Implications of Carotenoid Biosynthetic Genes in Apocarotenoid Formation during the Stigma Development of Crocus sativus and Its Closer Relatives

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Crocus sativus is a triploid sterile plant characterized by its long red stigmas, which produce and store significant quantities of the apocarotenoids crocetin and crocin, formed from the oxidative cleavage of zeaxanthin. Here, we investigate the accumulation and the molecular mechanisms that regulate the synthesis of these apocarotenoids during stigma development in C. sativus. We cloned the cDNAs for phytoene synthase, lycopene-β-cyclase, and β-ring hydroxylase from C. sativus. With the transition of yellow undeveloped to red fully developed stigmas, an accumulation of zeaxanthin was observed, accompanying the expression of CsPSY, phytoene desaturase, and CsLYCb, and the massive accumulation of CsBCH and CsZCD transcripts. We analyzed the expression of these two transcripts in relation to zeaxanthin and apocarotenoid accumulation in other Crocus species. We observed that only the relative levels of zeaxanthin in the stigma of each cultivar were correlated with the level of CsBCH transcripts. By contrast, the expression levels of CsZCD were not mirrored by changes in the apocarotenoid content, suggesting that the reaction catalyzed by the CsBCH enzyme could be the limiting step in the formation of saffron apocarotenoids in the stigma tissue. Phylogenetic analysis of the CsBCH intron sequences allowed us to determine the relationships among 19 Crocus species and to identify the closely related diploids of C. sativus. In addition, we examined the levels of the carotenoid and apocarotenoid biosynthetic genes in the triploid C. sativus and its closer relatives to determine whether the quantities of these specific mRNAs were additive or not in C. sativus. Transcript levels in saffron were clearly higher and nonadditive, suggesting that, in the triploid gene, regulatory interactions that produce novel effects on carotenoid biosynthesis genes are involved.

Carotenoids are terpenoid compounds that are ubiquitous in nature. In all photosynthetic organisms, they carry out essential functions in light-harvesting systems and in photosynthetic reaction centers (Horton et al., 1996). In higher plants, carotenoids play additional roles in providing distinct yellow, orange, and red colors to certain organs, such as flowers and fruits, to attract animals for pollination and dispersal of seeds. In those tissues, unique carotenoids synthesized as secondary metabolites accumulate to high concentrations and are stored within the chromoplasts.

All isoprenoids, including carotenoids, are derived from the ubiquitous C5 building blocks isopentenyl diphosphate and dimethylallyl diphosphate. These precursors can be synthesized through two different routes: the classical mevalonate pathway in the cytoplasm or the alternative nonmevalonate pathway in plastids (Rohmer et al., 1993; Arigoni et al., 1997). Metabolic cross-talk between the pathways can be observed under certain conditions (Hemmerlin et al., 2003). The plastidial pathway, now known as the 2-C-methyl-d-erythritol 4-phosphate pathway, has been fully elucidated by a combination of biochemical and genomic approaches (Rodríguez-Concepción and Boronat, 2002), and it provides the precursors for carotenoids. The first committing step of carotenoid biosynthesis is a head-to-head coupling of two molecules of geranylgeranylpyrophosphate to yield colorless phytoene by phytoene synthase (PSY). Subsequently, four additional double bonds are introduced by phytoene desaturase (PDS) and zeaxanthin desaturase (ZDS) producing the colored carotenes phytofluene, ζ-carotene, neurosporene, and lycopene. Recently, Park et al. (2002) and Isaacs et al. (2002) isolated the gene encoding carotenoid isomerase from Arabidopsis (Arabidopsis thaliana) and tomato (Lycopersicon esculentum), respectively, by map-based cloning. Carotenoid isomerase functions as the isomerization of polyisocarotenoids to all-trans-carotenoids. The cyclization of lycopene creates a series of carotenoids that have one or two rings of either the β- or ε-type. Lycopene β-cyclase (LYCb) catalyzes a two-step reaction that leads to β-carotene (two β-rings), whereas lycopene ε-cyclase creates one ε-ring to produce δ-carotene. It is thought that α-carotene (β, ε-carotene) is formed by the action of both enzymes (Cunningham et al., 1996). The resulting compounds are further modified by the introduction of hydroxyl groups onto the ionone rings.

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One ω-ring and one β-ring hydroxylation of α-carotene yield lutein in reactions catalyzed by the ω-hydroxylase, a member of the cytochrome P450 (Tian et al., 2004) and the β-hydroxylase (BCH; Cunningham and Gantt, 1998) family, respectively. Two β-ring hydroxylations of β-carotene yield zeaxanthin in a reaction catalyzed by BCH. These carotenoids can be further modified and processed to generate, among others, apocarotenoids that vary considerably among species (Britton, 1998). The importance of these compounds is apparent in some of the earliest annals of human history (Fraser and Bramley, 2004). For example, *Crocus sativus*, the source of saffron, was one of the first plants to be cultivated for a reason other than its caloric or nutritive value. The intense color of saffron is caused by apocarotenoids, especially crocetin esters (crocetin is a dicarboxylic acid with a carotenoid-like C-18 backbone), with gentiofucose (Tarantilis et al., 1995; Tarantilis and Polissiou, 1997). These saffron apocarotenoids are formed by zeaxanthin cleavage (Pfander and Schurtenberger, 1982), followed by specific glycosylation steps. Recently, two enzymes involved in saffron apocarotenoid formation have been isolated: the *Crocus zeaxanthin 7,8(7',8')-cleavage dioxygenase gene* (*CzZCD*), which codes for a chromoplast enzyme that initiates the biogenesis of crocetin glycosides and picrocrocin (Bouvier et al., 2003), and UGTCs2, which transfers Glc molecules to crocetin (Rubio et al., 2004). However, the genes and the corresponding enzymes involved in the previous biosynthesis steps remain uncharacterized, and, therefore, the regulation of carotenoid biosynthesis in this tissue is poorly understood. Understanding the regulation of apocarotenoid production during stigma development opens up the possibility of manipulating the timing of gene expression and, thus, the evolution of more interesting functions better adapted for the different uses of saffron (Fernandez, 2004; Giaccio, 2004), without changing the basic chemical signature of the plant.

