by an UGT, and then to safranal. In the other branch point, zeaxanthin is converted into violaxanthin and neoxanthin by *ZEP* and *NSX*, respectively. *Neoxanthin* is cleaved by *NCED* to produce *xanthoxin*, precursor of abscisic acid (*ABA*).
ALDH, aldehyde dehydrogenase; *β-LCY*, β-cyclase; *β-OHase*, β-carotene hydroxylase; *CCD*, carotenoid cleavage dioxygenase; *CRTISO*, carotenoid isomerase; *ε-LCY*, ε-cyclase; *ε-OHase*, ε-carotene hydroxylase, *NCED*, 9-*cis*-epoxycarotenoid dioxygenase; *NXS*, neoxanthin synthase; *PDS*, phytoene desaturase; *PSY*, phytoene synthase; *UGTs*, UDPG-glucosyltransferase; *ZDS*, ζ-carotene desaturase; *ZEP*, zeaxanthin epoxidase; *Z-ISO*, ζ-carotene isomerase. *Modified from Baba, S.A., Ashraf, N., 2016. Apocarotenoids of* Crocus sativus *L: From Biosynthesis to Pharmacology. Springer, Singapore.*

15.4.2 Carotenoid biosynthesis in saffron

Saffron has many volatile and nonvolatile active compounds belonging to different groups of secondary metabolites, including carotenoids, monoterpenoids, and flavonoids. Carotenoids, which are the most important pigments in saffron stigmas, are mainly C_{40} isoprenoids including α- and β-carotene and zeaxanthin (Gresta et al., 2008). C_{20} apocarotenoids such as crocetin and its ester derivatives are produced from the oxidative cleavage of carotenoids. Crocin is the glycosylated form of crocetin, both of which are responsible for the red color of saffron (Carmona et al., 2006). The taste and aroma of saffron are mainly due to the accumulation of picrocrocin and safranal, respectively. Picrocrocin is a colorless glycoside precursor of safranal and is formed from the enzymatic degradation of zeaxanthin (Srivastava et al., 2010).

The cDNAs encoding PSY and PDS are cloned in saffron (ScPYS and CsPDS), and expression analysis showed that the highest level of their expression occurs in the orange stage of the stigma (Castillo et al., 2005). Two lycopene cyclases have been identified in saffron (CsLCYB1 and CsLCYB2a). The *CsLcyB2a* gene is absent in *Crocus kotschyanus*, while it is present in *Crocus goulimyi* and *Crocus cancellatus* (Ahrazem et al., 2009). Two saffron genes encoding BCH, *CsBCH1*, *CsBCH2*, have been identified (Castillo et al., 2005). An ortholog of the CRTISO has been identified in saffron, but it needs further characterization (Baba et al., 2015b). Both β-carotene and zeaxanthin are substrates for the biosynthesis of unique apocarotenoids in saffron.

15.4.3 Apocarotenoid biosynthesis

The catalytic activity of carotenoid cleavage dioxygenases (CCDs) along with enzymatic oxidation via peroxidases/lipoxygenases or nonenzymatic photochemical oxidation in photosynthetic tissues under high light stress are responsible for formation of various apocarotenoid compounds, which have diverse roles in plants as phytohormones, signal molecules, and volatile/flavor compounds (Auldridge et al., 2006; Nisar et al., 2015). According to their substrate preference and/or the cleavage position, plant carotenoid cleavage oxygenases are grouped into two big families: 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) and CCDs (Auldridge et al., 2006; Walter and Strack, 2011).

An ortholog of the *NCED* gene was identified in saffron. The expression of *CsNCED* in floral and corm tissues correlated with ABA levels, suggesting a possible role of the gene in regulation of stigma senescence and corm dormancy in *C. sativus* (Ahrazem et al., 2011). Two isoforms of *CCD1* have been isolated from *C. sativus* (*CsCCD1a* and *CCD1b*) that cleave zeaxanthin and β-carotene at the 9, 10 double bond to produce C_{14} dialdehyde and β-ionone, respectively. *CsCCD1a* is ubiquitously expressed while *CsCCD1b* expression is specific to stigma (Rubio et al., 2008). Except for CCD1, which is located in cytosol, other CCDs have a plastid-targeting transit peptide and act in plastids (Rubio et al., 2008; Ahrazem et al., 2016). Through transcriptome sequencing *CsCCD2* was identified and characterized (Frusciante et al., 2014). The gene is expressed early during stigma development and is able to cleave zeaxanthin at sequentially the 7, 8 and 7′, 8′ double bonds adjacent to a 3-OH β-ionone ring, converting it to crocetin dialdehyde. *CsCCD2* is closely related to *CsCCD1* (with 97% identity) and its preferred substrates are lutein and zeaxanthin, but not β-carotene, as shown by in vitro cleavage assay.

According to the sequence of *CsCCD2* gene, the authors suggested that this gene lacks a plastid transit peptide, and thus is localized in the chromoplast outer envelope (Frusciante et al., 2014). However, another research group characterized *CaCCD2* from *Crocus ancyrensis* and compared its sequence with *CsCCD2*. There was a clear length difference between these two genes at the 5′ end, suggesting that the sequence of *CsCCD2* was not reported correctly originally. The 5′-RACE PCR analysis of *CsCCD2* and cloning the longest cDNA isolated from *C. sativus* stigma revealed that the full sequence, named *CsCCD2L*, encodes a protein, which is 60 amino acids longer than the CsCCD2, with a plastid transit peptide (Ahrazem et al., 2016) and localized in plastids (Demurtas et al., 2018). Further analysis resulted in identification of three *CsCCD2* paralogs in *C. sativus*. The longest gene, *CsCCD2a*, consisted of nine introns and ten exons. The *CsCCD2b* has nine exons and eight introns. The shortest one, *CsCCD2-t*, is a truncated gene. RNA-seq studies in three developmental stages of saffron stigma revealed that intron retention is the common form of alternative splicing in *CsCCD2* (Ahrazem et al., 2016).

