RESEARCH PAPER

The expression of a chromoplast-specific lycopene beta cyclase gene is involved in the high production of saffron’s apocarotenoid precursors

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Abstract

*Crocus sativus* is a triploid sterile plant characterized by its long red stigmas, which produce and store significant quantities of carotenoid derivatives formed from the oxidative cleavage of β-carotene and zeaxanthin. The present study reports on the genomic structures of two lycopene-β-cyclase genes, *CstLcyB1* and *CstLcyB2a*, and on their transcription patterns in different *C. sativus* tissues. Phylogenetic analysis showed that both proteins are located in different groups: *CstLcyB2a* encodes chromoplast-specific lycopene cyclases, with an expression analysis showing strongly in flower stigmas where it activates and boosts β-carotene accumulation. The *CstLcyB1* transcript, however, was present in leaves, tepals, and stigmas at lower levels. *In vivo* assays in transgenic *Arabidopsis* demonstrated lycopene β-cyclase activity of *CstLcyB2a*. *CstLcyB2a* is a *CstLcyB1* parologue derived through a gene duplication event, while promoter analysis showed that both genes have diverged in their regulatory sequences after duplication. Furthermore, it was found that the *CstLcyB2a* gene was absent from *Crocus kotschyanus* and, although present in *C. goulimi* and *C. cancellatus*, the absence of transcripts suggests that transcriptional regulation of *CstLcyB2a* is responsible for the low apocarotenoid content in these species.

Key words: Apocarotenoids, *Crocus sativus*, gene expression, lycopene, lycopene β-cyclase, promoter, stigma.

Introduction

Carotenoids are widely distributed isoprenoid pigments fulfilling diverse functions in all taxa (Britton, 1998). Due to their vital role in protecting the photosynthetic apparatus from photo-oxidation, carotenoids are synthesized in all photosynthetic organisms. Moreover, carotenoids represent essential structural components of the light-harvesting antenna and reaction centre complexes (Horton *et al.*, 1996) and are accumulated in many flowers and fruits providing distinct yellow, orange, and red colours, thus contributing substantially to plant–animal communication (Hirschberg, 2001; DellaPenna and Pogson, 2006). In addition, the colours of many carotenoid-accumulating fruits and flowers also contribute to an increase in their economic value (Fraser and Bramley, 2004; Botella-Pavia and Rodriguez-Concepción, 2006; Giuliano *et al.*, 2008). Apart from these functions, carotenoids serve as precursors of several physiologically important compounds, synthesized through oxidative cleavage and generally known as apocarotenoids. Representative examples are the ubiquitous chromophore retinal, the phytohormone abscisic acid, and strigolactones (DellaPenna and Pogson, 2006; Leyser, 2008).

**Solanum lycopersicum** (Giuliano et al., 1993; Fraser et al., 1994; Ronen et al., 1999; Isaacson et al., 2002), **Capsicum annuum** (Romer et al., 1993), **Nicotiana tabacum** (Busch et al., 2002), and in an alga (Steinbrenner and Linden, 2001). These studies have demonstrated that carotenoid accumulation is mainly controlled by the transcriptional regulation of carotenoid biosynthetic genes. Carotenoid biosynthesis and sequestration take place within the plastids of higher plants (reviewed by Cunningham, 2001). These studies have demonstrated that carotenoid derivatives, formed from the oxidative cleavage of β-carotene and zeaxanthin (Rubio et al., 2009) (Fig. 1). The conversion of lycopene to β-carotene is catalysed by the β cyclase enzyme (Cunningham et al., 1994; Hugueneu et al., 1995; Pecker et al., 1996). The ε and β cyclase enzymes are products of related genes, but the ε cyclase only adds one ε ring to lycopene, whereas the β cyclase can add two β rings. Carotenoids with two ε rings are not commonly found in plants. An exception is luteoxanthin from lettuce. The lettuce Lεε produces the bicyclic ε,ε-carotene (Cunningham et al., 1996). The lettuce enzyme, together with the ε and β cyclases, is similar in sequence to cyanobacterial β cyclase enzymes, and these cyclases are related to another group of carotenoid cyclase enzymes which includes such chromoplast-targeted Lcyβ enzymes as the tomato (Ronen et al., 2000) and Citrus enzymes (Alquézar et al., 2009), the capsanthin-capsorubin synthase (Cs) of pepper (Bouvier et al., 2000), and the neoxanthin synthase (NSY) gene from potato (Al-Babili et al., 2000). Recent data demonstrate a central role for gene duplication in the development of a chromoplast-specific carotenoid biosynthesis pathway (Galpaz et al., 2006; Giorio et al., 2008). Gene and genome duplications have been shown to be particularly prominent in plant genomes and have greatly influenced genomes organization and evolution (Otto and Whitton, 2000; Wendel, 2000; De Bodt et al., 2005; Maere et al., 2005). These duplication events also have profound effects on gene function and regulation. Gene duplication, as an important driving force for generating evolutionary novelty, enables genes to diverge in function or expression through neofunctionalization, where the gene acquires a new function or expression pattern, or subfunctionalization, a process in which functions or expression patterns are partitioned between duplicate genes (Prince and Pickett, 2002), as seems to be the case for the carotenogenic genes in tissues containing chromoplasts. Thus, in order to study the effects of gene duplication and their influence on gene regulation, it is of interest to carry out a comparative analysis of the promoters of these duplicated genes.