*C. sativus* is a triploid sterile plant, which is propagated by corms. During the past 5,000 years, early farmers selected *C. sativus* for its long, colorful, carotenoid-pigmented stigmas. This vegetative cultivation offers advantages in maintaining the genetic characteristics of the plants, but impedes any genetic improvement. In *C. sativus*, many experiments have been performed in order to obtain seeds (Grilli-Caiola et al., 2004) without obtaining further insight than those obtained by morphological studies. Although RAPDs have been used in many studies of genetic diversity (Karp et al., 1996), the limitations of these markers for phylogenetic analysis have been summarized by several authors (Jones et al., 1997; Pejic et al., 1998; Harris, 1999), including problems associated with reproducibility and product homology. By contrast, for studies of congeneric species, noncoding sequences are often sources of phylogenetically informative characters and, for the various noncoding regions, introns are being used for such purposes in plants. Palumbi and Baker (1994) described a method to find variable regions of the genome, which they called exon-primer, intron-crossing. The method entails using data from two or more related species to find conserved exons that flank variable introns. Primers can then be designed within the flanking exons that will amplify introns in a broader array of plant species. This method has been widely used to identify the origins of hybrids or allopolyploid plant taxa (Frohlich and Meyerowitz, 1997; Bailey and Doyle, 1999; Ge et al., 1999; Olsen and Schaal, 1999; Tank and Sang, 2001; Doyle et al., 2002; Lee et al., 2002; Oh and Potter, 2003; Small et al., 2004). Furthermore, because of biparental inheritance of these markers, recent hybrids initially possess both divergent parental genotypes, as evidenced by DNA sequence polymorphisms. In the absence of sexual reproduction, concerted evolutionary homogenization of sequences by interchromosomal crossing over or gene conversion during chromosome pairing at meiosis would not be expected (Sang, 2002). Consequently, hybrids reproducing strictly vegetatively, as *C. sativus*, should retain copies of both divergent sequences. Considering the origin of saffron directly associated with the selection of plants with exceptional long and red stigmas and therefore higher apocarotenoid content, it is possible to suggest that early farmers favored by this manner the selection of specific carotenogenic alleles. Because BCH is directly involved in the production of saffron apocarotenoids, we addressed the origin of saffron by analyzing the genomic sequences of *CsBCH1* from several Crocus species and cultivars.

In order to gain insight into the molecular basis of saffron apocarotenoid synthesis and accumulation, we studied the composition of carotenoids and the expression of carotenoid and apocarotenoid biosynthetic genes during stigma development. Partial cDNA sequences related to *PSY, PDS*, and *LYC* were cloned, and two full-length transcripts coding for β-carotene hydroxylase *CsBCH1* and *CsBCH2* were isolated. Their expression was followed during the stigma development of saffron, in addition to the expression of the carotenoid cleavage genes *CzZCD* and the Crocus carotenoid 9,10(9′,10′)-cleavage dioxygenase gene
(CsCCD) that cleaves a broader range of precursors. These results show the simultaneous expression of genes participating in the synthesis of the saffron apocarotenoid precursor zeaxanthin. We also provide information on differences among Crocus species at the chemical and molecular level. The utility of these data for Crocus species classification and for C. sativus origin determination is discussed.

RESULTS

Identification of Carotenoids in C. sativus Stigmas

To identify and quantify saffron carotenoids in fully developed stigmas, two different extraction methods were used. The first analysis involved methanol-water extraction of the stigma tissue followed by acetone extraction. This extract was subjected to HPLC separation, and then the structural identities of the separated metabolites based on their diode array spectra were evaluated. This revealed that the main pigments present in the extract were saffron apocarotenoids, crocin glucosides, and picrocrocin. On the contrary, the carotenoid fraction was a minor fraction of the total pigments (data not shown). The carotenoid composition of saffron stigmas was consistent with previous reports (Pfander and Schurtenberger, 1982), with the exception of zeaxanthin, which we did not detect. Several peaks were identified and tentatively assigned as phytoene (λmax nm, 278, 282), phytofluene (λmax nm, 334, 350, 370), ζ-carotene (λmax nm, 380, 402, 428), and β-carotene (λmax nm, 432, 458, 484), based on their retention times and spectral properties. For the second extraction method, we followed Fraser et al. (2000). This method seemed more efficient at extracting carotenoids. The total amount of carotenoids extracted by chloroform from saffron stigma tissue was greater than by acetone. These differences could be related to the release of carotenoids from the stigma plastoglobules (Grilli-Caiola and Canini, 2004) that are soluble in chloroform and not in acetone. Using this extraction procedure, in addition to the previously detected compounds, it was possible to detect the presence of zeaxanthin (λmax nm, 452, 480) at higher concentrations than β-carotene (λmax nm, 432, 458, 484), and β-cryptoxanthin (λmax nm, 454, 482) was also detected at higher levels (Fig. 1). Therefore, Fraser et al.’s (2000) method was used for the determination of carotenoids during the previous stigma developmental stages.

Eight stages of development have been defined for C. sativus stigmas based on the length of the tissue, pigmentation, and apocarotenoid content (Himeno and Sano, 1987). Stage II corresponds to a yellow undeveloped stigma and stage IV represents an orange undeveloped stigma. Amounts of carotenoids were extremely low in these tissues in comparison with the carotenoid content present in the fully developed stigma (Fig. 1). Nevertheless, the detection of phytoene (λmax nm, 278, 282) and ζ-carotene (λmax nm, 380, 402, 428) was possible in the yellow stigma, whereas β-carotene (λmax nm, 432, 458, 484) and β-cryptoxanthin (λmax nm, 454, 482) were the main carotenoids detected in the orange stigma.