The CCD4 family is the largest family of plant CCDs and plays a significant role in the level of organ pigmentation including citrus peel (Rodrigo et al., 2013), *Arabidopsis* seeds (Gonzalez-Jorge et al., 2013), and potato tubers (Campbell et al., 2010), and in volatile emission during flowering in saffron (Rubio et al., 2008). Rubio et al. (2008) showed that expression patterns of *CsCCD4a* and *CsCCD4b* are consistent with the highest levels of β-carotene and emission of β-ionone during the stigma development. This volatile compound plays a role in attracting insect pollinator especially in those *Crocus* species that are self-incompatible and have heavy pollen grains to transfer with air

(Rubio et al., 2008). A new member of the *CCD4* family from *C. sativus*, named *CsCCD4c*, was isolated later (Frusciante et al., 2014). The expression of this intronless gene is restricted to stigmas and induced by heat, osmotic stress, and wounding, suggesting the role of its products in the adaptation of saffron to environmental stresses (Frusciante et al., 2014). The expression level of *CsCCD4a* and *CsCCD4b* genes increases in response to dehydration, salt, and methylviologen (Baba et al., 2015a). Transgenic *Arabidopsis* plants overexpressing *CsCCD4b* developed longer roots with a higher number of lateral roots and displayed higher activity and expression of reactive oxygen species (ROS) scavenging enzymes. These results indicate that β-ionone and β-cyclocitral, products of CsCCD4b, may be involved in plant responses to dehydration, salinity, and oxidative stresses (Baba et al., 2015a).

Some CCD4 enzymes in other plants, like VcCCD4a and VcCCD4b in *Vitis vinifera*, cleave carotenoids at 5, 6 (5′, 6′) double bonds (Lashbrooke et al., 2013), or like citrus CCD4 at 7′, 8′ double bonds (Gonzalez-Jorge et al., 2013; Rodrigo et al., 2013). In saffron, zeaxanthin cleavage dioxygenase (*CsZCD*) was first reported to cleave zeaxanthin at the 7, 8/7′, 8′ positions to produce crocetin dialdehyde (Bouvier et al., 2003). However, sequence comparison and structure prediction revealed that CsZCD is an N-truncated form of CsCCD4, missing one blade of the β-propeller structure, and therefore does not have cleavage activity (Rubio et al., 2008).

CCD7 works in sequence with CCD8 to synthesize strigolactone, an apocarotenoid hormone that inhibits plant shoot branching. First, β-carotene is cleaved by CCD7 to produce 10′-apo-β-carotenal and β-ionone. Then, the cleavage of 10′-apo-β-carotenal by CCD8 leads to formation of C_{18}-ketone β-apo-13-carotenone that, via several different reactions, is converted to strigolactone precursor carlactone (Seto et al., 2014). These molecules also stimulate the growth of symbiotic mycorrhizal fungi (Akiyama et al., 2005) and the germination of parasitic plant seeds (Cook et al., 1972). In *C. sativus*, *CsCCD8* is highly expressed in quiescent axillary buds, and its expression is significantly decreased by decapitation indicating its involvement in axillary bud outgrowth. In addition, *CsCCD8* may have a significant function in the control of apical dominance. The abundance of CCD7 and CCD8 in immature orange stigmas and reduction of both genes in the senescent stigma suggests an interesting novel function for the enzymes and of strigolactones (Frusciante et al., 2014).

15.4.4 Crocin, crocetin, picrocrocin, and safranal

As described above, production of bioactive compounds in saffron engages the MEP pathway from pyruvate and glyceraldehyde-3-phosphate to GGPP, the carotenoid pathway from GGPP to zeaxanthin, and the crocin pathway from zeaxanthin to crocin. In the latter one, symmetric cleavage of zeaxanthin at the 7, 8/7′, 8 positions by CsCCD2 yields to crocetin dialdehyde and 3-OH-β-cyclocitral. On one hand, crocetin dialdehyde is further dehydrogenated and glycosylated to crocetin and crocin by an aldehyde dehydrogenase (ALDH) and UDPG-glucosyltransferase (UGT), respectively. On the other hand, hydroxy-β-cyclocitral is converted to picrocrocin by an UGT, and then to safranal (Frusciante et al., 2014). Five different ALDHs were identified in saffron; expression of two of them (*ADH2946* and *ADH11367*) had a high correlation with crocetin concentration, and showed the highest expression level at the anthesis stage. Also, there was a third candidate (*ADH54788*) with an expression pattern like crocetin (Gómez-Gómez et al., 2017). The ADLH enzymes are localized in the endoplasmic reticulum (Demurtas et al., 2018).

Crocin is the most important metabolite of saffron stigma and quickly dissolves in water. Besides the *Crocus* genus, crocin has also been identified in other plants such as *Buddleja officinalis*, *Nyctanthes arbor-tristis*, and *Gardenia jasminoides* (Gadgoli and Shelke, 2010; Liao et al., 1999; Pfister et al., 1996). Depending on the number of sugar molecules, different forms of crocin are produced. Different forms of crocin including crocetin β-D-glucosyl ester, crocetin β-D-gentiobiosyl ester, di-(β-D-glucosyl) ester, crocetin β-D-gentiobiosyl-β-D-glucosyl ester, and crocetin di-(β-D-gentiobiosyl) ester are discernable in HPLC analysis (Moraga et al., 2004). The crocetin di-(β-D-gentiobiosyl) ester (α-crocin) is the most abundant form of crocin in saffron and because of high water solubility it has the maximum coloring capacity, making it a good candidate for applications in foods as colorant (Tarantilis et al., 1995). β-Crocetin (mono methyl ester of crocetin) and γ-crocetin (dimethyl ester of crocetin) are minor components among water-soluble C_{20} apocarotenoids of saffron (Fernández, 2004).

