

A Chromoplast-Specific Carotenoid Biosynthesis Pathway Is Revealed by Cloning of the Tomato *white-flower* Locus ^W

Navot Galpaz,^{a,1} Gil Ronen,^{a,1} Zehava Khalifa,^a Dani Zamir,^b and Joseph Hirschberg^{a,2}

^aDepartment of Genetics, Alexander Silberman Life Sciences Institute, Hebrew University of Jerusalem, Jerusalem, 91904 Israel

^bRobert H. Smith Institute of Plant Sciences and Genetics in Agriculture, Hebrew University of Jerusalem, Jerusalem, 91904 Israel

Carotenoids and their oxygenated derivatives xanthophylls play essential roles in the pigmentation of flowers and fruits. Wild-type tomato (*Solanum lycopersicum*) flowers are intensely yellow due to accumulation of the xanthophylls neoxanthin and violaxanthin. To study the regulation of xanthophyll biosynthesis, we analyzed the mutant *white-flower* (*wf*). It was found that the recessive *wf* phenotype is caused by mutations in a flower-specific β -ring carotene hydroxylase gene (*CrtR-b2*). Two deletions and one exon-skipping mutation in different *CrtR-b2 wf* alleles abolish carotenoid biosynthesis in flowers but not leaves, where the homologous *CrtR-b1* is constitutively expressed. A second β -carotene hydroxylase enzyme as well as flower- and fruit-specific geranylgeranyl diphosphate synthase, phytoene synthase, and lycopene β -cyclase together define a carotenoid biosynthesis pathway active in chromoplasts only, underscoring the crucial role of gene duplication in specialized plant metabolic pathways. We hypothesize that this pathway in tomato was initially selected during evolution to enhance flower coloration and only later recruited to enhance fruit pigmentation. The elimination of β -carotene hydroxylation in *wf* petals results in an 80% reduction in total carotenoid concentration, possibly caused by the inability of petals to store high concentrations of carotenoids other than xanthophylls and by degradation of β -carotene, which accumulates as a result of the *wf* mutation but is not due to altered expression of genes in the biosynthetic pathway.

INTRODUCTION

Plant carotenoids are C₄₀ carbohydrates with a chain of conjugated double bonds, which creates a chromophore that absorbs light in the blue range of the spectrum. Flowers and fruits of many species are colored due to the accumulation in the chromoplasts of carotenoid pigments that provide distinct colors to the tissues, ranging from yellow to orange and red, to visually attract pollinators and facilitate seed dispersal by animals. Many plant species, including tomato (*Solanum lycopersicum*), accumulate yellow pigments in flowers because insects are preferentially attracted to this color (Kevan, 1983). Carotenoids, mainly xanthophylls, are the most prevalent yellow pigments found in flowers. Carotenoids are also synthesized in chloroplasts, where they play essential roles in photosynthesis in the light-harvesting systems and in the photosynthetic reaction centers (Frank et al., 1999; Demmig-Adams and Adams, 2002; Holt et al., 2004; Robert et al., 2004; Horton and Ruban, 2005; Standfuss et al., 2005).

In the last decade, carotenoid biosynthesis in plants has been described at the molecular level (reviewed in Cunningham and

Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). The carotenoid biosynthesis pathway begins with the formation of phytoene from geranylgeranyl diphosphate in the central isoprenoid pathway. Four dehydrogenation (desaturation) steps lead to the linear molecule lycopene, which is then cyclized at each end by either ϵ - or β -cyclase to yield β -carotene (β,β -carotene) or α -carotene (β,ϵ -carotene). Hydroxylation of these carotenes at C3 and C3' gives rise to the xanthophylls zeaxanthin and lutein, respectively. Epoxidation at C5,C6 converts zeaxanthin to violaxanthin via the intermediate antheraxanthin. Subsequent opening of the cyclohexenyl 5-6-epoxide ring in violaxanthin gives rise to neoxanthin.

Tomato is an important model plant for the study of carotenoid biosynthesis in chromoplast-containing tissues, such as fruits. The fruits of the cultivated tomato are red owing to the accumulation of lycopene, and its flowers are yellow due to the xanthophylls violaxanthin and neoxanthin. The accumulation of lycopene in fruits is determined by differential expression of genes encoding biosynthetic enzymes during the breaker stage of fruit development (reviewed in Hirschberg, 2001). At this stage, transcription of the genes encoding phytoene synthase (*Psy*), phytoene desaturase (*Pds*), ζ -carotene desaturase (*Zds*), and carotene isomerase (*CrtISO*) is upregulated, whereas the genes for lycopene β -cyclase (*Lcy-b*) and lycopene ϵ -cyclase (*Lcy-e*) are not transcribed. Consequently, the enhanced flux of carotene in the pathway is arrested at lycopene. The substantial increase in carotenoid biosynthesis in tomato flowers is correlated with upregulation of expression of the carotenoid biosynthesis genes *Psy1*, *Pds*, *Lcy-b*, and *Cyc-b* (Giuliano et al., 1993; Corona et al., 1996; Ronen et al., 2000). The gene *Lcy-e* is expressed at a very low level in petals and anthers, resulting in the formation of only