Saffron, the dried red stigmas of *C. sativus*, is one of the oldest natural food additives used as a flavouring and a colouring agent. *C. sativus* is characterized by its long red stigmas, which produce and store significant quantities of carotenoids and the apocarotenoid profile in *C. sativus* stigmas, which include such chromoplast-targeted Lcyβ enzymes and these cyclases are related to another group of carotenoid cyclase enzymes which includes such chromoplast-targeted Lcyβ enzymes as the tomato (Ronen et al., 2000) and Citrus enzymes (Alquézar et al., 2009), the capsanthin-capsorubin synthase (Cs) of pepper (Bouvier et al., 2000), and the neoxanthin synthase (NSY) gene from potato (Al-Babili et al., 2000). Recent data demonstrate a central role for gene duplication in the development of a chromoplast-specific carotenoid biosynthesis pathway (Galpaz et al., 2006; Giorio et al., 2008). Gene and genome duplications have been shown to be particularly prominent in plant genomes and have greatly influenced genomes organization and evolution (Otto and Whitton, 2000; Wendel, 2000; De Bodt et al., 2005; Maere et al., 2005). These duplication events also have profound effects on gene function and regulation. Gene duplication, as an important driving force for generating evolutionary novelty, enables genes to diverge in function or expression through neofunctionalization, where the gene acquires a new function or expression pattern, or subfunctionalization, a process in which functions or expression patterns are partitioned between duplicate genes (Prince and Pickett, 2002), as seems to be the case for the carotenogenic genes in tissues containing chromoplasts. Thus, in order to study the effects of gene duplication and their influence on gene regulation, it is of interest to carry out a comparative analysis of the promoters of these duplicated genes.
of β-carotene in *C. sativus* stigmas and, therefore, in apocarotenoid accumulation. The expression patterns of both genes were followed during stigma development and compared with the changes in the carotenoid levels. In addition, the genomic structure of these genes and their regulation in other *Crocus* species, which differ in their carotenoid and apocarotenoid content have been studied.

### Materials and methods

#### Plant material

For this study, eight species of *Crocus* were used. All the specimens were obtained from private collections in the UK (Potterton Nursery). Plant tissues were independently harvested and frozen in liquid nitrogen and stored at –80 °C until required. Stigmas were collected at seven developmental stages defined according to Rubio et al. (2008).

Seeds of *Arabidopsis* wild-type, ecotype Columbia (Col), and transgenic lines were sown in pots containing vermiculite and watered with nutrient solution under a controlled environment with 16/8 h light/dark cycles at 22 °C and 72 °C for 2 min, 30 cycles at 94 °C for 20 s, 64 °C for 20 s, and a final extension at 72 °C for 5 min. The amplified PCR products were analysed by electrophoresis in 1% agarose gel. The PCR products were then cloned into pGEM-T (Promega, Madison, WI, USA). The ligated DNA was transformed into *E. coli* strain JM109. The clones (50 colonies) were picked individually and amplified in 3 ml of LB medium at 37 °C over night. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep kit (Promega, Madison, WI, USA). The ligated DNA was trans- formed into *E. coli* strain JM109. The clones (50 colonies) were picked individually and amplified in 3 ml of LB medium at 37 °C overnight. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep kit (Promega, Madison, WI, USA) and then analysed by EcoRI restriction digestion.

#### Cloning of *C. sativus* lycopene cyclase coding genes

Genomic DNA extracts were prepared from *Crocus* leaves by using a CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1990). The genomic sequences were obtained by genome walking with the Universal Genome Walker Kit (Clontech, Palo Alto, CA) using the primers described in Table 1. PCR with gene-specific primers from the identified gDNA clones were used to isolate the full open reading frames. Total RNA was isolated from developing *C. sativus* stigmas by using Ambion PolyATracker and following manufacturer’s protocols (Ambion, Inc.). This was done by using 1 μg of poly(A)+ RNA from stigmas to synthesize the first-strand cDNA with a Superscript II reverse transcriptase supplied in the SMART™ RACE cDNA Amplification Kit (Clontech). The gene-specific primers described in Table 1 were used with the following cycling program: one cycle at 94 °C for 3 min, 10 cycles at 94 °C for 20 s, 66 °C –0.2 °C/cycle for 20 s, and 72 °C for 2 min, 30 cycles at 94 °C for 20 s, 64 °C for 20 s, and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The amplified PCR products were analysed by electrophoresis in 1% agarose gel. The PCR products were then cloned into pGEM-T (Promega, Madison, WI, USA). The ligated DNA was transformed into *E. coli* strain JM109. The clones (50 colonies) were picked individually and amplified in 3 ml of LB medium at 37 °C overnight. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep kit (Promega, Madison, WI, USA) and then analysed by EcoRI restriction digestion.

#### DNA sequencing and analysis of DNA sequences

Plasmids were sequenced using an automated DNA sequencer (ABI PRISM 3730xl, Perkin Elmer) at Macrogen Inc. (Seoul, Korea). Computer analysis of the DNA and amino acid sequences were carried out using the Kalign multiple sequence alignment algorithm (Lassmann and Sonnhammer, 2005). Relative molecular masses were calculated from the deduced amino acid compositions with the Compute Mw tool available at the ExPASy Molecular Biology Web server (Geneva, Switzerland). Subcellular sorting was