PCR-Based Search for the Saffron BCH Gene Family

To identify genes encoding β-carotene hydroxylase enzymes in C. sativus, a PCR-based cloning strategy was adopted. Sequencing of PCR products revealed only one type of insert related to carotene hydroxylases. To isolate full-length cDNA clones, a RACE-PCR strategy was adopted. Comparison of the 3’ RACE-PCR nucleotide sequences sequenced from all 60 clones revealed two different classes of BCH sequences, arbitrarily named CsBCH1 and CsBCH2. CsBCH1 and CsBCH2 were represented by 58 and two clones, respectively. An additional RACE-PCR approach allowed the isolation of full-length cDNA clones for CsBCH1 and CsBCH2. The isolated CsBCH1 full-length cDNA is 1,461 bp, excluding the poly(A)+ tail. A 5’ untranslated region of 97 bp precedes the putative start codon, and 426 bp of 3’ untranslated DNA follow the termination codon and precede the poly(A)+ tail. A possible polyadenylation signal (the sequence AATAA at bp 1,415–1,421) occurs 20 bp before the poly(A)+ tail. The predicted amino acid sequence of the β-carotene hydroxylase 1 specifies a polypeptide of 291 amino acids in length with a molecular mass of 34 kD and a pI of 9.48. The isolated CsBCH2 full-length cDNA is 891 bp, excluding the poly(A)+ tail and the 3’ untranslated region. A 5’ untranslated region of 14 bp precedes the putative start codon. The predicted amino acid sequence of the β-carotene hydroxylase 2 specifies a polypeptide of 305 amino acids in length with a molecular mass of 32 kD and a pI of 10.4.

The substrate and product of the carotene hydroxylases are hydrophobic and many carotenogenic enzymes are tightly associated with plastid membranes (Cunningham, 2002). Transmembrane region analysis of the CsBCH1 and CsBCH2 amino acid sequences using the SMART algorithm indicated one main hydrophobic domain in each sequence. As has been previously described for other plant enzymes, the saffron protein includes a long N-terminal region that is absent in the bacterial enzymes. The presence of an N-terminal extension that has no counterpart in the bacterial enzymes is also present in plant β-cyclase enzymes that convert lycopene to carotene (Cunningham et al., 1996). This N-terminal extension might provide for specific interaction of the carotenoid biosynthetic enzymes among them and with components of the membrane (Hugueney et al., 1995). In addition, data analysis with the ChloroR program (Emanuelsson et al., 1999) predicted a plastid localization signal in the first 59 and 60 amino acids of CsBCH1 and CsBCH2, respectively.

Sequence comparison indicates that CsBCH1 and CsBCH2 are quite similar to other known carotene hydroxylases. The saffron enzymes share 72% to 88%
amino acid identity with β-carotene hydroxylases from various plants (data not shown). Phylogenetic analysis based on the alignment of all available full-length protein sequences of higher plant carotene hydroxylases showed the presence of three main sub-groups. One more divergent group consists of cDNAs encoding enzymes from the grasses (Poales), and the other two groups correspond to the other monocotyledonous enzymes and dicotyledonous plants (Fig. 2); this closer relationship between Asparagales and eudicots has recently been suggested by Kuhl et al. (2004). Inside each cluster, it was obvious that the proteins from the same family had the highest structural similarity to each other. In addition, phylogenetic analysis of BCH proteins from different plant species, for instance, tomato and pepper (Capsicum annuum), revealed grouping of tomato BCH1 and BCH2 with pepper BCH2 and BCH1, respectively (Fig. 2). One can assume a duplication event of an ancestral BCH gene that took place prior to separation of Lycopersicon and Capsicum into different genera, and the same can be inferred for Arabidopsis and Brassica.

**Gene Structure Predictions for Saffron BCH Genes**

Three different genomic clones were isolated from saffron by PCR amplification with several BCH oligonucleotide combinations. Comparison between the cDNA and the genomic DNA-sequencing products revealed the presence of four introns in the genomic clones (Table I), and all presented conservation of the GT...AG intron boundaries (Brown, 1986). Two of the genomic clones only differ in the sequence of intron I and were considered to be allelic variations of CsBCH1. Comparison among the intron sequences of the isolated saffron genomic clones revealed that intron I sequences showed a 71.9% identity in the last 64 nucleotides, which indicates a deletion or an insertion.
in the CsBCH1 or CsBCH2 introns, respectively. The presence of three inverted repeats in the nonoverlapping intron sequence of CsBCH2 suggested that length differences with the CsBCH1 intron could be the result of one or more insertion events. Intron II showed a 77.8% identity, intron III showed an 84.9% identity, and intron IV showed a 72.5% identity.

The currently available plant genomic clones coding for BCH show the presence of several introns (Fig. 3A). The Arabidopsis BCH genomic sequences showed differences at intron number and intron sizes (Tian and DellaPenna, 2001), five introns in the BCH gene on chromosome 4, and six introns in the BCH gene on chromosome 5. In rice (Oryza sativa), there are five introns (BCH clone from chromosome 4) and four introns (BCH genes in chromosomes 3 and 10). In the case of the only available maize (Zea mays) BCH genomic sequence, although three genes have been reported (Wurtzel, 2004), only four introns are present. Because the majority of the monocot BCH sequences contain four introns in conserved positions, we suggest that an ancestral monocot BCH gene contained four introns in these positions.

Given the variability in intron number and sequence, and in spite of the conservation of several intron positions in the currently available genomic BCH sequences, we investigated the presence of introns in related Crocus species in order to resolve C. sativus origin. The genus Crocus comprises 83 species and it is divided into two subgenera: the subgenus Crocus comprising all species except one, and Crocus banaticus, which is the sole member of the subgenus Crociris. The subgenus Crocus is further divided into two sections, Crocus and Nudiscapus, and each is further divided into series based on the nature of the style and corm tunic features. Crocus from different sections were analyzed for BCH genomic sequences. The detection of bands of different sizes showed that at least the 3’ region of the CsBCH1 genomic sequences was clearly divergent among the analyzed species (Fig. 3B). This variability opens the possibility of studying the phylogenetic relationships in the Crocus genus through the analysis of these 3’ regions. A total of 40 accessions from 19 species were analyzed (Table II). By RAPD analysis, we have observed a high level of intraspecific genetic variability.
with the exception of \textit{C. sativus}, from different locations (data not shown). Using \textit{CsBCH1}-specific oligonucleotides, we amplified and analyzed the sequence of all the accessions from the 5' end of intron I to the 3' end of intron II. Interestingly, we found major differences at the 3' end of the first intron, which in the series \textit{Crocus} presents a U-rich cluster, a feature that has been associated with an increase in expression of the respective gene due to mRNA accumulation (Ko et al., 1998; Rose, 2002). These data were combined with the apocarotenoid profiles, which allowed us to suggest a good correlation between the obtained molecular data