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed. E-mail hirschu@vms.huji.ac.il; fax 972-2-5633066.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Joseph Hirschberg (hirschu@vms.huji.ac.il).

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minute amounts of lutein in these organs. By contrast, *Lcy-e* is highly expressed in leaves, where lutein is the most prevalent carotenoid.

A number of mutations that alter carotenoid concentration or composition have been described in plants (reviewed in Hirschberg, 2001). Some of them affect carotenoid biosynthesis distinctively in flowers or fruits. For example, in tomato, *white-flower* (*wf*) abolishes xanthophyll accumulation in petals but does not change xanthophylls in leaves, and *yellow-flesh* (*r*) eliminates carotenoids in fruits only. These mutations suggest that carotenoid biosynthesis has unique characteristics in different organs.

To investigate the regulation of carotenoid synthesis in flowers, we analyzed the tomato mutant *wf* (Young and MacArthur, 1947). The recessive *wf* allele determines white to beige petals and pale anthers compared with intense yellow organs in the wild type (Figure 1). We report here on the cloning of two functional β -ring carotene hydroxylase genes (*CrtR-b*) in tomato that are responsible for the conversion of β -carotene to zeaxanthin. *CrtR-b1* is constitutively expressed in leaves, whereas *CrtR-b2* is active exclusively in flowers. We found that the *wf* phenotype is caused by a mutation in the gene *CrtR-b2*, thus confirming that

CRTR-B1 does not play a primary role in β -ring hydroxylation in flowers. These findings together with previous data demonstrate a central role for gene duplication in the development of a chromoplast-specific carotenoid biosynthesis pathway.

RESULTS

Phenotypic Characterization of *wf*

Flowers of the cultivated tomato accumulate high concentrations of the yellow xanthophylls violaxanthin and neoxanthin. By contrast, the corolla in the recessive mutant *wf* is whitish as a result of a decrease in total carotenoid content (Figure 1, Table 1). Two known *wf* lines, LA2370 (background genotype unknown) and LA3575 (background genotype: Ailsa Craig), were obtained from the Tomato Genetics Resource Center (University of California, Davis). In addition, we have isolated two novel *wf* alleles that were induced in the tomato cultivar M82 by ethyl methanesulfonate (line e1827) or fast neutron bombardment (line n5681; Menda et al., 2004; <http://zamir.sgn.cornell.edu/mutants/>). These single-gene recessive mutations were found to be allelic to *wf* by genetic crossing. We designated LA2370, e1827, and n5681 as *wf1-1*, *wf1-2*, and *wf1-3*, respectively. LA3575 turned out to carry the same allele as *wf1-1*. Both *wf1-2* and *wf1-3* are isogenic with the wild-type line M82.

Carotenoid analysis indicated a reduction of 80 to 84% in total carotenoids in petals of the various *wf* mutant alleles (Table 1). The sharp decrease in the xanthophylls neoxanthin and violaxanthin was accompanied by an increase of β -carotene, a carotenoid species that in the wild type appears in minute amounts only. No difference between *wf* and M82 was found in lutein, which occurs in flowers in small amounts (Table 1). The phenotype of the anthers of *wf* was similar to that of the petals (Table 1). By contrast, no differences in carotenoid content were detected in leaves or fruits of *wf* compared with M82 (Table 1), and no other phenotypic changes were observed in *wf* plants. The limited buildup of β -carotene in *wf* flowers suggested the possibility of a flower-specific inhibition of β -carotene hydroxylation.