### Table 1. Primer sequences used for *CsLcyB1* and *CsLcyB2* genes cloning and analysis

<table>
<thead>
<tr>
<th>Primers for <em>CsLcyB1</em> full-length isolation and RT-PCR expression analysis</th>
<th>cDNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence 5′ –3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>LcyB1</em>-f</td>
<td>ATGGATCCGCTCTTTGAGAAC</td>
<td></td>
</tr>
<tr>
<td><em>LcyB1</em>-r</td>
<td>CTATCCTCTCTGATATGTC</td>
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<tr>
<th>Primers for <em>CsLcyB2a/b</em> full-length isolation and RT-PCR expression analysis</th>
<th>cDNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence 5′ –3′</th>
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<td>TCCACTGTCAATACAGACAG</td>
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<tr>
<td><em>LcyB1</em>-f</td>
<td>GCCATGCGCTTCCCTTCA</td>
<td></td>
</tr>
<tr>
<td><em>LcyB1</em>-r</td>
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<tr>
<th>Primers for 3′ and 5′ RACE-PCRs</th>
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<tr>
<td><em>LcyB1</em>-f</td>
<td>ATGGATCCGCTCTTTGAGAAC</td>
<td></td>
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<tr>
<td><em>LcyB1</em>-r</td>
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<th>Primers for promoter isolation and analysis</th>
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<td><em>P-LcyB1</em>-r</td>
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</tr>
<tr>
<td><em>P-LcyB1</em>-r</td>
<td>GGCGGTTCCTCAGAAGGGCAATCAT</td>
<td></td>
</tr>
<tr>
<td><em>LcyB2</em>-f</td>
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</table>

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<tr>
<th>Primers for cloning <em>CsLcyB2a</em> in expression vector</th>
<th>cDNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence 5′ –3′</th>
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<tr>
<td><em>LcyB2</em>-att-sense</td>
<td>GGCGGTTCCTCAGAAGGGCAATCAT</td>
<td></td>
</tr>
<tr>
<td><em>LcyB2</em>-att-antisense</td>
<td>GGCGGTTCCTCAGAAGGGCAATCAT</td>
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<sup>a</sup>f, forward; r, reverse.
predicted at the PSORT Web server for analysing and predicting protein-sorting signals at the Institute for Molecular and Cellular Biology (Osaka, Japan). Phylogenetic analysis and tree construction was carried out using the Clustal W program at the EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the PhyloDendron program (http://nibio.bio.indiana.edu/treepapp/treeprint-form.html).

Nucleotide replacement (Ka) and synonymous (Ks) substitutions were estimated using the WSPMaker tool (Lee et al., 2008).

Expression analysis

For RT-PCR, total RNA was isolated from *C. sativus* anthers, leaves, tepals, and stigma (pre-anthesis), and from the other seven *Crocus* species stigmas (pre-anthesis) by grinding fresh tissue in liquid nitrogen to a fine powder and extracting in 1 ml of Trizol reagent (Gibco-BRL) per 100 mg of tissue fresh weight, according to the protocol of the manufacturer. The RNA was resuspended in 100 μl of RNase-free water and treated with RNase-free DNase (Promega, Madison, WI). The DNase was heat inactivated before RT-PCR. The RNA was quantified with a spectrophotometer at OD of 260 and 280 and stored at −80 °C. Various initial concentrations of treated RNA, ranging over 10-fold difference, were used to demonstrate the differential accumulation of the RNA in the tissues analysed in the RT-PCR experiments. Total RNA samples were reverse transcribed with a first-strand cDNA synthesis kit (Amersham Biosciences) and random primers (Promega, Madison, WI, USA). The gene expression levels were evaluated using specific primer pairs for each gene (Table 1). The PCR reactions were carried out using 10 μM of each primer, 200 μM dNTPs, and 2 units of *Taq* polymerase (Invitrogen). After an initial denaturation step for 2 min, the PCR reactions were performed for 30 cycles at 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 1 min. As an internal control, the mRNA level of the constitutively expressed ribosomal protein 18 was used (Moraga et al., 2004). The program PhotoCaptMw was used to quantify the intensity of the ethidium bromide-stained DNA bands from the positive images of the gel.

Vector construction and Arabidopsis transformation

To produce transgenic plants in which the CstLcyB2a protein was expressed under the control of the 35S promoter, the vector pGWB8 was used (Nakagawa et al., 2007). The strategy followed for cloning CstLcyB2a in this vector was based on Gateway Technology and the oligonucleotides used are indicated in Table 1. The CstLcyB2a cDNA was amplified with the att-primers (Table 1) and introduced into the vector pDONR™221 (Invitrogen) by a BP recombination reaction, and from this vector to the plant expression vector pGWB8 by an LR recombination reaction, using Gateway Technology (Invitrogen, Carlsbad, CA). The recombinant pGWB8 vector was transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation and bacteria were selected on YEB agar with 50 μg ml⁻¹ kanamycin and hygromycin and 100 μg ml⁻¹ rifampicin and 25 μg ml⁻¹ gentamicin. Arabidopsis plants were transformed by floral dipping (Clough and Bent, 1998) and transformants selected on Murashige and Skoog agar with 50 μg ml⁻¹ kanamycin and hygromycin.

HPLC analysis of carotenoids extracted from transgenic Arabidopsis lines

Extraction of carotenoid pigments was performed as described by Fiore et al. (2006), using canthaxanthin as the internal standard. Carotenoid composition of each sample was analysed by reverse phase HPLC using a Hewlett Packard 1100 HPLC (Palo Alto, CA) connected on line with a photodiode array detector, with a dynamic range from ultraviolet to visible region (190–700 nm) set to scan from 250 nm to 540 nm, along with a Sugerlabor Inertsil ODS-2 5-μm C18 column (250 x 4.6 mm), and developed using a methanol, tert-methyl butyl ether gradient system (Fraser et al., 2000).