### Table 1. Intron sequences corresponding to the genomic clones coding for BCH isolated from \textit{C. sativus}

<table>
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<th>Intron No.</th>
<th>BCH1 Intron Sequences</th>
<th>BCH2 Intron Sequences</th>
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<td>GTAGCTGCGACATATTGTATGTTATGTCACGGTTTCCAGATCTTG-ATACCTCGTATCCAGGATTGGGGATTCTTTTTTTTTTAG</td>
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<td>IV</td>
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<td>GGTAGCTTCCACGAGGACTGGAATTTCTGGTTGGAATTACAG</td>
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</table>

### Figure 3.

**A**

Diagrammatic representation of the \textit{BCH} genes of \textit{Arabidopsis}, maize, rice, and \textit{C. sativus}. The position of the ATG translation start and the translation stop are indicated. Boxes and lines denote coding regions and introns, respectively. The position of the primers used for intron analysis and sequencing are indicated on the diagrammatic representation of the genomic clone \textit{CsBCH1}.

**B**

and the production and accumulation of these metabolites in the stigma tissue of these species. The highest levels of genetic diversity varied greatly among the different populations. There are two main divisions in this series. Both can be discriminated by the presence of a molecular feature in the 3’ end of the second intron, which is the number of repetitions of the TTTA motif. This motif is repeated twice in the group that contains both *C. sativus* alleles, while in the other group it is repeated four times. Furthermore, these two groups can be correlated with the geographical distribution of the populations. The second group includes populations from the islands of Crete and Lesbos. In the *C. cartwrightianus* populations from Crete (GKO00-8 and GKO00-44), the alleles are more closely related to each other than to those from other *C. cartwrightianus* populations, suggesting population divergence probably due to geographical isolation. The same is observed for all the *C. pallasii* alleles from Lesbos. However, for the rest of the alleles present in this subgroup that group together, there was no correspondence between alleles to

<table>
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<th>Nomenclature</th>
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</tbody>
</table>
population identity, which suggests that these populations maintain an ancestral allelic variation. In addition, hybridization might be another cause of this allele sharing (Small et al., 2004). In contrast, the other main group in the series Crocus includes only continental populations of *C. cartwrightianus* and *C. hadriaticus*, along with a *C. pallasii* population and all the *C. sativus* populations. The *C. sativus* alleles were related to specific accessions of *C. cartwrightianus* and *C. hadriaticus* (Fig. 4), with which *C. sativus* sequences share polymorphism. These data suggest that *C. sativus* arose from a hybridization event involving both species and therefore confirms its origin as an allopolyploid.

**High Expression Levels in Saffron Stigma**

During the development of saffron, the stigma changes in color from white to scarlet, passing through...
yellow and orange stages, parallel stigma growth, and apocarotenoid accumulation. Apocarotenoids increased 7% from the yellow to the orange stage and up to 88% in the scarlet stage (Himeno and Sano, 1987). We have also shown the accumulation of carotenoids during this process, which reach their maximum levels at the time of anthesis. β-Carotene and zeaxanthin increased by 60.5% and 85%, respectively, from the orange to the scarlet stage. Due to this active accumulation of carotenoids, as a first step to evaluate the importance of the BCH genes in the control of zeaxanthin synthesis, we choose three developmental stages in saffron stigmas corresponding to stages II and IV (yellow and orange stigma, both corresponding to developing tissue), and stage VIII (scarlet, mature tissue; Fig. 5A). If either CsBCH1 or CsBCH2 transcript accumulation correlates with zeaxanthin accumulation in saffron stigma, we would expect that transcript levels would vary during stigma development. Reverse transcription (RT)-PCR was performed with RNA purified from stigmas in stages II, IV, and VIII. Under the same PCR conditions, CsBCH1 transcripts were detected at much higher levels than CsBCH2. The expression levels of CsBCH1 were higher in developed stigma (Fig. 5B), in accordance with the highest levels of carotenoids detected in this developmental stage. We compared the CsBCH1 expression with the transcript levels of other genes from the pathway. Based on the conserved amino acid sequences among plant species in carotenoid biosynthetic genes, two sets of degenerated primers were designed for each PSY and LYCb. RT-PCRs were performed with saffron stigmas. The cDNA fragments for these carotenoid biosynthetic genes were designated as CsPSY (GenBank accession no. AJ888514) and CsLYCb (GenBank accession no. AJ888515). Specific oligonucleotides for each of these cDNA fragments and for the CsPDS full-length cDNA (GenBank accession no. AY183118) were designed and used in the expression analysis. The RT-PCR analysis revealed that accumulation of the mRNAs corresponding to the carotenogenic genes studied during stigma development followed different patterns (Fig. 5B). The CsPSY and CsPDS genes reached the maximal levels in the orange stage and then remained relatively constant through development. The pattern of transcript accumulation for CsLYCb, CsBCH2, and CsBCH1 was similar, reaching a maximum that was coincident with the accumulation of saffron carotenoids. These results indicate that carotenoid accumulation in stigma tissue correlates with expression of CsBCH1 and other carotenogenic genes. Furthermore, we investigated the expression levels of the genes CsCCD and CsZCD, coding for cleavage carotenoid enzymes in saffron stigmas (Bouvier et al., 2003). The CsCCD enzyme acts on a broader range of precursors such as β-carotene and zeaxanthin. The CsCCD gene expression increased from the yellow to the orange and scarlet stages following the accumulation of β-carotene and zeaxanthin during stigma development, which suggests a depletion of these compounds in the latter phases of development. The gene CsZCD, which codes for a chromoplast enzyme that initiates the biosynthesis of crocetin glycosides and picrocrocin, was highly expressed in the scarlet stigma when the highest levels of zeaxanthin and apocarotenoids have been detected.