Moraga et al. (2004) reported the cloning of two *GTase* genes from *C. sativus*. *CsUGT2* was expressed only in stigma of *Crocus* species that synthesize crocin and the expression pattern was consistent with accumulation of crocetin with higher sugar moieties. Transcripts of *CsUGT3* and other structural genes for carotenoid biosynthesis were detected in the stigma tissue of all tested *Crocus* species. Expression of *CsUGT2* in *Escherichia coli* demonstrated the glucosylation activity of recombinant protein against crocetin, crocetin β-D-glucosyl ester, and crocetin β-D-gentibiosyl ester (Moraga et al., 2004). Interestingly, *CsUGT2* is expressed in stigma-like structures (Namin et al., 2009). In *C.*

ancyrensis, high expression of an ortholog of *CsUGT2* was correlated with high levels of crocin accumulation in stigma and tepals (Ahrazem et al., 2015).

There is no UGT identified yet for conversion of hydroxy-β-cyclocitral to picrocrocin, a monoterpene glycoside, which is considered the main bitter taste of saffron. Hydrolysis of picrocrocin gives rise to safranal, the main constituent of the volatile oil fraction (30%−70% of the essential oil). Besides safranal, other aromatic compounds with the same skeleton such as isophorone, 2,2,6-trimethyl-1,4-cyclohexanedione, 4-ketoisophorone, 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one, and 2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde add to the aroma of the spice and seem to be derived from picrocrocin (Maggi et al., 2009). However, identification of several new glycosides indicates their role as the glycosidic aroma precursor along with picrocrocin (Carmona et al., 2006). Although studies show that dehydration is important for production of safranal (Himeno and Sano, 1987; Raina et al., 1996), it is not exactly clear yet whether this reaction happens enzymatically or nonenzymatically.

15.4.5 Genetic regulation of carotenoids biosynthesis

The steady-state level of carotenoids is dependent on the rate of their biosynthesis, degradation, and storage capacity of the cell. Plants have multiple mechanisms controlling production and accumulation of carotenoids including regulation of the expression of key carotenoid biosynthesis pathway genes and CCDs. During the development of saffron, the color of stigma changes from white to scarlet, passing through yellow, orange, and red stages, accompanying carotenoid accumulation. For example, in one study β-carotene and zeaxanthin reached 60.5% and 85%, respectively, in the scarlet stage (Castillo et al., 2005).

Phytoene biosynthesis is a bottleneck in the carotenoid pathway. *PSY* gene is induced in response to different factors including temperature, drought, salt, high light, ABA, development, photoperiod, and posttranscriptional feedback regulation (Cazzonelli and Pogson, 2010). In *Arabidopsis*, RAP2.2 transcription factor binds to the promotion of *PSY* and *PDS* and overexpression of RAP2.2 results in reduction of *PSY* and *PDS* transcription and carotenoid levels (Welsch et al., 2007). Another transcription factor in *Arabidopsis* named RIF was also shown to bind to the *PSY* promoter under dark conditions and repress its expression (Toledo-Ortiz et al., 2010). In *C. sativus*, the lowest level of *PSY* and *PDS* transcripts, which is at the early developmental stages the abundance of *PSY* and *PDS* transcripts is low and then reaches to the highest level in the orange stage of the stigma, and then remained relatively constant through development (Castillo et al., 2005).

The cyclization of lycopene is the other regulatory node in carotenoid biosynthesis pathway that is catalyzed by two lycopene cyclases, Ɛ-LCY and β-LCY. In *Arabidopsis*, silencing of Ɛ-LCY by cosuppression results in alteration of the ratios of lutein to β-carotene (Pogson et al., 1996). Two genes encoding lycopene β-cyclase are characterized in saffron: *CsLcyB2a* and *CsLcyB2a* (Ahrazem et al., 2009). The stable expression of *CsLcyB2a* in transgenic *Arabidopsis* showed lycopene β-cyclase activity and caused an increase of β-carotene. *CsLcyB2a* is expressed only in stigma tissue, where β-carotene accumulated, with the highest levels of expression in the days before anthesis. However, *CsLcyB1* transcript is present in leaves, tepals, and stigmas at lower levels (Ahrazem et al., 2009).

BCHs are key enzymes in the accumulation of apocarotenoids in *C. sativus* as they are directly involved in production of zeaxanthin as precursor of saffron apocarotenoids (Bouvier et al., 2003). Detection of *BCH* transcripts by RT-PCR in fully developed stigma at the anthesis stage showed that massive accumulation of both *CsBCH1* and *CsBCH2* transcripts correlated with zeaxanthin and the accumulation of its derivations in saffron stigma (Castillo et al., 2005). Moreover, the abundance of *CsBCH1* transcripts in different *Crocus* species was related to the their zeaxanthin content, suggesting that the reaction catalyzed by CsBCH1 enzyme could be the limiting step in the production of apocarotenoids in the saffron stigma (Castillo et al., 2005). The expression of the carotenogenic genes including *PSY*, *ZDS-V*, *BCH*, and *LCY-II* was correlated with accumulation of crocins in *C. ancyrensis* (Ahrazem et al., 2015).

There are cis-regulatory motifs in the *CCD2* promoter responding to light, temperature, and circadian regulation that cause higher expression levels during the night and under low temperatures (Ahrazem et al., 2016). Similar behavior was observed for the chromoplast-specific carotenogenic genes, *CsLycB2a* and *CsBCH1*, suggesting coregulation of these genes during the development of the stigma in saffron. In addition, the accumulation pattern of cyclocitral was correlated with the expression level of *CsCCD2* and thus it could be assumed as a signal molecule from chromoplast to nucleus, which coordinates the expression of *CsCCD2* with the developmental state of the chromoplast during stigma growth (Ahrazem et al., 2009, 2016). The maximum expression of *CCD2* is at the orange stage, coincident with accumulation of crocetin and crocin (Frusciante et al., 2014).