Cloning and Sequence Analysis of *CrtR-b* Genes

To find out whether a mutation in β -carotene hydroxylase could account for the *wf* phenotype, we cloned the β -carotene hydroxylase gene, *CrtR-b*, from tomato. A cDNA library from tomato was screened with the *CrtR-b* cDNA from *Arabidopsis thaliana*. Restriction endonuclease analysis revealed 18 positive phages with a similar digestion pattern and one positive phage that was different. Two different *CrtR-b* genes were subcloned into the plasmid vector pBluescript KS-. The new plasmids were designated pCrtR-b1 and pCrtR-b2, respectively. Nucleotide sequence analysis of *CrtR-b1* and *CrtR-b2* cDNA revealed open reading frames of 309 and 314 codons, respectively, that encode polypeptides of calculated molecular masses of 34.5 and 35.1 kD. The predicted β -carotene hydroxylase polypeptides share overall similarity of 80% and identity of 73 and 62% with the β -carotene hydroxylases from *Arabidopsis*. Both putative polypeptides begin with a MetAlaAla sequence, which suggests a plastid transit sequence (Gavel and von Heijne, 1990). Indeed, chloroplast transit peptide prediction software, ChloroP v1.1 (<http://www.cbs.dtu.dk/services/ChloroP/>;

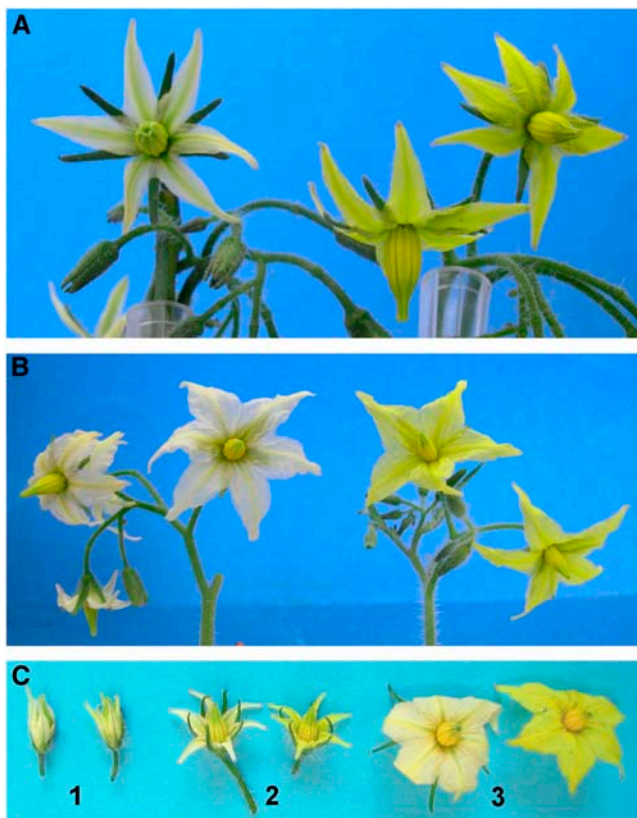


Figure 1. Flowers of *wf* Mutants.

(A) *wf1-1* (F2 of a cross LA2370 \times IL3-2) (left) and its nearly isogenic wild-type line (F2 of the same cross).

(B) *wf1-2* (e1827) (left) and its isogenic line M82.

(C) Flower of *wf1-2* (left) and the wild type (right) in developmental stages 1 to 3 analyzed in this work.

Table 1. Carotenoid Accumulation in Petals, Anthers, Fruits, and Leaves of the Wild Type and *wf* Mutants

	Neoxanthin	Violaxanthin	Anteraxanthin	Lutein	Zeaxanthin	β -Carotene	Lycopene	Phytoene	Total
Petals									
Wild type (M82)	427.4 \pm 32	192.6 \pm 24		2.4 \pm 1					622.4
<i>wf1-1</i> (LA2370) ^a	24.3 \pm 9	5.6 \pm 3		9.6 \pm 3		65.7 \pm 18		3.9 \pm 1	109.1
<i>wf1-2</i> (e1827) ^b	33.1 \pm 6	7.9 \pm 1		3.3 \pm 1		73.7 \pm 11		5.5 \pm 2	123.5
<i>wf1-3</i> (n5681) ^b	57.3	7.0		4.5		31.6			100.4
Anthers									
Wild type (M82)	197.4 \pm 11	93.6 \pm 8		12.0 \pm 2		3.9 \pm 3			306.9
<i>wf1-1</i> (LA2370) ^a	42.5 \pm 3	14.4 \pm 3		13.8 \pm 5		22.3 \pm 7			93.0
<i>wf1-2</i> (e1827) ^b	51.1 \pm 5	16.0 \pm 1		8.9 \pm 1		32.2 \pm 4			108.2
<i>wf1-3</i> (n5681) ^b	56.6	4.2		2.7		29.2			92.7
Fruit									
Wild type (M82)				0.9 \pm 0.6		1.2 \pm 0.4	40.6 \pm 3.0	4.7 \pm 1.0 ^c	47.4
<i>wf1-2</i> (e1827) ^b				0.7 \pm 0.1		1.7 \pm 1.5	35.0 \pm 5.2	4.8 \pm 2.0 ^c	42.2
<i>wf1-1</i> (LA2370) ^a				0.6 \pm 0.5		8.1 \pm 5.3	30.3 \pm 5.1	8.1 \pm 2.1 ^c	47.1
<i>wf1-3</i> (n5681) ^b				2.7		3.2	18.7	5.8 ^c	30.4
Leaves									
Wild type (M82)	12.5 \pm 3	28.4 \pm 10	3.2 \pm 1	50.6 \pm 13	2.7	19.1 \pm 5			116.6
<i>wf1-1</i> (LA2370) ^a	14.7 \pm 1	24.3 \pm 3	1.5 \pm 1	55.4 \pm 6	0.8 \pm 1	19.5 \pm 2			116.2
<i>wf1-2</i> (e1827) ^b	16.8 \pm 2	32.1 \pm 11	3.0 \pm 1	59.9 \pm 10	2.0 \pm 1	25.8 \pm 3			139.6
<i>wf1-3</i> (n5681) ^b	15.3	31.0	<0.1	52.6	<0.1	22.3			121.2