The expression levels were also analyzed in other saffron tissues (Fig. 5B). In petals, the expression level of CsBCH1 was low. Saffron petals are purple and mainly accumulate anthocyanins (Norbaek and Kondo, 1998). CsBCH1 expression levels were also studied in stamens and roots, where very low levels of expression were detected (data not shown).

**Figure 5.** Expression levels of saffron carotenogenic genes during stigma development. The mRNA levels were determined by RT-PCR amplification using specific oligonucleotides for each carotenogenic gene. A, Stigma tissue of *C. sativus* in different developmental stages. B, CsBCH1 and CsBCH2 transcript expression in undeveloped (StII and StIV) and fully developed (StVIII) stigma, petals, and leaves. The expression pattern of CsPSY, CsPDS, CsLYCb, CsCCD, and CsZDS was monitored during stigma development. Equal amounts of total RNA were used in each reaction. The levels of constitutively expressed RPS18 coding genes were assayed as controls. The PCR products were separated by 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

**Correlation between CsBCH1 Transcript Levels and Zeaxanthin Production**

We have shown that apocarotenoid concentration and composition vary greatly among Crocus species (Fig. 4). We also analyzed the carotenoid content in Crocus species that greatly vary in their stigma coloration (Fig. 6A). *Crocus goulimyi* has a pale yellow stigma; this light coloration was primarily because of a low concentration of total carotenoids (Fig. 6B). Only phytofluene and ζ-carotene were detected after HPLC profile magnification. In contrast, the stigma tissue showed a high content in flavonoids. *Crocus imperati* has an orange stigma, phytofluene, ζ-carotene, and...
β-carotene were detected in the mature stigma after HPLC profile magnification. *C. hadriaticus*, *C. cartwrightianus*, and *C. sativus* all have a red stigma (Fig. 6A). *C. cartwrightianus* and *C. hadriaticus* showed a high carotenoid content in the stigmas. β-Carotene was the main carotenoid followed by zeaxanthin (Fig. 6B), while in *C. sativus*, zeaxanthin was the main carotenoid followed by β-carotene (Fig. 6B). Thus, these species are useful experimental models to investigate the molecular mechanism regulating carotenoid concentration and composition in the stigma. We determined the expression levels of *CsBCH1* in these tissues. Although transcripts were detected in all the tested tissues, the expression levels were quite different. Correlation between zeaxanthin accumulation and *BCH* expression in fully developed stigmas was found (Fig. 6C), which suggests that zeaxanthin production in the stigma tissue of *C. sativus* is regulated at the gene expression level. In addition, the expression levels of *CsZCD* were analyzed in each sample (Fig. 6C). The expression levels were not correlated with the accumulation of apocarotenoids in these tissues (shown in Fig. 4) and with their crocin content (Fig. 6C). Therefore, although *CsZCD* was expressed in the mature stigma tissue of these Crocus species at relatively high levels, it seems that the biosynthesis of
zeaxanthin is the limiting step for the accumulation of CsZCD products.

**Polyploidy and Carotenogenic Gene Expression Levels**

*C. sativus* is characterized from the other species within the series Crocus by its long stigmas and its high apocarotenoid content. New phenotypes often rise with polyploidy formation and can contribute to the success of polyploids in nature or their selection for use in agriculture. The causes of variation in polyploids could involve changes in gene expression through increased variation in dosage-regulated gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes during organ development (Osborn et al., 2003; Otto, 2003). Due to the triploid nature of *C. sativus*, we decided to test whether the high expression level observed for CsBCH1 in this plant was correlated with genome dosage or the result of other trans-acting regulatory effects on the target genes. In addition, we also tested the expression levels of the carotenogenic genes, CsPSY, CsPDS, CsLYCb, and CsZCD, in order to determine the existence of a coordinate regulation in the apocarotenoid biosynthetic pathway. When performing this kind of study, it is desirable to use for comparison the most closely related diploid species (Osborn et al., 2003). Therefore, we compared the expression levels of these genes in *C. sativus* with those in its closer diploid relatives, *C. cartwrightianus* accession 12629 and *C. hadriaticus* accessions J93-26 and ØJ01-32. The mRNA of the ribosomal protein S18 was used as an internal standard in the semiquantitative RT-PCR analysis. A direct gene dosage effect will give an equal transcript level per genome among ploidies, or per cell values of 50% for dosage effect will give an equal transcript level per genome among ploidies, or per cell values of 50% for polyploids could involve changes in gene expression through increased variation in dosage-regulated gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes during organ development (Osborn et al., 2003; Otto, 2003). Due to the triploid nature of *C. sativus*, we decided to test whether the high expression level observed for CsBCH1 in this plant was correlated with genome dosage or the result of other trans-acting regulatory effects on the target genes. In addition, we also tested the expression levels of the carotenogenic genes, CsPSY, CsPDS, CsLYCb, and CsZCD, in order to determine the existence of a coordinate regulation in the apocarotenoid biosynthetic pathway. When performing this kind of study, it is desirable to use for comparison the most closely related diploid species (Osborn et al., 2003). Therefore, we compared the expression levels of these genes in *C. sativus* with those in its closer diploid relatives, *C. cartwrightianus* accession 12629 and *C. hadriaticus* accessions J93-26 and ØJ01-32. The mRNA of the ribosomal protein S18 was used as an internal standard in the semiquantitative RT-PCR analysis. A direct gene dosage effect will give an equal transcript level per genome among ploidies, or per cell values of 50% for monoploid, 100% for diploid, and 150% for triploid. The transcript levels for CsPSY showed a close positive correlation to ploidy. For CsPDS, CsLYCb, CsBCH, and CsZCD, the positive correlation of transcript level to ploidy went over the level predicted from a strict gene dosage effect (Table III). These observations indicate an interaction of carotenogenic genes dosage and their regulation genes in producing the net effects on gene expression, a phenomenon that is quite common in various types of hybrid situations (Guo et al., 1996; Wendel, 2000; Auger et al., 2005). Furthermore, gene products involved in the same pathway or macromolecular complex are often coregulated, responding basically to the same regulators (Veitia, 2004), and this is likely to be the case for the carotenogenic genes in *C. sativus*.