Carotenoids were identified by their retention time, absorption, and fine spectra (Roueff et al., 1996; Britton, 1998). The carotenoid peaks were integrated at their individual maximum wavelength and their content was calculated using calibration curves of β-carotene (Sigma), lutein (Sigma), and neoxanthin (Extrasynthese).

Promoter isolation and comparison

Promoters of CstLcyB1 and CstLcyB2a were obtained by genome walking using the Universal Genome Walker Kit (Clontech, Palo Alto, CA) and the primers described in Table 1. Promoters were analysed by the Mobyle programme CONSENSUS for the identification of consensus patterns in unaligned DNA sequences (Hertz and Storino, 1995) and by FOOTPRINTER (Blanchette and Tompa, 2003). The FOOTPRINTER takes into account the evolutionary relationships and distances between the genes compared (based on a phylogenetic tree). In order to choose adequate motif sizes, we proceeded as previously reported (De Bodt et al., 2005). To compare known sites for transcription factors with those detected by our approach, the Plant-Care (Lescot et al., 2002) database was scanned.

For promoter amplification from different *Crocus* species, the gene-specific primers p-lycB2-f and p-LycB2-r2 described in Table 1 were used, with the following cycling program: one cycle at 94 °C for 3 min, 35 cycles at 94 °C for 20 s, 50 °C for 20 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified PCR products were analysed by electrophoresis in 1% agarose gel.

Results

Cloning and sequence analysis of *C. sativus* cyclases

As part of an ongoing effort to isolate genes involved in carotenoid biosynthesis in *C. sativus* stigmas, two partial sequences with homology to carotenoid cyclases were isolated: *LcyB1* (GenBank accession number AJ888515) and *LcyB2* (this work). A genome walker approach allowed the isolation of three full-length gDNA clones, one for *LcyB1*, *CstLcyB1* (GenBank accession number GQ202143) and two for *LcyB2*, *CstLcyB2a* (GenBank accession number GQ202141) and *CstLcyB2b* (GenBank accession number GQ202142). Specific oligonucleotides were generated from the predicted coding region of these three clones and used for RT-PCR experiments on different tissues. Expression was observed only for *CstLcyB1* and *CstLcyB2a*. Comparison of the obtained cDNA sequences with the gDNA sequences revealed no introns.

The isolated *CstLcyB1* cDNA is 1491 bp, and the predicted amino acid sequence specifies a polypeptide of 497 amino acids in length with a molecular mass of 56.7 kDa. The isolated *CstLcyB2a* full-length cDNA is 1538 bp. The predicted amino acid sequence specifies a polypeptide of 469 amino acids in length with a molecular mass of 51.94 kDa. The CstLcyB2b shows high homology with CstLcyB2a, but contains a deletion of 78 amino acids close
to the N-terminal region (Fig. 2A). The predicted amino acid sequence specifies a polypeptide of 391 amino acids in length with a molecular mass of 43.6 kDa.

The substrate and product of lycopene cyclases are hydrophobic, and many carotenogenic enzymes are tightly associated with plastid membranes (Bonk et al., 1997; Cunningham, 2002). Transmembrane region analysis of *C. sativus* proteins using the SOSU1 (Hirokawa et al., 1998) and the TMHMM (Moller et al., 2001) programmes predicted the presence of two transmembrane helices of 23 amino acids (368–390) and (453–475) in CstLcyB1, whereas CstLcyB2a and CstLcyB2b amino acid sequences have no hydrophobic domains. Using other empirically based analyses employing databases of known helical membrane-spanning domains (Hofmann and Stoffel, 1993) it seems that two regions of the plant cyclases are likely to form transmembrane helices in CstLcyB2a and CstLcyB2b. The outer boundaries or limits of the predicted transmembrane helical regions are approximate, and we do not mean to imply that these two regions are certain to form transmembrane helices or that they are the only regions that may do so.

Data analysis with the PSORT programme (Horton et al., 2006) predicted a plastid location for both proteins. Transit peptides of 54 bp and 23 bp were predicted for CstLcyB1 and CstLcyB2a, respectively (Fig. 2A). The *C. sativus* proteins CstLcyB1 and CstLcyB2a contain a domain close to the N-terminus that bind dinucleotides as NAD(P) or FAD, with CstLcyB2a and CstLcyB2b amino acid sequences have no hydrophobic domains. Using other empirically based analyses employing databases of known helical membrane-spanning domains (Hofmann and Stoffel, 1993) it seems that two regions of the plant cyclases are likely to form transmembrane helices in CstLcyB2a and CstLcyB2b. The outer boundaries or limits of the predicted transmembrane helical regions are approximate, and we do not mean to imply that these two regions are certain to form transmembrane helices or that they are the only regions that may do so.

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CstLcyB1 exhibited 79% identity at the amino acid level with a *Vitis vinifera* lycopene β-cyclase (CAN69313) and 77% and 71% identity with the characterized citrus and tomato lycopene β-cyclases. However, CstLcyB2a and CstLcyB2b are similar to chromoplast specific lycopene-β-cyclases such as the tomato (Ronen et al., 2000) and *Citrus* (Alquézar et al., 2009), to the pepper capsanthin–capsorubin synthase (Ccs) (Bouvier et al., 1994), and to the neoxanthin synthase gene from potato (Al-Babili et al., 2000) (Fig. 2B). Phylogenetic analysis based on the alignment of available full-length protein sequences of plant lycopene cyclases show three main branches corresponding to β-cyclases, ε-cyclases, and a third branch containing Ccs (Bouvier et al., 1994), which also possesses a lycopene β-cyclase activity (Hugueney et al., 1995), the neoxanthin synthase enzyme from *Solanum tuberosum* (Al-Babili et al., 2000), the tomato chromoplast-specific β-cyclase gene (Ronen et al., 2000), and the chromoplast-specific lycopene β-cyclase from *Citrus* (Alquézar et al., 2009), in addition to other plant enzymes with activities yet to be characterized (Fig. 2B).