For the first time, D'Agostino and coworkers developed an expressed sequence tag (EST) collection from saffron mature stigma comprising 6603 high-quality ESTs, which facilitated identification of key genes involved in

apocarotenoid metabolism and regulation (D'Agostino et al., 2007). Expression profiling from five different tissues/organs of *C. sativus* revealed that key enzymes involved in apocarotenoid biosynthesis including CCD, glucosyltransferases, ALDHs and beta glucosidases expressed higher in stigma compare to other tissues, suggesting that apocarotenoids are produced mainly in stigma (Jain et al., 2016). Similar expression patterns were reported earlier for PSY, PDS, and CCD2 (Baba et al., 2015b). Another comprehensive expressions study of candidate genes involved carotenoid/apocarotenoid biogenesis throughout five developmental stages revealed that the expression of MEP/carotenoid transcripts, together with *CCD2* and *UGT2*, but not *PDS-II* and *ZDS*, have very high positive correlations (Gómez-Gómez et al., 2017).

Differential expression of transcription factors such as MYB, MYB-related, WRKY, C2C2-YABBY, and bHLH involved in secondary metabolism indicated that they might have regulatory roles in apocarotenoid biosynthesis and accumulation in *C. sativus* in a spatio-temporal manner (Baba et al., 2015b; Jain et al., 2016). The expression of CsULT1, an ultrapetala transcription factor, increases gradually in saffron stigma until the anthesis stage, whic is similar to the trend of accumulation of crocin, and therefore, suggesting a regulatory role for the novel transcription factor in apocarotenoid biosynthesis (Ashraf et al., 2015). This anticipation was confirmed by overexpression of *CsULT1* in saffron calli (Ashraf et al., 2015).

A zinc finger transcription factor, called CsSAP09, was identified in *C. sativus*; its upstream region contains light and stress responsive elements. *CsSAP09* expression was the highest in stigma tissue, the site of apocarotenoid accumulation, at the anthesis stage, suggesting this transcription factor as a potential candidate for regulation of apocarotenoid biosynthesis (Malik and Ashraf, 2017).

Using the EST library from mature saffron stigmas generated by D'Agostino et al. (2007) and available mature miRNAs from miRBase (http://www.mirbase.org/), Zinati and coworkers reported two putative miRNAs (miR414 and miR837-5p) that target the genes that coexpressed with genes such as β-LCY and Ɛ-LCY as well as transcription factors and protein kinase in *C. sativus*. This indicates that these miRNAs may be involved in a regulatory pathway of carotenoid/apocarotenoid biosynthesis in saffron stigma (Zinati et al., 2016).

15.5 Production of saffron metabolites in microorganisms

Synthetic biology strategies have been adopted to produce valuable saffron apocarotenoids in microorganisms. The expression of β-OHase and *ZCD1* genes in *Chlorella vulgaris*, using the *Agrobacterium tumefaciens*-mediated transformation method, resulted in production of crocetin in the transgenic microalgae (Lou et al., 2016). Some strains of *Saccharomyces cerevisiae* produce low levels of β-carotene. By introducing the three key enzymes of crocetin biosynthesis, β-OHase, CCD, and ALDH, from different sources of a β-carotene-producing *S. cerevisiae* strain, the highest amount of crocetin produced in eukaryotic cells was achieved (Chai et al., 2017). Characterization of the key enzymes involved in apocarotenoid metabolism in saffron provides a platform for future biotechnological applications of these genes in other species, especially those with substrates for crocetin formation (Ahrazem et al., 2015).

The EVOLVA company was the first to use *S. cerevisiae* as the host to produce saffron bioactive compounds. There are several patents granted to EVOLVA including WO2011146833, US20140248668, WO2013021261, WO2015162283, WO2015132411, and US20170044552.

Due to low amounts of acetyl-CoA enzyme and the low activity of cellular prenyl phosphate in yeast, MEV pathway is heavily regulated and consequently results in very low levels of prenyl phosphate for commercial production of terpenoid molecule in yeast. Patent document WO2011146833 (Hansen, 2016) disclosed the method for producing isoprenoid compounds including zeaxanthin-3-diglucoside, zeaxanthin, and C_{30} carotenoids in yeast by coexpressing multiple MEV pathway gene analogs that increase prenyl phosphates, or by expressing the nonendogenous enzyme ATP citrate lyase, which leads to high concentrations of acetyl-CoA in cytosol. It claims that the yeast host cell produces at least 25-fold more isoprenoid compound (150 mg g^{-1} dry weight) than unaltered yeast cells. Patent documents US20140248668 (Raghavan et al., 2017) reveal a recombinant, carotenoid-producing host such as *S. cerevisiae*, expressing *CsZCD* alone or in combination with recombinant UGTs. It is clear that *S. cerevisiae* does not have carotenoid pathway, and therefore genes involved in β-carotene synthesis including *PDS*, *GGDP synthase*, and *β-carotene synthase* were also introduced into the yeast. It claims that the host can produce detectable amounts of one or more of the saffron bioactive compounds like crocetin, crocetin dialdehyde, crocin, or picrocrocin. Patent documents WO2015162283 and US20170044552 (Kumar, 2017) disclosed microorganisms producing saffron compounds including hydroxy-β-cylcocitral and picrocrocin. The hosts are engineered to express exogenous genes encoding cytochrome P450, truncated ferredoxin, flavin-dependent ferredoxin reductase, UGT polypeptides. Patent document WO2015132411

(Kumar, 2017) gives detailed descriptions of the genes arranged in expression cassettes involved in synthesis of saffron compounds including crocetin, crocetin dialdehyde, and crocin or picrocrocin.