**DISCUSSION**

As apocarotenoid biosynthesis can be limited by the rate at which the carotenoid pathway supplies substrate zeaxanthin, we have used a combination of approaches to identify the repertoire of carotenogenic genes in *C. sativus* and to study the expression of each gene during stigma development in relation to the carotenoid accumulation in this tissue.

To identify the *PSY, LYCb*, and *BCH* genes in *C. sativus*, we initially used degenerate PCR primers that target evolutionarily conserved sequences in these genes. By sequencing multiple PCR-derived clones, we identified partial fragments of *PSY, LYCb*, and *BCH*. Due to the importance of zeaxanthin as the precursor of saffron apocarotenoids, we consider BCH to be a key enzyme for the production and accumulation of apocarotenoid in the saffron stigma. Therefore, we approached the cloning and characterization of the two *CsBCH* genes by using various PCR-based methods. The cloned genes encode active proteins (data not shown) with homologies higher than 78% identity to the respective genes from other plant species and share conserved sequence motifs. Phylogenetic analysis showed that both were close to the Narcissus enzyme. The topology of the phylogenetic tree shows that BCH sequences do not fall into two primary clades as predicted by the ancient gene duplication hypothesis. The analysis suggests that duplication events have apparently occurred at several levels. For example, the BCH sequences of the grass family are more closely related to each other than they are to other monocot sequences, indicating that a recent gene duplication event is responsible for this arrangement, as has been observed for the carotenogenic gene *PSY* (Busch et al., 2002; Gallagher et al., 2004). A similar history is evident for sequences from the Brassicaceae and the Solanaceae. Even more recent gene duplication seemed to give rise to *CsBCH1* and *CsBCH2*. Both sequences are closely related, are alignable throughout their length, and showed the same intron number and position. Because normally introns evolve more rapidly than coding sequences and the

**Table III. Expression levels of carotenogenic RNA transcripts in *C. sativus* relative to expression of *C. hadriaticus* and *C. cartwrightianus* diploids**

<table>
<thead>
<tr>
<th>Accession</th>
<th>CsPSY Mean</th>
<th>CsPSY se</th>
<th>CsPDS Mean</th>
<th>CsPDS se</th>
<th>CsLYCb Mean</th>
<th>CsLYCb se</th>
<th>CsBCH Mean</th>
<th>CsBCH se</th>
<th>CsZCD Mean</th>
<th>CsZCD se</th>
</tr>
</thead>
<tbody>
<tr>
<td>J93-26</td>
<td>1.48</td>
<td>0.03</td>
<td>1.8</td>
<td>0.04</td>
<td>2.00</td>
<td>0.03</td>
<td>1.62</td>
<td>0.04</td>
<td>1.56</td>
<td>0.02</td>
</tr>
<tr>
<td>ØJ01-32</td>
<td>1.55</td>
<td>0.04</td>
<td>1.7</td>
<td>0.02</td>
<td>2.03</td>
<td>0.07</td>
<td>1.63</td>
<td>0.03</td>
<td>2.8</td>
<td>0.06</td>
</tr>
<tr>
<td>12629</td>
<td>1.47</td>
<td>0.01</td>
<td>1.85</td>
<td>0.02</td>
<td>2.01</td>
<td>0.03</td>
<td>1.74</td>
<td>0.03</td>
<td>1.59</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Mean is an average value of the relative mRNA levels from three replicates. Mean values in boldface are significantly different from diploids (P < 0.05 in t test).
intron sequence divergence between loci is a measure of evolutionary distance, the observed data suggest a history of recent gene duplication for the saffron BCH genes.

We detected two cDNA populations based on sequence analysis of the 3′ region obtained by RACE-PCR. The finding of two BCH genes in saffron was not surprising, as this has been reported in several plants, including Arabidopsis (Tian and DellaPenna, 2001), tomato (Hirschberg, 1998), pepper (Bouvier et al., 1998), and Citrus (Kim et al., 2001). In tomato, the two enzymes are organ specific; one expressed in green tissue and the other in the flower (Hirschberg, 2001). Interestingly, in the phylogenetic analysis, the BCH fruit-specific enzymes from tomato and pepper cluster together. Both plants produce fruits where the carotenoid biosynthetic pathway is extremely active and accumulate the resulting carotenoids in the chromoplast at high concentrations (Fraser et al., 1994; Ronen et al., 2000; Iglesias et al., 2001). The lack of enough full amino acid sequences in the monocot group did not allow us determine whether chromoplast-specific enzymes are present inside Asparagales BCH, as seems to be the case in the Solanales. On the contrary, in Arabidopsis, both hydroxylases are expressed in all tissues, but β-hydroxylase I mRNA is always present at higher levels (Tian and DellaPenna, 2001), indicating that both enzymes are redundant.