Carotenoids were measured in fully developed (stage 3) flowers.

^a Background genotype unknown or hybrid.

^b Isogenic with M82.

^c Including phytofluene.

Emanuelsson and Nielsen, 1999), identified the existence of a plastid transit peptide with a proposed cleavage site between Val-59 and Cys-60 in CRTR-B1 and Val-57 and Cys-58 in CRTR-B2. The sequences of the predicted mature polypeptides after cleavage of the transit sequences are 94% similar and 86% identical.

To analyze the structure of the β -carotene hydroxylase genes, we cloned the genomic DNA of both *CrtR-b* genes by screening a BAC library of tomato (cv Heinz 1706) (Budiman et al., 2000). Using the inserts of pCrtR-b1 and pCrtR-b2 as probes, we selected a single positive BAC clone from each probe. DNA of these BACs was digested with *EcoRI* (*CrtR-b1*) or *XbaI* (*CrtR-b2*), and the resulting fragments were cloned using the plasmid pBluescript SK-. The DNA inserts of these plasmids were sequenced, resulting in 3076 bp of *CrtR-b1* and 3570 bp for the *CrtR-b2* genomic sequences. Comparison with the cDNA sequence revealed seven exons in each gene, interrupted by six introns (Figure 2). Complete conservation in exon organization and splicing sites was found between tomato and *Arabidopsis* (Figure 2); however, there was low conservation in the length and sequence of the introns.

CrtR-b2 Maps to *wf*

To test for possible linkage between the β -carotene hydroxylase gene and the *wf* phenotype, we mapped both hydroxylase genes to the tomato introgression lines (ILs; Eshed and Zamir, 1995). Restriction fragment length polymorphism (RFLP) analysis using either *CrtR-b1* or *CrtR-b2* as probes revealed that the first mapped to IL6-1, and *CrtR-b2* was mapped exclusively to IL3-2 on the short arm of chromosome 3 but not to IL3-1 (Figure 3; Liu

et al., 2003). The mutation *wf* was previously mapped to a similar position on chromosome 3 as *CrtR-b2* (van der Biezen et al., 1994). To verify and refine previous mapping, we crossed the *wf* line LA2370 with IL3-2 and used 540 F2 plants for RFLP mapping and determination of flower phenotypes (Figure 3); complete cosegregation was established between *wf* and *CrtR-b2*.

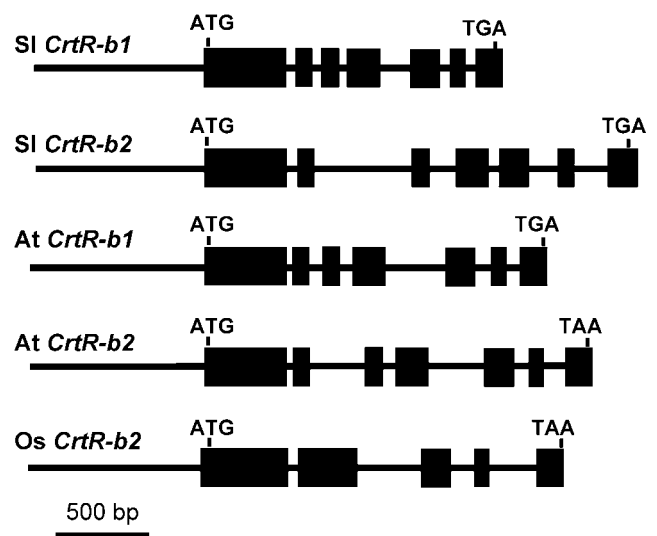


Figure 2. Gene Structure of β -Carotene Hydroxylase Genes (*CrtR-b*).