15.6 Saffron–microbe interactions

The mutualistic interactions between saffron roots and arbuscular mycorrhizal fungi (AMF) were detected in different regions of saffron cultivation in Khorasan province, Iran. There were mainly *Glomus macrocarpus* and less frequently *Glomus mosseae* species. Artificial inoculation of saffron corms with *G. macrocarpus* resulted in a 26% increase in dry plant weight. Interestingly this inoculation had a similar effect on onion, but not on the bulbs of narcissus and gladiolus, indicating species-dependent interaction (Kianmehr, 1981). Positive effects of *Glomus aggregatum*, *G. mosseae*, and *Glomus etunicatum* were also reported on saffron (Mohebi-Anabat et al., 2015). AMF colonization on saffron increased corm diameter and flower yield (Aimo et al., 2010), and the amounts of carbohydrates, proteins, phenolic compounds, and minerals in the corms (Lone et al., 2016).

There are about 40 times more bacteria in the rhizosphere of *C. sativus* compared with bulk soil (Ambardar and Vakhlu, 2013). Further analysis detected 22 different genera of bacteria in rhizosphere and cormosphere (the soil around the corms); *Pseudomonas* was the dominant genus among eight different genera in rhizosphere and *Pantoea* was the dominant genus among six different genera in cormosphere (Ambardar et al., 2014).

Bacillus subtilis has been shown to have beneficial effects on saffron production. Inoculation of saffron corms with spores of FZB24 strain of the bacteria sped up corm growth, increased stigma biomass by 12%, and increased picrocrocin, crocetin, and safranal. Interestingly the manner of bacterial inoculation affected the beneficial effects and thus the optimum application method to increase both qualitative and quantitative traits needs further optimization (Sharaf-Eldin et al., 2008). In another experiment, *Bacillus amyloliquefaciens* was reported to be effective at controlling corm rot caused by *Fusarium oxysporum* (Gupta and Vakhlu, 2015).

A cDNA encoding a novel class of chitinase, *Safchi A*, was characterized in saffron. This cDNA is mainly expressed in roots and corms, and its expression is induced by elicitor treatment, methyl jasmonate, wounding, and by the fungi *F. oxysporum*, *Beauveria*, and *Phoma* species, suggesting a defense role for the protein. Furthermore, in vitro assays with the recombinant native protein showed chitinolytic and antifungal activity of the Safchia protein (López and Gómez-Gómez, 2009).

CsPR10 encoding an ortholog of *Arabidopsis* PR10 protein was identified in saffron stigmas. The CsPR10 protein showed inhibitory effects on the growth of *F. oxysporum* but not on *Verticillium dahlia* and *Penicillium* species (Gómez-Gómez et al., 2011). Although it is suggested that CsPR10 is involved in defense responses to pathogens, its expression pattern does not support this speculation. Unlike most defense-related genes, *CsPR10* is not expressed in leaves and roots, the main organs that are affected by the pathogens; instead, the highest level of its expression was detected in anthers and tepals (Gómez-Gómez et al., 2011).

15.7 Molecular response to abiotic stresses

CCDs are enzymes that cleave carotenoids to produce apocarotenoids. Isoforms of this enzyme in *C. sativus* (CsCCD4) are expressed in response to dehydration stress, and their heterologous expression in *Arabidopsis* resulted in transgenic plants with longer roots, higher number of lateral roots, more tolerance to salt, oxidative, and dehydration stresses, along with higher expression of ROS-metabolizing enzymes (Baba et al., 2015b). A β-glucosidase from *C. sativus*, CsBGlu12, which is a vacuole-localized enzyme, results in accumulation of flavonols, and thereby confers resistance to abiotic stresses through ROS scavenging (Baba et al., 2017).

An stress-inducible glycosyltransferase (*CsGT*) was identified in saffron that its ectopic expression in *Arabidopsis thaliana* enhanced salt and oxidative stress tolerance (Ahrazem et al., 2015). Characterization of *CsGT* showed that the gene regulates root growth by modulating auxin signaling and cell cycle, thereby, enhancing survival to salt and oxidative stresses (Ahrazem et al., 2015).

15.8 Conclusion

Researchers have started to investigate the developmental biology and biochemistry of saffron, however, these efforts are still not proportional to the high economic importance of this cash crop. Sterility and very low genetic variation, lack of genome sequence, and lack of functional genomics tools are probably the main reasons for slow progress in saffron molecular research. By the fast progresses in the next generation sequencing technologies, it is expected to have

more genomic and transcriptomic data for saffron available in the near future. Then, developing fast and efficient functional genomics tools including transient and permanent gene silencing and overexpression techniques are necessary. Studies on microbiome assembly in rhizosphere and cormosphere of saffron will help uncover saffron−microbe interactions and also to develop biofertilizers for this valuable crop.

References

Ahrazem, O., Rubio-Moraga, A., López, R.C., Gómez-Gómez, L., 2009. The expression of a chromoplast-specific *Lycopene beta cyclase* gene is involved in the high production of saffron's apocarotenoid precursors. J. Exp. Bot. 61, 105−119.

Ahrazem, O., Rubio-Moraga, A., Trapero, A., Gómez-Gómez, L., 2011. Developmental and stress regulation of gene expression for a 9-cis-epoxycarotenoid dioxygenase, *CstNCED*, isolated from *Crocus sativus* stigmas. J. Exp. Bot. 63, 681−694.

Ahrazem, O., Rubio Moraga, A., Jimeno, M.L., Gómez-Gómez, L., 2015. Structural characterization of highly glucosylated crocins and regulation of their biosynthesis during flower development in *Crocus*. Front. Plant Sci. 6, 971.

Ahrazem, O., Rubio-Moraga, A., Berman, J., Capell, T., Christou, P., Zhu, C., et al., 2016. The carotenoid cleavage dioxygenase CCD2 catalysing the synthesis of crocetin in spring crocuses and saffron is a plastidial enzyme. New Phytol. 209, 650−663.