**Driving forces for genetic divergence**

A common origin by duplication of an ancestral Lcy-B gene has been proposed for chromoplast-specific β-cyclases (Krubasik and Sandmann, 2000). To explore whether Darwinian positive selection was involved in driving gene divergence of CstLcyB1 and CstLcyB2a after duplication, the number of synonymous substitutions per synonymous site, *K*<sub>s</sub>, and the number of non-synonymous substitutions per non-synonymous site, *K*<sub>a</sub>, between both genes were analysed. The level of replacement and synonymous site nucleotide divergence ratio (*K*<sub>a</sub>/*K*<sub>s</sub> = 0.1943) indicates that this gene pair is likely to be undergoing a purifying selection, which strongly indicates high function constraint of protein evolution. To detect interesting evolutionary signatures, subregions of both protein-coding DNA sequences were scanned and the selection pressure calculated (estimated by *K*<sub>a</sub>/*K*<sub>s</sub>) (Fig. 3). The output showed many grey-coloured regions, indicating that these regions have undergone neutral evolution. However, there were exceptions in some regions, where the red colour indicated *K*<sub>a</sub>/*K*<sub>s</sub> values higher than one, hence suggesting that these regions have undergone amino acid fixations during evolution, a fact that indicates that these particular positions are functionally important. These regions do in fact codify for domains involved in catalytic activity (Fig. 2A).

**Stable expression of the CstLcyB2a gene in Arabidopsis thaliana**

The function of lycopene-β-cyclase has been demonstrated for many of the enzymes shown in the phylogenetic tree (Fig. 2B). However, the activity of the enzymes present in the CstLcyB2a and CstLcyB2b group seems to be more heterogeneous. Therefore, the function of the CstLcyB2a enzyme in transgenic *Arabidopsis* plants was assayed. In a first approach, lycopene-accumulating *E. coli* cells, carrying a lycopene biosynthetic plasmid (pACCRT-EIB; Misawa and Shimada, 1998) were cotransfected with plasmids expressing the cDNA of CstLcyB2a and CstLcyB2b. Carotenoids were extracted and analysed by HPLC. Expression of CstLcyB2a brought about synthesis of β-carotene in *E. coli*, whereas the CstLcyB2b was unable to transform lycopene into β-carotene (data not shown). Due to these results, *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* flowers (Col-0) was performed only with the plasmid CstLcyB2a-pGW8, in which CstLcyB2a was driven by the cauliflower mosaic virus (CaMV) 35S constitutive promoter. Transgenic plants showed no morphological difference from untransformed plants. In order to determine the carotenoid alteration generated by the overexpression of CstLcyB2a, carotenoids were extracted from 6-d-old transgenic seedlings (15 seedlings per line in three batches of five) and analysed by HPLC. Eight transgenic (*T<sub>3</sub>* lines were analysed, with two of them showing carotenoid levels similar to the wild type; whereas the other six showed increase accumulation of β-carotene, with three of these six showing an increase in lutein content (Fig. 4). In none of the transgenic lines were the levels of neoxanthin and violaxanthin significantly affected (data not shown) suggesting the lack of neoxanthin synthase activity for CstLcyB2a.
Fig. 2. Characteristics of the saffron beta lycopene cyclase proteins. (A) Alignment of deduced amino acid sequences of CstLcyB2a, CstLcyB2b, and CstLcyB1 using the Kalign multiple sequence alignment algorithm. Numbers on the left denote the number of amino acid residues. Residues identical for all sequences in a given position are in white text on a black background, those identical in two of the three or similar in the three sequences are on a grey background. The most likely points for chloroplast precursor cleavage are indicated with arrows. Characteristic regions of plant LCYs are indicated as boxes, the di-nucleotide binding signature, motifs (M) I to IV (Hugueney et al., 1995; Cunningham et al., 1996). (B) Unrooted phylogenetic tree based on the amino acid sequences of algae and plant lycopene cyclase proteins. Only full-length members of the family are included. The predicted protein sequences were initially clustered using ClustalW. Accession numbers for non-photosynthetic plant β-cyclases: Capsicum annum, Q42435; Citrus sinensis AAF18389; Daucus carota, ABB52072; Solanum lycopersicum, AAG21133; Solanum tuberosum, CAB92977; Crocus sativus-a (CstLcyB2a), GQ202141; Crocus sativus-b (CstLcyB2b), GQ202142. Accession numbers for alga β-cyclases: Haematococcus pluvialis, AY182008; Dunaliella salina, ACA34344. Accession numbers for plant e-cyclases: Arabidopsis thaliana, AAF82838; Vitis vinifera, CAN68182.1; Citrus sinensis, AA548096; Adonis palestina, AAK07431; Solanum lycopersicum, ACB28618. Accession numbers for plant
Expression analysis of CstLcyB1 and CstLcyB2a

The expression pattern of both genes was examined in different organs. CstLcyB1 was highly expressed in leaves and stigma tissue, and at lower levels in tepals (Fig. 5A). By contrast, CstLcyB2a was only detected in the stigma tissue (Fig. 5A). Under the conditions tested the expression of CstLcyB2b could not be detected.