Tomato (SI) *CrtR-b1* and *CrtR-b2*, *Arabidopsis* (At) *CrtR-b1* and *CrtR-b2*, and rice (Os) *CrtR-b2*. Exons are depicted as boxes.

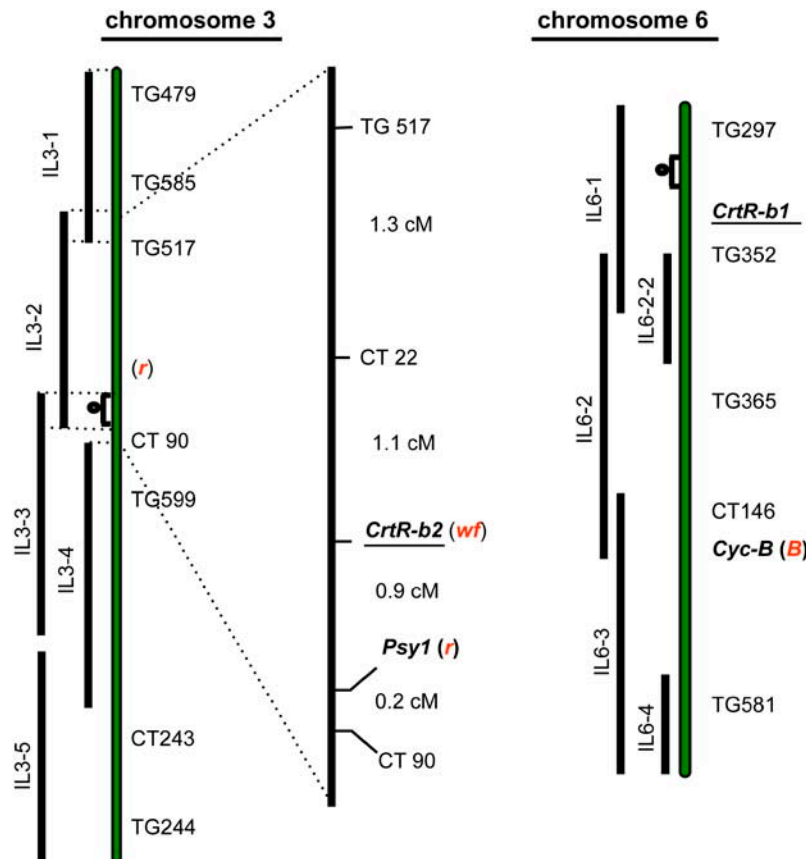


Figure 3. Genetic Mapping of *CrtR-b1*, *CrtR-b2*, and *wf* on the Tomato Linkage Map.

The linkage map was adapted from Eshed and Zamir (1995). The relevant chromosomal segments from *Solanum pennellii* that were introgressed into *S. lycopersicum* are represented by black bars with the names of the ILS that carry them. The high-resolution map of IL3-2, which includes the *wf* locus, is displayed next to chromosome 3. RFLP markers used in the fine mapping of *wf* and genetic distances between each pair are presented. *r* and *B* represents the loci of *yellow-flesh* (*Psy1*) and *Beta* (*Cyc-B*) mutations, respectively. cM, centimorgans.

The Molecular Basis of *wf* Alleles

Quantitative RT-PCR analysis of *CrtR-b2* mRNA in flowers indicated a similar level of expression in *wf1-1*, *wf1-2*, and the wild-type M82 (data not shown). However, the *CrtR-b2* transcripts in *wf1-1* and *wf1-2* were shorter than that of M82 by ~60 nucleotides (Figure 4). Sequence analysis of the *CrtR-b2* cDNA from M82 (wild type) and mutant *wf1-2* confirmed that the mRNA of the latter had a deletion of 60 nucleotides, which encompassed the entire length of the predicted exon 2. A point mutation from G to A was identified in the genomic sequence of *CrtR-b2* from *wf1-2* (Figure 5). The mutation occurred in the intron-exon junction consensus sequence at the donor site of the second intron and is expected to lead to exon skipping during RNA processing. Sequence analysis of genomic DNA and cDNA sequences of *CrtR-b2* from *wf1-1* revealed a 59 bp deletion at the very beginning of exon 1 (nucleotides 303 to 362; Figure 5), resulting in a short open reading frame of 29 codons due to a premature stop codon. Allele *wf1-3*, which was generated by fast neutron bombardment (Menda et al., 2004), carries a DNA deletion encompassing the entire *CrtR-b2*, as indicated by the failure to amplify this gene by PCR using a combination of specific primers.