Aimo, S., Gosetti, F., D'Agostino, G., Gamalero, E., Gianotti, V., Bottaro, M., et al., 2010. Use of arbuscular mycorrhizal fungi and beneficial soil bacteria to improve yield and quality of saffron (*Crocus sativus* L.). Acta Hortic. 850, 159−164.

Akiyama, K., Matsuzaki, K.-i, Hayashi, H., 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435, 824−827.

Ambardar, S., Vakhlu, J., 2013. Plant growth promoting bacteria from *Crocus sativus* rhizosphere. World J. Microbiol. Biotechnol. 29, 2271−2279.

Ambardar, S., Sangwan, N., Manjula, A., Rajendhran, J., Gunasekaran, P., Lal, R., et al., 2014. Identification of bacteria associated with underground parts of *Crocus sativus* by 16S rRNA gene targeted metagenomic approach. World J. Microbiol. Biotechnol. 30, 2701−2709.

Ashraf, N., Jain, D., Vishwakarma, R.A., 2015. Identification, cloning and characterization of an ultrapetala transcription factor CsULT1 from *Crocus*: a novel regulator of apocarotenoid biosynthesis. BMC Plant Biol. 15, 25.

Auldridge, M.E., McCarty, D.R., Klee, H.J., 2006. Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr. Opin. Plant Biol. 9, 315−321.

Baba, S.A., Ashraf, N., 2016. Apocarotenoids of *Crocus sativus* L: From Biosynthesis to Pharmacology. Springer, Singapore.

Baba, S.A., Jain, D., Abbas, N., Ashraf, N., 2015a. Overexpression of *Crocus* carotenoid cleavage dioxygenase, *CsCCD4b*, in *Arabidopsis* imparts tolerance to dehydration, salt and oxidative stresses by modulating ROS machinery. J. Plant Physiol. 189, 114−125.

Baba, S.A., Mohiuddin, T., Basu, S., Swarnkar, M.K., Malik, A.H., Wani, Z.A., et al., 2015b. Comprehensive transcriptome analysis of *Crocus sativus* for discovery and expression of genes involved in apocarotenoid biosynthesis. BMC Genomics 16, 698.

Baba, S.A., Vishwakarma, R.A., Ashraf, N., 2017. Functional characterization of CsBGlu12, a β-Glucosidase from *Crocus sativus*, provides insights into its role in abiotic stress through accumulation of antioxidant flavonols. J. Biol. Chem. 292, 4700−4713.

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, 47−62.

Buchanan, B.B., Jones, R.L., 2007. Biochemistry and Molecular Biology of Plants. I.K. International Publishing House Pvt. Limited.

Caiola, M.G., 2005. Embryo origin and development in *Crocus sativus* L.(*Iridaceae*). Plant Biosyst. 139, 335−343.

Caiola, M.G., Canini, A., 2010. Looking for saffron's (*Crocus sativus* L.) parents. *Saffron* (AM Husaini, ed.). Funct. Plant Sci. Biotechnol. 4, 1−14.

Campbell, R., Ducreux, L.J., Morris, W.L., Morris, J.A., Suttle, J.C., Ramsay, G., et al., 2010. The metabolic and developmental roles of carotenoid cleavage dioxygenase4 from potato. Plant Physiol. 154, 656−664.

Carmona, M., Zalacain, A., Sánchez, A.M., Novella, J.L., Alonso, G.L., 2006. Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS. J. Agric. Food Chem. 54, 973−979.

Castillo, R., Fernández, J.-A., Gómez-Gómez, 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.

Cazzonelli, C.I., Pogson, B.J., 2010. Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant Sci. 15, 266−274.

Chai, F., Wang, Y., Mei, X., Yao, M., Chen, Y., Liu, H., et al., 2017. Heterologous biosynthesis and manipulation of crocetin in *Saccharomyces cerevisiae*. Microb. Cell Fact. 16, 54.

Cheynier, V., 2012. Phenolic compounds: from plants to foods. Phytochem. Rev. 11, 153−177.

Chichiricco, G., Caiola, M.G., 1986. *Crocus sativus* pollen germination and pollen tube growth in vitro and after intraspecific and interspecific pollination. Can. J. Bot. 64, 2774−2777.

Cook, C., Whichard, L.P., Wall, M., Egley, G.H., Coggon, P., Luhan, P.A., et al., 1972. Germination stimulants. II. Structure of strigol, a potent seed germination stimulant for witchweed (*Striga lutea*). J. Am. Chem. Soc. 94, 6198−6199.

Croteau, R., Kutchan, T.M., Lewis, N.G., 2000. Natural products (secondary metabolites). Biochem. Mol. Biol Plants 24, 1250−1319.

D'Agostino, N., Pizzichini, D., Chiusano, M.L., Giuliano, G., 2007. An EST database from saffron stigmas. BMC Plant Biol. 7, 53.

Demurtas, O.C., Frusciante, S., Ferrante, P., Diretto, G., Azad, N.H., Pietrella, M., et al., 2018. Candidate enzymes for saffron crocin biosynthesis are localized in multiple cellular compartments. Plant Physiol. 177, 990−1006.

Eisenreich, W., Bacher, A., Arigoni, D., Rohdich, F., 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell. Mol. Life Sci. 61, 1401–1426.

Estilai, A., 1978. Variability in saffron (*Crocus sativus* L.). Experientia 34, 725.

Fernández, J.-A., 2004. Biology, biotechnology and biomedicine of saffron. Recent Res. Dev. Plant Sci. 2, 127–159.

Frusciante, S., Diretto, G., Bruno, M., Ferrante, P., Pietrella, M., Prado-Cabrero, A., et al., 2014. Novel carotenoid cleavage dioxygenase catalyzes the first dedicated step in saffron crocin biosynthesis. Proc. Natl. Acad. Sci. 111, 12246–12251.