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. However, in C. sativus, the development of the stigma occurs concomitantly with the amyloplast-to-chromoplast transition and the stigma never turns green during this process (Grilli-Caiola and Canini, 2004). We also showed that stigma development parallels carotenoid accumulation. Therefore, this tissue is a good model system to study the carotenoid and apocarotenoid formation and accumulation during this transition process. We determined the relationship between carotenogenic gene expression and the process of carotenoid accumulation. We examined the expression patterns of CsPSY, CsPDS, CsLYCβ, and both CsBCH gene family members during stigma development. This analysis shows that the transcript levels of genes are modulated during development, displaying their lowest levels at early developmental stages. The CsBCH1 transcript accumulates at high levels in saffron stigma, which is consistent with the production and accumulation in this tissue of the zeaxanthin-derivate apocarotenoids crocin, crocetin, and picrocrocin. These apocarotenoids reach the highest levels before the time of anthesis. At this stage, we have detected that β-carotene and zeaxanthin, which is the precursor of saffron apocarotenoids, are the main carotenoids present in the stigma extract. However, the levels of carotenoids in the developed stigma are much lower compared with the massive accumulation of apocarotenoids, suggesting a high flux rate in the carotenoid pathway. Furthermore, the CsCCD gene increased its expression concomitantly with the accumulation of its carotenoid substrates. The CsZCD gene, which codes for the enzyme catalyzing the zeaxanthin cleavage reaction to produce crocetin (Bouvier et al., 2003), was highly expressed in fully developed stigma of several Crocus species. However, the transcript levels were not mirrored by changes in the apocarotenoid content of these species. By contrast, differences between the species in terms of relative BCH transcript abundance mirrored the differences in the zeaxanthin content. All these data suggest that the reaction catalyzed by the CsBCH1 enzyme could be the limiting step in the formation of saffron apocarotenoids in the stigma tissue due to the accumulation of the respective substrate and product. A similar situation has been observed in other secondary metabolic pathways, where the levels of precursors are ultimately the rate-limiting step (Theis and Lerdau, 2003). Moreover, the analysis of the stigmas of several Crocus species showed that quantitative and qualitative changes of the carotenoid pigments are related to the expression levels of CsBCH1, thus supporting the hypothesis that the major mechanism controlling carotenoid accumulation is transcriptional regulation of carotenogenic genes (Wurtzel, 2004).

This study also shows the utility of CsBCH1 intron sequences for phylogenetic analysis at the species level. In addition, this intron has several of the attributes expected for nuclear sequences used for resolving relationships at lower taxonomic levels, such as the presence of one or more sizeable noncoding regions flanked by conserved regions and single- or low-copy number sequences (Sang, 2002; Soltis and Soltis, 2003). BCH genes are generally members of small gene families (often only two or three loci) and maintain at least four conserved intron positions across all members. These introns are typically flanked by conserved exons that provide the opportunity to develop universal primers for direct sequencing.

Traditional classification of the subgenus Crocus mostly relied on similarities in morphology such as floral and corm structures (Mathew, 1999). This subgenus is divided into two sections, which are subdivided in the series using the nature of the style and corm tunic. The molecular data based on BCH1 intron sequences support such groupings. Basically, we found two main clades, one containing sequences from all the genera included in the section Crocus and the other corresponding to the section Nudiscapus. Furthermore, we found a good correlation at the series level. The exception was Crocus medius located out of the cluster of the series Longiflori and present in the series Versicolori. In fact, the apocarotenoid profile of C. medius clearly differs from the one obtained for C. goulimyi. Furthermore, recent studies showed similar results when flower pigment composition has been studied (Nørbaek et al., 2002); the authors consider that C. medius should be included in the series.
Versicolores rather than in the series Longiflori. In addition, in a study with repetitive DNA, Frello et al. (2004) showed that the species included in the series Longiflori does not form a monophyletic group. Thus, considering the data obtained from these molecular studies, the taxonomic classification should be reevaluated.

C. sativus BCH1 alleles grouped together with C. cartwrightianus and C. hadriaticus accessions. Thus, C. cartwrightianus, C. hadriaticus, and C. sativus showed a close resemblance in chromosome morphology (Brighton, 1977); they also showed the most similar apocarotenoid profile. Although C. cartwrightianus and C. hadriaticus are native from Greece, they are not known to inhabit the same areas because C. hadriaticus is a western Greek taxon and C. cartwrightianus is a southeastern taxon. Considering different geographic origins for these species, the data obtained suggest that a human-mediated event might have been responsible for the formation of C. sativus from a hybridization event between C. cartwrightianus and C. hadriaticus. However, the possibility of a natural hybridization event could be considered assuming an overlapping distribution for both species in the past. In addition, sometimes misclassification occurs due to the fact that, in many cases, flowers of C. hadriaticus and C. cartwrightianus may look alike and therefore both species could be simultaneously present in specific areas. Nevertheless, our data support the proposed allotriploid origin of C. sativus (Agaye, 2002; Nørbak et al., 2002) as the result of crossing between C. sativus and C. hadriaticus. The resynthesis from the presumptive parental diploids C. hadriaticus and C. cartwrightianus could help not only to demonstrate the origin of saffron, but also possibly to provide a good tool to obtain fertile saffron plants that could be improved.

**MATERIALS AND METHODS**

**Plant Material**

For this study, we included 19 species of Crocus. Specimens were obtained from Dr. U. Jacobsen from the Agricultural University of Denmark and referred to by the number in parentheses, The Royal Botanic Gardens, Kew (London), and private collections in the United Kingdom (Potterton Nursery; Table II). Three individuals representing each population were used. Plant tissues were independently harvested and frozen in liquid nitrogen and stored at −80°C until required. Stigmas were collected at three developmental stages defined according to Himeno and Sano (1987): stage II, closed with the first signs of yellow pigmentation; stage IV, closed with orange color; and stage VIII, dark red pigment.

**Sample Preparation and HPLC Analysis**

Carotenoids and apocarotenoids were analyzed by reverse-phase HPLC using a Hewlett-Packard 1100 HPLC connected online to a photodiode array detector with a dynamic range from UV to visible region (190–700 nm) and a Sugarlab Inertsil ODS 2 5 µm C18 column (250 × 4.6 mm). The mobile phase delivered at a flow rate of 0.8 mL/min was buffer A, 10% acetonitrile with 0.05% trifluoroacetic acid; buffer B, 90% acetonitrile with 0.05% trifluoroacetic acid; buffer C, 100% acetonitrile with 0.05% trifluoroacetic acid; and buffer D, 100% tetrahydrofuran with 0.05% trifluoroacetic acid. The elution conditions used to separate the carotenoids were as follows: 82.5% buffer A and 12.5% buffer B for 5 min; 100% buffer C for 25 min; 60% buffer C and 40% buffer B for 5 min; and 100% buffer C for 2 min and 82.5% buffer A and 12.5% buffer B for 10 min at 0.8 mL/min flow rate. Apocarotenoid extraction and analysis conditions were performed as previously described (Rubio et al., 2004). Crocin content was estimated spectrophotometrically at 466 nm using an extinction coefficient of crocetin, E2° = 4,320 (Britton, 1995).