Functional Expression of *CrtR-b* cDNAs in *Escherichia coli*

Although tomato has two *CrtR-b* sequences, all the *wf* mutations were identified in the *CrtR-b2* gene. To ascertain that both *CrtR-b* genes code for an active β -carotene hydroxylase, we tested their

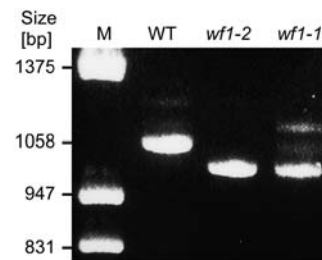


Figure 4. Deletions in *CrtR-b2* mRNA of *wf1-2* (e1827) and *wf1-1* (LA2370).

Total RNA was extracted from stage 3 flowers and used as template for RT-PCR amplification. The resulting DNA products were separated by electrophoresis on 1.0% agarose gel and stained with ethidium bromide. A full-length DNA fragment of 1058 bp was generated in the wild type. Small deletions of ~60 bp were observed in the mutants. M, DNA size markers.

ATTTGGATCCTCCACCGTCC ATGGCGGCCGGAATTCAGC	280
CTCCGCTAGTTCCCGAACCATTCGCCTCCGTCATAACCCG	320
<u>TTTCTCAGTCCAAAATCCGCCTCAACCGCCCCGCCGTTT</u>	360
<u>TGTTCTTCTCTCCGTTAACTCGCAATTTTGGCGCAATTTT</u>	400
GCTGTCTAGAAGAAAGCCGAGATTGGCGGTTTGT	440
CTGGAGAATGAGAAATTGAATAGTACTATCGAAAGTGAGA	480
GTGAAGTAATAGAGGATCGGATACAAGTAGAGATTAATGA	520
GGAGAAGAGTTTAGCTGCCAGTTGGCTGGCGGAGAAATTG	560
GCGAGGAAGAAATCGGAGAGGTTACTTATCTTGTGGCAG	600
CTGTGATGTCTAGTTTGGGGATTACTTCTATGGCGATTTT	640
GGCGGTTTATTACAGATTTTCATGGCAAATGGAGGTACTA	680
ATTAATGAATCACTCCATTCTTCTTTCTTCTTTCTTAAGC	720
TAAATCTTAAAGCTTTTTGATAGGGTGGAGAAGTGCCTTT	760
TTCTGAAATGTTAGCTACATTCACTCTCTCGTTTGGCGCT	800
A	
GCCGTAAGTTCCTTTTTTTTTTCTTCTTTTACATGTTTCGAT	840
TCTTCTTCCATTTTGTCTAATTTGTGTGATATAATTTAA	880
ATTTTGAGAATTAATAATTTTGATTTTGTATTTTTTTTCA	920
ACTCAAATTCAAACTATGTGATATAAATTACATATAATA	960
CATAAATATGTACTTTAACTTGGCTTCAAATCACATTTAT	1000
GTCATTCAATTTTGGATGTGTTCAAGTAGATACTTAAATT	1040
TGTATAAAATTGAACAAATGGACACACATGTTCTACATGT	1080
GATCCTACATGTCATTTTTTTGTCTTACATTATGTCTTATG	1120
TGTATTATGTCATGTAGGATTCATGTGTTTATTTTTTTTAA	1160
ATGTTAGATAATCAAAGTGTTTGTGTTTGTGCATTATGAAAG	1200
TTGGAGGTCAAATTTAAAATTCAATATATCTATAATAAAA	1240
TGTTTAAAAAGTAATAGAAGTTTCAAAAATGAGTTGATTT	1280
GCTGTAATTTAATGCTGTGGTCCCTCAGGTAGGAATGGAG	1320
TACTGGGCGAGATGGGCTCATAGAGCACTATGGCATGCTT	1360
CCTTATGGCACATGCACGAGGTTAATTTCAAATCTTTAC	1400
ACTAAATTATTAGGCACTAATTTGGATTCTTATTTAAAAA	1400

Figure 5. Mutations in the *CrtR-b2* Gene from *wf1-1* and *wf1-2*.