Gadgoli, C., Shelke, S., 2010. Crocetin from the tubular calyx of *Nyctanthes arbor-tristis*. Nat. Prod. Res. 24, 1610–1615.

Ghaffari, S.M., Bagheri, A., 2009. Stigma variability in saffron (*Crocus sativus* L.). Afr. J. Biotechnol. 8, 601–604.

Gómez-Gómez, L., Rubio-Moraga, A., Ahrazem, O., 2011. Molecular cloning and characterisation of a pathogenesis-related protein *CsPR10* from *Crocus sativus*. Plant Biol. 13, 297–303.

Gómez-Gómez, L., Trapero-Mozos, A., Gómez, M.D., Rubio-Moraga, A., Ahrazem, O., 2012. Identification and possible role of a MYB transcription factor from saffron (*Crocus sativus*). J. Plant Physiol. 169, 509–515.

Gómez-Gómez, L., Parra-Vega, V., Rivas-Sendra, A., Seguí-Simarro, J., Molina, R., Pallotti, C., et al., 2017. Unraveling massive crocins transport and accumulation through proteome and microscopy tools during the development of saffron stigma. Int. J. Mol. Sci. 18, 76.

Gonzalez-Jorge, S., Ha, S.-H., Magallanes-Lundback, M., Gilliland, L.U., Zhou, A., Lipka, A.E., et al., 2013. Carotenoid cleavage dioxygenase4 is a negative regulator of β-carotene content in *Arabidopsis* seeds. Plant Cell 25, 4812–4826.

Gresta, F., Lombardo, G., Siracusa, L., Ruberto, G., 2008. Saffron, an alternative crop for sustainable agricultural systems. A review. Agron. Sustain. Dev. 28, 95–112.

Gupta, R., Vakhlu, J., 2015. Native *Bacillus amyloliquefaciens* W2 as a potential biocontrol for *Fusarium oxysporum* R1 causing corm rot of *Crocus sativus*. Eur. J. Plant Pathol. 143, 123–131.

Hansen, J., 2016. Method of Producing Isoprenoid Compounds in Yeast. Google Patents.

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.

Jain, M., Srivastava, P.L., Verma, M., Ghangal, R., Garg, R., 2016. De novo transcriptome assembly and comprehensive expression profiling in *Crocus sativus* to gain insights into apocarotenoid biosynthesis. Sci. Rep. 6, 22456.

Kalivas, A., Pasentsis, K., Polidoros, A.N., Tsaftaris, A.S., 2007. Heterotopic expression of B-class floral homeotic genes *PISTILLATA*/*GLOBOSA* supports a modified model for crocus (*Crocus sativus* L.) flower formation. DNA Seq. 18, 120–130.

Kianmehr, H., 1981. Vesicular—arbuscular mycorrhizal spore population and infectivity of saffron (*Crocus sativus*) in Iran. New Phytol. 88, 79–82.

Kirby, J., Keasling, J.D., 2008. Metabolic engineering of microorganisms for isoprenoid production. Nat. Prod. Rep. 25, 656–661.

Kitazawa, M.S., Fujimoto, K., 2014. A developmental basis for stochasticity in floral organ numbers. Front. Plant Sci. 5, 545.

Kumar, A.S., 2017. Methods for Recombinant Production of Saffron Compounds. Google Patents.

Lashbrooke, J.G., Young, P.R., Dockrall, S.J., Vasanth, K., Vivier, M.A., 2013. Functional characterisation of three members of the *Vitis vinifera* L. carotenoid cleavage dioxygenase gene family. BMC Plant Biol. 13, 156.

Liao, Y.-H., Houghton, P.J., Hoult, J., 1999. Novel and known constituents from *Buddleja* species and their activity against leukocyte eicosanoid generation. J. Nat. Prod. 62, 1241–1245.

Lone, R., Shuab, R., Koul, K., 2016. AMF association and their effect on metabolite mobilization, mineral nutrition and nitrogen assimilating enzymes in saffron (*Crocus sativus*) plant. J. Plant Nutr. 39, 1852–1862.

López, R.C., Gómez-Gómez, L., 2009. Isolation of a new fungi and wound-induced chitinase class in corms of *Crocus sativus*. Plant Physiol. Biochem. 47, 426–434.

Lou, S., Wang, L., He, L., Wang, Z., Wang, G., Lin, X., 2016. Production of crocetin in transgenic *Chlorella vulgaris* expressing genes *crtRB* and *ZCD1*. J. Appl. Phycol. 28, 1657–1665.

Maggi, L., Carmona, M., Del Campo, C.P., Kanakis, C.D., Anastasaki, E., Tarantilis, P.A., et al., 2009. Worldwide market screening of saffron volatile composition. J. Sci. Food Agric. 89, 1950–1954.

Malik, A.H., Ashraf, N., 2017. Transcriptome wide identification, phylogenetic analysis, and expression profiling of zinc-finger transcription factors from *Crocus sativus* L. Mol. Genet. Genomics 292, 619–633.

Mohebi-Anabat, M., Riahi, H., Zanganeh, S., Sadeghnezhad, E., 2015. Effects of arbuscular mycorrhizal inoculation on the growth, photosynthetic pigments and soluble sugar of *Crocus sativus* (saffron) in autoclaved soil. Int. J. Agron. Agric. Res. 6, 296–304.

Moraga, A.R., Nohales, P.F., Pérez, J.A.F., Gómez-Gómez, L., 2004. Glucosylation of the saffron apocarotenoid crocetin by a glucosyltransferase isolated from *Crocus sativus* stigmas. Planta 219, 955–966.

Namin, M.H., Ebrahimzadeh, H., Ghareyazie, B., Radjabian, T., Gharavi, S., Tafreshi, N., 2009. In vitro expression of apocarotenoid genes in *Crocus sativus* L. Afr. J. Biotechnol. 8, 5378–5382.