For the analysis of carotenoids, two extraction methods were used: Crocus stigma carotenoids were extracted in a microcentrifuge tube by grinding with a micropestle in methanol (100 µL) and incubated for 5 min on ice. Tris-HCl (50 mM, pH 7.5; containing 1 µL NaCl) was then added (100 µL) and a further incubation for 10 min in ice was carried out. The precipitate was collected by centrifugation at 3,000g for 5 min at 4°C. The pellet was then regrinded in acetone (400 µL) and incubated on ice for 10 min. The mixture was centrifuged at 3,000g for 5 min at 4°C. This last step was repeated until no pigments were detected in the pellet. The supernatants were pooled and evaporated and the dried residues were stored at −80°C until HPLC analysis. The second method of extraction was performed according to Fraser et al. (2000). The assays were performed in triplicate. Peak identification was based on comparison with standards purified from bacteria expressing carotenoid biosynthetic genes (Cunningham and Ganot, 1998). In particular, retention time and UV/visible light spectra determinations were undertaken and compared with standards and published data (Britton, 1995).

**Cloning of Saffron BCH cDNAs**

Total RNA and mRNA were isolated from developed saffron stigma by using Ambion PolyATrack and following the manufacturer’s protocols. Conserved regions from plant genes coding β-carotene hydroxylase were used for the design of degenerated primers used in the PCR amplification of β-carotene hydroxylase genes from cDNA that was prepared by RT of poly(A) from total RNA isolated from saffron stigma. The first-strand cDNAs were synthesized by RT from 2 µg of total RNA using a first-strand cDNA synthesis kit (Pharmacia) and an oligo(dT) primer. These cDNAs were used as templates for PCR using the follow primers: BCH-E, 5′-GACCAGAAGTTCGGAGAAAGT-3′ and BCH-R, 5′-ACCACCTCTCAAACTTCT-3′. The 3′ and 5′ regions of the cDNAs were amplified by two nested reactions using the 5′-CDS primer and SMARTIII A oligo for the 5′-RACE reaction and the 3′-CDS primer A for the 5′-RACE reaction supplied in the SMART RACE cDNA amplification kit (CLONTECH) and the BCH primers BCH-199as, 5′-ATGAGCGGAACGCTCGTGAGG-3′, BCH-39as, 5′-GTGGACAGCCGACCG-GC-3′, BCH-30as, 5′-CCGACGTGCCTAATCCGAG-3′, and BCH-438s, 5′-GTCGCCCTATGAGCCTTCT-3′, and the reverse primer 5′-GTCTTT-GATCTTCCTCACC-3′. A touch-down PCR program was run using the following conditions: 94°C for 3 min, 10 cycles at 94°C for 30 s, 66°C to 0.2°C/cycle for 30 s and 72°C for 2 min, and 30 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 2 min. Only the expected amplification products for CsBCH1 were used as targets of a second PCR nested reaction. The products were purified by agarose gel electrophoresis with the Wizard SV gel and PCR cleanup system (Promega), cloned in the pGEM-T easy vector (Promega), and sequenced in both directions using primers from the vector. Sequencing steps were carried out via automated cycle sequencing using dRhodamine dye termination and an ABI PRISM 3100 genetic analyzer. Computer-aided sequence similarity searches were made using BLAST (National Center for Biotechnology Information [NCBI]; http://www.ncbi.nlm.nih.gov), motif searches were made by using PROSITE (http://expasy.hcuge.ch/sprot/prosite.html), TMRpred (http://www.isrec.isb-sib.ch), and PSORT II (http://psort.nibb.ac.jp).

The full-length saffron CsBCH1 and CsBCH2 cDNAs were amplified from cDNA obtained from stigma tissue using the forward primers 5′-CTGCA-GAAGTCTCGGCAAAAAT-3′ for CsBCH1 and 5′-CCCATGACGCGCCAG-TATCC-3′ for CsBCH2, and the reverse primer 5′-TGCCTTTAATCTCC-CTGTACC-3′ for both amplifications.

**Isolation and Analysis of Nucleic Acids**

Total DNA was isolated from plant leaves using the NucleoSpin plant kit (Macherey-Nagel). The isolated DNA was quantified and the target regions were amplified via PCR in a Perkin-Elmer GeneAmp 2400. In order to survey the levels of sequence variation of the three first introns of CsBCH1 in Crocus species, we determined the nucleotide sequences of these regions using the above-described primers in addition to the following primers:
Measurement of mRNA by RT-PCR

Total RNA was isolated from corms, roots, leaves, petals, and stigma by using the TRIzol reagent (Invitrogen). From total RNA, the cDNA was synthesized using the Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). For tissue analysis of DNA intron sequences from several Crocus species were obtained in this study. Both amino acid and nucleotide sequences were aligned using ClustalW (Thompson et al., 1994) and manually adjusted to minimize substitutions and to maximize gaps. The data from peptides and genomic sequences were both analyzed using the program package Geneline (Brosdy et al., 1995).

Phylogenetic Analysis

Full-length protein sequences of putative BCH homologs were obtained from GenBank. Truncated BCH homologs from Sandersonia aurantiaca, Citrus sinensis, and Citrus limon are also present in the databases, but were not used for phylogenetic analysis because full-length sequences were not possible. DNA intron sequences from several Crocus species were obtained in this study. Both amino acid and nucleotide sequences were aligned using ClustalW (Thompson et al., 1994) and manually adjusted to minimize substitutions and to maximize gaps. The data from peptides and genomic sequences were both analyzed using the program package Geneline (Brosdy et al., 1995).

Accession Numbers

The GenBank accession numbers of the amino acid sequences of BCH used for intron analysis in Figure 3A are as follows: Arabidopsis ChIV, AL161563; Arabidopsis ChV, ABO25606; rice ChIV, AL606624; rice ChII, AC119747; rice ChX, AE017115; and maize, AY104495.

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LITERATURE CITED


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