Genomic sequence of the first three exons of *CrtR-b2* from tomato is presented. Exons are in boldface. The sequence deleted in allele *wf1-1* is underlined. A transition mutation of G to A that causes skipping of the second exon in allele *wf1-2* is shown at nucleotide 804.

enzymatic activity in *E. coli* cells carrying plasmid pBCAR-T (Ronen et al., 1999). These bacteria that produce β -carotene were cotransfected with plasmids pCrtR-b1 or pCrtR-b2 or with the empty vector pBluescript SK⁻. Carotenoids were extracted from the bacteria and analyzed by HPLC. Cells carrying pBCAR-T and pBluescript SK⁻ yielded β -carotene, whereas cells carrying both pBCAR-T and pCrtR-b1 or pCrtR-b2 produced, in addition, zeaxanthin (β , β -carotene-3,3'-diol) and the intermediate β -cryptoxanthin (β , β -carotene-3-diol) (Figure 6, Table 2). These results indicate that both *CrtR-b1* and *CrtR-b2* encode active isoforms of β -carotene hydroxylase that catalyzes the hydroxylation of carbons 3 and 3' on the β -rings of β -carotene.

To determine whether the tomato CRTR-B enzymes could add hydroxyl groups also to ϵ -ring carotenoids, we cotransfected

pCrtR-b1 or pCrtR-b2 to a δ -carotene accumulating *E. coli* cells that carried the plasmid pDCAR (Ronen et al., 1999). No alteration in carotenoid composition was observed in these cells, indicating that both CRTR-B enzymes in tomato were incapable of hydroxylation of ϵ -ring in δ -carotene in *E. coli* cells (Table 2).

Expression of *CrtR-b* Genes in Tomato

The steady state mRNA level of the two *CrtR-b* genes was measured in various tissues by RT-PCR and quantitative real-time RT-PCR. Total RNA was extracted from roots, leaves, flowers, and fruits of tomato (cv VF-36 and M82), and the relative amount of mRNA of *CrtR-b1* and *CrtR-b2* was determined with gene-specific primers. Results presented in Figure 7 indicate

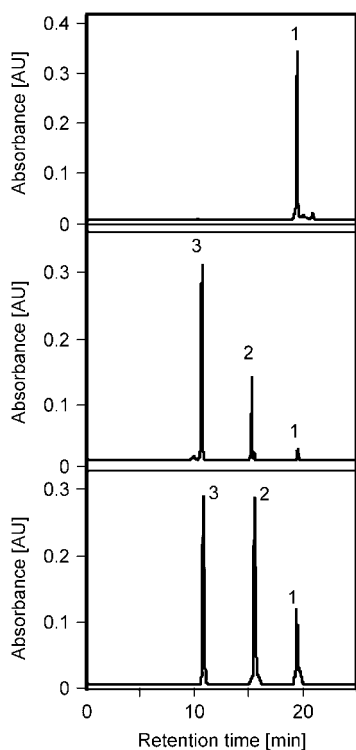


Figure 6. HPLC Analysis of Carotenoids in *E. coli* Cells Expressing *CrtR-b1* and *CrtR-b2* from Tomato.

Carotenoids were extracted from *E. coli* cells carrying plasmid pBETA (top); pBETA + pCrtR-b1 expressing cDNA of *CrtR-b1* from tomato (middle); or pBETA + pCrtR-b2 expressing cDNA of *CrtR-b2* from tomato (bottom). Peak identification: 1, β -carotene; 2, β -cryptoxanthin; 3, zeaxanthin.

that the two *CrtR-b* genes are expressed in a tissue-specific manner. *CrtR-b1* is expressed in leaves and sepals, but in flower tissues, it is expressed at low levels. By contrast, *CrtR-b2* is highly expressed in petals and anthers, where yellow xanthophylls accumulate at high concentrations, and is relatively low in carpels and sepals (Figure 7). We could not detect *CrtR-b2* mRNA in tomato leaves. In roots, *CrtR-b2* is expressed at low levels and *CrtR-b1* is hardly detected.

Expression of Isoprenoid and Carotenoid Biosynthesis Genes in *wf*

The phenotype of *wf* is caused by a significant reduction in total flower carotenoids, mainly due to a 90 to 95% decrease in the concentration of the yellow xanthophylls neoxanthin and violaxanthin in the petals. Since the mutations in *wf* occur in the β -carotene hydroxylase gene, the decrease in total carotenoids is not easily explained by a block of the biosynthetic pathway at the β -carotene hydroxylation step. To explore the possibility of alterations in transcription of genes in the pathway leading to β -carotene, mRNA levels of the genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (*Dxs*), geranylgeranyl diphosphate synthase (*Ggps2*), phytoene synthase (*Psy1* and *Psy2*), phytoene desaturase (*Pds*), and ζ -carotene desaturase (*Zds*) were determined by quantitative real-time RT-PCR at three stages of flower development. No significant differences in gene expression were found between *wf1-2* and the wild type (Figure 8). These results cancel out the possibility of a negative effect of the mutation on expression of key enzymes in the isoprenoid pathway.