During the development of C. sativus, stigmas change in colour from white to scarlet, passing through the yellow and orange stages. These changes parallel stigma growth and carotenoid and apocarotenoid accumulation (Castillo et al., 2005; Rubio et al., 2009). The carotenoid β-carotene was already detected in the yellow and orange stages, but reached its highest levels in the scarlet stages. Due to the active accumulation of β-carotene in C. sativus stigmas, and the high expression levels detected in the tissue for CstLcyB2a, RT-PCR analysis was performed with RNA isolated from different stages of stigma development. The CstLcyB2a transcript was detected in all the stages, with the highest levels of expression in the days previous to anthesis (Fig. 5B). The expression levels of CstLcyB1 were also followed and transcripts were detected in the red and pre-anthesis stages (Fig. 5B). The levels were much lower than the ones observed for CstLcyB2a.

Promoter divergence after duplication in C. sativus

In order to determine the origin of the differential expression of both genes, the promoter region of CstLcyB1 and CstLcyB2a genes was isolated and analysed. In silico promoter analysis (Table 2) showed that CstLcyB1 possesses several motifs involved in stress response such as defense/stress responsiveness, MeJA, ABA, anoxia, and light responsiveness. By contrast, the CstLcyB2a promoter possesses motifs involved in ABA, light responsiveness, and circadian regulation. This result suggests that while both genes are involved in β-carotene biosynthesis, CstLcyB1 expression might be modulated under biotic stress conditions. In addition, these promoters were compared with other promoter sequences from different LycB genes and the Ccs gene (Fig. 6A). Sequence analysis of CstLycB1 and CstLycB2a promoters revealed the lack of similarity between both promoters. Interestingly, the phylogenetic analysis showed that promoters of chromoplast specific Lycβ genes grouped together, with the exception of Citrus, where both promoters showed a 51% identity. The promoters for the chromoplast-specific genes were extracted and analysed for conserved motifs. The number of motifs uncovered among Citrus, Crocus, and tomato or pepper reflects the difference in the overall degree of conservation (Fig. 6B). No motifs of size 11 bp or 12 bp were detected among the promoters analysed due to the increase in nucleotide substitutions, reflecting promoter divergence. In addition, the promoters of CYC-β from tomato and Ccs from pepper were clearly more conserved than the other two promoters showing a one-to-one relationship, as seven motifs of size 10 bp can be uncovered, while only five and two of these seven conserved motifs can be identified in the other two promoters (Fig. 6B).

Promoter structure and expression of the specific lycopene cyclase in Crocus species with different stigma colours

Due to the specific expression of CstLcyB2a in the stigma tissue, its expression patterns were analysed in different Crocus species before anthesis. These expression patterns are characterized by their different carotenoid concentration and composition (Castillo et al., 2005) along with a great...
C. goulimyi reveals that the Crocus determine whether defects in the promoter structure could yellow stigma primarily due to a low concentration of total magnification. By contrast, C. pallasii C. cartwrightianus stigmas and showed a high carotenoid content in CstLcyB2a (Castillo et al., 2005). The pattern for C. medius is similar but low levels of β-carotene have been detected without HPLC profile magnification (Castillo et al., 2005). By contrast, the C. kotschyanus was undetectable in C. cancellatus, and low levels of β-carotene were detected only after HPLC profile magnification (Castillo et al., 2005). The same was observed for C. kotschyanus (data not shown), which has a stigma similar in colour to C. goulimyi (Fig. 7A). C. cancellatus showed high levels of phytofluene and ζ-carotene, and low levels of β-carotene were detected after HPLC profile magnification (Castillo et al., 2005). The pattern for C. medius was the same was observed for C. kotschyanus, and C. sativus, which has a stigma similar in colour to C. cancellatus –carotene, and low levels of phytofluene were detected without HPLC profile magnification (Castillo et al., 2005). The pattern for C. medius was similar but low levels of β-carotene have been detected without HPLC profile magnification. By contrast, C. pallasi, C. hadriaticus, C. cartwrightianus, and C. sativus all have a dark red stigma and showed a high carotenoid content in β-carotene (Castillo et al., 2005). Transcripts for CstLcyB1 were detected in all the stigmas tested with the exception of C. goulimyi and C. kotschyanus (Fig. 7B). High expression levels of CstLcyB2a were detected in C. hadriaticus, C. cartwrightianus, C. pallasi, and C. sativus. By contrast, the transcript of CstLcyB2a was undetectable in C. goulimyi, C. kotschyanus, and C. cancellatus (Fig. 7B). The presence of CstLcyB2a homologues in these species was determined by PCR amplification over gDNA, and only for C. kotschyanus was no amplification product obtained. By contrast, the CstLcyB1 gene was detected in all the samples tested (Fig. 7C). The PCR products obtained for both genes from the analysed species were sequenced and compared with the products obtained by RT-PCR, which do correspond to the expected genes.

To investigate further the absence of expression of CstLcyB2a in C. goulimyi and C. cancellatus, the promoter regions of different Crocus species were examined to determine whether defects in the promoter structure could account for the observed differences. As shown in Fig. 7D, genomic PCR analysis of the different Crocus samples reveals that the CstLcyB2a promoter region is equivalently sized in C. sativus, C. hadriaticus, C. pallasi, C. cartwrightianus, and C. medius but differs in C. goulimyi and C. cancellatus, whose promoter regions showed multiple deletions and changes (data not shown). The promoter region was not amplified from C. kotschyanus.