Nisar, N., Li, L., Lu, S., Khin, N.C., Pogson, B.J., 2015. Carotenoid metabolism in plants. Mol. Plant 8, 68–82.

Pfister, S., Meyer, P., Steck, A., Pfander, H., 1996. Isolation and structure elucidation of carotenoid − glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne). J. Agric. Food Chem. 44, 2612–2615.

Pogson, B., McDonald, K.A., Truong, M., Britton, G., DellaPenna, D., 1996. Arabidopsis carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. Plant Cell 8, 1627–1639.

Prunet, N., Meyerowitz, E.M., 2016. Genetics and plant development. C. R. Biol. 339, 240–246.

Raghavan, S., Hansen, J., Sonkar, S., Kumar, S., Kumar, K., Panchapagesa, M., et al., 2017. Methods and Materials for Recombinant Production of Saffron Compounds. Google Patents.

Raina, B.L., Agarwal, S.G., Bhatia, A.K., Gaur, G.S., 1996. Changes in pigments and volatiles of saffron (*Crocus sativus* L.) during processing and storage. J. Sci. Food Agric. 71, 27–32.

Rodrigo, M.J., Alquézar, B., Alós, E., Medina, V., Carmona, L., Bruno, M., et al., 2013. A novel carotenoid cleavage activity involved in the biosynthesis of Citrus fruit-specific apocarotenoid pigments. J. Exp. Bot. 64, 4461–4478.

Rodrıguez-Concepción, M., Boronat, A., 2002. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol. 130, 1079–1089.

Rubio, A., Rambla, J.L., Santaella, M., Gómez, M.D., Orzaez, D., Granell, A., et al., 2008. Cytosolic and plastoglobule-targeted carotenoid dioxygenases from *Crocus sativus* are both involved in β-ionone release. J. Biol. Chem. 283, 24816–24825.

Sandmann, G., Römer, S., Fraser, P.D., 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metab. Eng. 8, 291–302.

Seto, Y., Sado, A., Asami, K., Hanada, A., Umehara, M., Akiyama, K., et al., 2014. Carlactone is an endogenous biosynthetic precursor for strigolactones. Proc. Natl. Acad. Sci. 111, 1640–1645.

Sharaf-Eldin, M., Elkholy, S., Fernández, J.-A., Junge, H., Cheetham, R., Guardiola, J., et al., 2008. *Bacillus subtilis* FZB24® affects flower quantity and quality of saffron (*Crocus sativus*). Planta Med. 74, 1316–1320.

Srivastava, R., Ahmed, H., Dixit, R., 2010. *Crocus sativus* L.: a comprehensive review. Pharmacogn. Rev. 4, 200–208.

Tarantilis, P.A., Tsoupras, G., Polissiou, M., 1995. Determination of saffron (*Crocus sativus* L.) components in crude plant extract using high-performance liquid chromatography-UV-visible photodiode-array detection-mass spectrometry. J. Chromatogr. 699, 107–118.

Theißen, G., Melzer, R., Rümpler, F., 2016. MADS-domain transcription factors and the floral quartet model of flower development: linking plant development and evolution. Development 143, 3259–3271.

Toledo-Ortiz, G., Huq, E., Rodríguez-Concepción, M., 2010. Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. Proc. Natl. Acad. Sci. 107, 11626–11631.

Tsaftaris, A., Pasentsis, K., Polidoros, A., 2005. Isolation of a differentially spliced C-type flower specific AG-like MADS-box gene from *Crocus sativus* and characterization of its expression. Biol. Plant. 49, 499–504.

Tsaftaris, A., Pasentsis, K., Makris, A., Darzentas, N., Polidoros, A., Kalivas, A., et al., 2011. The study of the E-class *SEPALLATA3-like* MADS-box genes in wild-type and mutant flowers of cultivated saffron crocus (*Crocus sativus* L.) and its putative progenitors. J. Plant Physiol. 168, 1675–1684.

Tsaftaris, A., Pasentsis, K., Kalivas, A., Michailidou, S., Madesis, P., Argiriou, A., 2012a. Isolation of a *CENTRORADIALIS/TERMINAL FLOWER1* homolog in saffron (*Crocus sativus* L.): characterization and expression analysis. Mol. Biol. Rep. 39, 7899–7910.

Tsaftaris, A.S., Pasentsis, K., Madesis, P., Argiriou, A., 2012b. Sequence characterization and expression analysis of three *APETALA2-like* genes from saffron crocus. Plant Mol. Biol. Rep. 30, 443–452.

Tsaftaris, A.S., Pasentsis, K., Iliopoulos, I., Polidoros, A.N., 2004. Isolation of three homologous AP1-like MADS-box genes in crocus (*Crocus sativus* L.) and characterization of their expression. Plant Sci. 166, 1235–1243.

Wafai, A.H., Bukhari, S., Mokhdomi, T.A., Amin, A., Wani, Z., Hussaini, A., et al., 2015. Comparative expression analysis of senescence gene *CsNAP* and B-class floral development gene *CsAP3* during different stages of flower development in Saffron (*Crocus sativus* L.). Physiol. Mol. Biol. Plants 21, 459–463.

Walter, M.H., Strack, D., 2011. Carotenoids and their cleavage products: biosynthesis and functions. Nat. Prod. Rep. 28, 663–692.

Welsch, R., Maass, D., Voegel, T., DellaPenna, D., Beyer, P., 2007. Transcription factor RAP2. 2 and its interacting partner SINAT2: stable elements in the carotenogenesis of *Arabidopsis* leaves. Plant Physiol. 145, 1073–1085.

Zinati, Z., Shamloo-Dashtpagerdi, R., Behpouri, A., 2016. In silico identification of miRNAs and their target genes and analysis of gene co-expression network in saffron (*Crocus sativus* L.) stigma. Mol. Biol. Res. Commun. 5, 233–246.