**Discussion**

The increasing characterization of carotenogenic enzymes that are specifically expressed in chromoplastic tissues represents an important breakthrough for the study of carotenogenesis regulation in these tissues of high economic value. In the case of lycopene cyclation, it represents a key branching point in the carotenoid biosynthetic pathway that profoundly affects carotenoid composition (Cunningham, 2002). C. sativus stigmas are characterized by the massive accumulation of apocarotenoids derived from zeaxanthin and β-carotene cleavage (Rubio et al., 2009). In this work, the isolation of two different lycopene cyclase genes, namely CstLcyB1 and CstLcyB2a is reported. CstLcyB2a was expressed in the stigma tissue where it seems to play a major function in the massive apocarotenoid biosynthesis and accumulation. The expression analysis of CstLcyB2a in different Crocus species indicates that transcriptional regulation of this gene affects carotenoid and apocarotenoid content in the stigma tissue. Furthermore, analysis of the promoter region indicates that its structure determines whether or not high levels of total apocarotenoids are produced in the different Crocus species. Thus, these species are useful experimental models to investigate the molecular mechanism regulating carotenoid concentration and composition in the stigma.

The polypeptides of CstLcyB1 and CstLcyB2a differ in size, and their amino acid sequence is only 47% identical. Phylogenetic analysis of CstLcyB1 and CstLcyB2a showed that both are present in different groups: CstLcyB1 was related to the initially characterized group of LcyBs whereas CstLcyB2 was more closely related to chromoplastical cyclases (CYC-B and CCS). Although two different CstLcyB2 genes were isolated, CstLycB2a and CstLycB2b, the CstLycB2b protein showed a deletion of 78 aa in the N-terminal region, which contains the dinucleotide binding signature, an important domain present in all the LycB enzymes. Furthermore, expression for CstLycB2b in the tissues analysed could not be detected, and protein expression assays did not show any activity, thus suggesting that CstLycB2b is not a functional gene in C. sativus. By contrast, CstLycB2a showed lycopene β-cyclase activity in transgenic Arabidopsis plants, which showed increased levels of β-carotene. In several transgenic lines, an increase in lutein levels was observed, which is in agreement with previous observations suggesting that LCYɛ activity is not rate-limiting for lutein levels (Direto et al., 2006; Yu et al., 2008).

The expression of CstLcyB2a was restricted to the stigma tissue, while the up-regulation of the CstLcyB2a gene parallels β-carotene accumulation and the massive accumulation of apocarotenoids in the stigma tissue of C. sativus (Castillo et al., 2005; Rubio et al., 2009). The presence of two LcyB genes, one with a chromoplast-specific expression, has previously been reported in other carotenogenic tissues, mainly in fruits. The CYC-B gene from tomato is
transiently expressed in fruit and petals, whereas it is undetectable in roots, leaves, and stem (Ronen et al., 2000). The orthologous CYC-B gene from watermelon might be an essential flesh colour determinant in this fruit (Tadmor et al., 2005), while in orange the Cs b-LCY2 plays a key role in the carotenogenesis of citrus fruits by redirecting the flux of carotenes into the b, b-branch to lead to the accumulation of xanthophylls characteristic of orange fruit ripening (Alquezar et al., 2009). Therefore, the involvement of a second chromoplast-specific LcyB gene in the regulation of carotenoid composition appears to be a frequent mechanism in carotenoid rich tissues. Interestingly, no homologues to this chromoplast specific lycopene b-cyclases have been identified in Arabidopsis and rice whose genomes are fully sequenced, probably due to the absence of tissues rich in carotenoids in these species. Although the complete genome has been duplicated several times in both plants in their evolutionary past, fewer than 27% of A. thaliana (Blanc et al., 2003) and 15.4% of rice genes (Wu et al., 2008) have been retained as duplicates. In fact, loss is the most likely fate of a duplicated gene (Walsh, 1995; Lynch and Connery, 2003), and retention has often been explained by functional divergence occurring by either neo- or subfunctionalization. Among the groups of duplicated genes with high retention rates are those involved in secondary metabolism (Maere et al., 2005). Evolutionarily, a common origin by duplication of an ancestral Lcy-B gene has been proposed for chromoplast-specific b-cyclases (Krubasik and Sandmann, 2000). Retention of both genes could be explained by a selective advantage acquired through the functionally redundant activity of duplicated genes (Osborn et al., 2003) increasing the levels of the gene product in

Fig. 5. Expression analysis of CstLcyB1 and CstLcyB2a by RT-PCR in different tissues. (A) Expression levels in leaves, stigma, tepals, and anthers and relative transcript levels (normalized to 18S rRNA) were determined by reverse transcriptase (RT)-PCR. (B) Stigma tissue of C. sativus in different developmental stages: yellow (Y), orange (O), red (R), one day before anthesis (–1da), anthesis (da), two days after anthesis (+2da), and three days after anthesis (+3da) and relative transcript levels (normalized to 18S rRNA) determined by reverse transcriptase (RT)-PCR. All the RT-PCR experiments were repeated three times, and representative results are shown. The RPS18 gene for 18S RNA was amplified as a control. The PCR products were separated by 1% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.
a particular tissue or time (Force et al., 1999; Osborn et al., 2003). Therefore, recruitment of carotenoid metabolism in plant tissues with a high carotenoid content could have occurred later on in evolution through gene subfunctionalization, suggesting it was the case for CstLcyB2a during stigma development and for CYC-B and CsLCY2 during...
fruit development. The acquisition of distinct expression patterns could be explained by the analysis of the respective promoters. The results obtained indicate that similar gene expression patterns should be expected for the most conserved promoters, a fact which has been experimentally confirmed for the tomato and pepper genes (Hugueney et al., 1995; Ronen et al., 2000). The poor degree of conservation of the analysed promoters with the *Crocus* sequence suggests that it has acquired distinct expression patterns, as confirmed in the present study, with high expression levels in the stigma tissue.

In previous studies, it was found that *Crocus* species showing dark red stigmas accumulate increasing levels of total carotenoids and apocarotenoids during development, whereas *Crocus* species with non-dark red stigmas accumulate lower levels of total carotenoids and apocarotenoids. The observed expression patterns of *CstLcyB2a* in different *Crocus* species suggest that previously observed β-carotene and apocarotenoid accumulation (Castillo et al., 2005; Rubio et al., 2009) were regulated, in part, at *CstLcyB2a* transcriptional level, together with β-carotene hydroxylase 1 (Castillo et al., 2005). Nevertheless, it should be noted that, in the case of the species in which *CstLycB2a* transcripts were absence, the accumulation of phytofluene and ζ-carotene suggests that upstream enzymes in the pathway are also affected, and that the chromoplast-specific pathway in these species has been lost during evolution. Our current data, which indicates that the expression levels of the carotenoid biosynthetic genes are the key regulators of the high levels of total carotenoid accumulation in *Crocus* stigmas, are in good agreement with previous results obtained in other tissues of different plants. In other carotenogenic tissues such as fruits, the regulation of chromoplast specific lycopene β-cyclase has been shown to be critical in the specific accumulation of β-carotene in tomato (Ronen et al., 2000). The pale orange coloration of *Beta* mutant fruits is due to an important increase in the transcription of this gene, which leads to a higher accumulation of β-carotene than in wild-type fruit. By contrast, the old-gold mutant carries a null allele of this chromoplast specific LcyB resulting in a reduction of β-carotene (Ronen et al., 2000). In pepper, the high expression levels of the *Ccs* gene are correlated with high levels of capsanthin (Bouvier et al., 1994). In *Citrus*, the up-regulation of a chromoplast specific lycopene cyclase gene, *Csf-LCY2*, parallels the massive accumulation of β,β-xanthophylls accompanying orange fruit maturation (Rodrigo et al., 2004).

Several genetic studies have shown the relationship between the presence of structural genes for carotenoid biosynthesis and the phenotypic variability in carotenoid accumulation (Thorup et al., 2000; Wong et al., 2004; Tadmor et al., 2005; Harjes et al., 2008; Alquézar et al., 2009). In *Crocus*, the relationship between the presence of the *CstLcyB2* transcript and the phenotypic variability in stigma colours is due to alterations in the gene structure. A similar phenomenon has been found in the tomato *CYC-B* gene (Ronen et al., 2000). In pepper, several studies have reported that the *Ccs* gene is either deleted or mutated in yellow cultivars (Popovsky and Paran, 2000; Ha et al.,

![Fig. 7. Pigmentation levels of stigmas from different *Crocus* species are associated with *CstLcyB2a* transcript accumulation. (A) Photographs showing the external pigmentation of stigmas of several *Crocus* species: from left to right and from top to bottom: *C. cancellatus*, *C. hadiaticus*, *C. cartwrightianus*, *C. sativus*, *C. pallasi*, *C. goulimyi*, *C. medius*, and *C. kotschyanus*. (B) Detection of *CstLcyB1* and *CstLcyB2a* transcripts by RT-PCR in preanthesis stigmas in different *Crocus* species and relative transcript levels (normalized to 18S rRNA). Number from 1 to 8 correspond to: *C. cancellatus*, *C. hadiaticus*, *C. cartwrightianus*, *C. sativus*, *C. pallasi*, *C. goulimyi*, *C. medius*, and *C. kotschyanus*. The levels of constitutively expressed *RPS18* coding gene were assayed as controls. (C) PCR amplification of *CstLcyB1* and *CstLcyB2a* genes from the genomes of the indicated *Crocus* species. Both genes were amplified in the genomic region corresponding to their cDNAs. (D) PCR amplification of the *CstLcyB2a* promoter from the genomes of the indicated *Crocus* species. Promoters were amplified from a region corresponding to 759 bp upstream of the translational start site (GenBank GQ202144) of *C. sativus* promoter. The PCR products were separated by 1% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.
carotenoids in specific tissues. In the stigma tissue, trans-
expression pattern by subfunctionalization, which
involves the loss of activity of a β-LCY2b Citrus allele (Alquézar et al., 2009).
The absence of coding and genomic regions of LcyB2a in
C. kotschyanus has no phenotypic manifestation in leaves and stems, indicating that LcyB2a plays a dispensable role
in vegetative tissues under normal growth conditions, as
has been observed in the old gold tomato mutant (Ronen et al.,
2000) and in Star Ruby grapefruit (Alquézar et al., 2009).
In C. goulmiyi and C. cancellatus, although the coding
sequence of LcyB2a was present, multiple deletions in the
promoter seem to impair the promoter activity.

In summary, two lycopene cyclase genes from C. sativus,
CstLcyB1 and CstLcyB2a have been identified and charac-
terized. Based on the low sequence identity to each other, it
is likely that this gene pair arose from an old gene
duplication event. The absence of LcyB2 orthologues in
plants that do not accumulate high levels of carotenoids
suggests that LcyB2 is a LcyB1 parologue that acquired a
new expression pattern by subfunctionalization, which
allows a tight and timely control over the synthesis of
carotenoids in specific tissues. In the stigma tissue, tran-
script levels of CsLcyB2a were found to correlate positively
with stigma carotenoid content in genetically diverse Crocus,
suggesting that the major regulatory control of
carotenogenesis in this tissue is at the transcriptional level.

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