DISCUSSION

Regulation of Carotenoid Biosynthesis in Flowers

In tomato, the concentration of total carotenoids in petals and anthers is 3- to 4-fold higher than in leaves and 8- to 10-fold higher than in mature tomato fruit. The carotenoids in flowers and fruits are considered secondary metabolites as they contribute to plant fitness but are not necessarily essential for the physiology in these tissues. On the other hand, in green leaves, carotenoids are indispensable for photosynthesis. Constitutive expression of carotenoid biosynthesis genes has been observed in all green tissues that have been examined. By contrast, accumulation of high concentrations of carotenoids in flowers and fruits is correlated with upregulation of genes that enhance the flux of the biosynthetic pathway (reviewed in Hirschberg, 2001; Bramley, 2002). Thus, the enhanced expression during tomato flower development of *CrtR-b2*, *Psy1* (Giuliano et al., 1993; this work), *Pds* (Giuliano et al., 1993; Corona et al. 1996), *Cyc-b*, and *Lcy-b* (Ronen et al., 2000) results in increased carotenoid biosynthesis. Lack of lycopene ϵ -cyclase activity due to downregulation of

Table 2. Functional Expression of Tomato β -Carotene Hydroxylases *CrtR-b1* and *CrtR-b2* in *E. coli* Cells

Plasmids	β -Carotene	β -Cryptoxanthin	Zeaxanthin	Others ^a
pBETA + KS-	98			2
pBETA + pTCrtR-b1	4	25	71	
pBETA + pTCrtR-b2	10	45	45	
Plasmids	Lycopene	δ -Carotene	ϵ -Carotene	
pDELTA + KS-	2	94	4	
pDELTA + pTCrtR-b1	2	94	4	
pDELTA + pTCrtR-b2	2	94	4	

Carotenoid composition (%) was determined in *E. coli* cells carrying various combinations of plasmids: pBETA, carrying *CrtE*, *CrtB*, and *CrtI* from *Erwinia uredovora* and *Lcy-b* from tomato; pTCrtR-b1, expressing the tomato cDNA of *CrtR-b1*; pTCrtR-b1 and pTCrtR-b1, expressing truncated cDNA clones of *CrtR-b1*; pTCrtR-b2, expressing the cDNA of *CrtR-b2*. KS-, pBluescript KS-.

^aA mixture of unidentified carotenes upstream to lycopene.

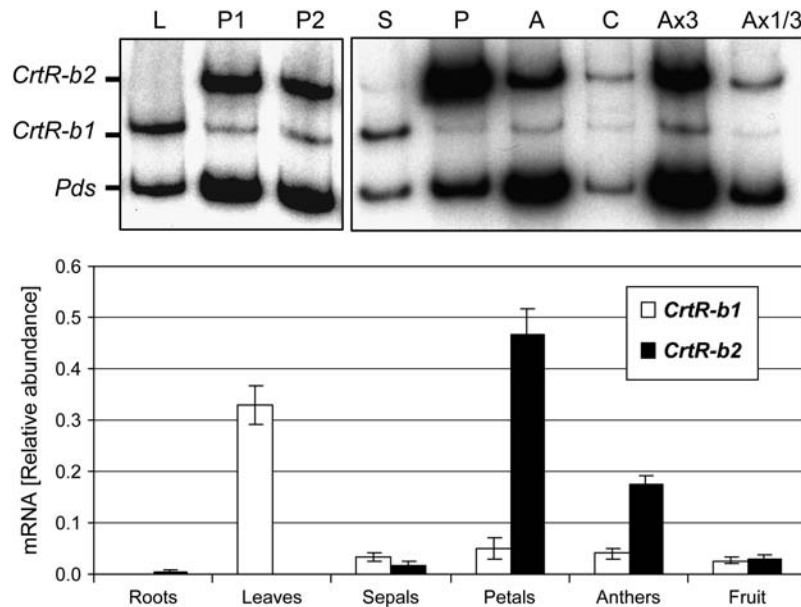


Figure 7. Expression of *CrtR-b1* and *CrtR-b2* in Various Tomato Organs.

Top panel: Steady state levels of mRNA of *CrtR-b1*, *CrtR-b2*, and *Pds* were measured concomitantly by RT-PCR amplification from the same samples of total RNA. PCR was performed with ^{32}P -labeled dCTP. Autoradiograms of PCR-amplified DNA fragments that were separated by polyacrylamide gel electrophoresis are exhibited. RNA from leaves (L) and petals from flower stages 1 (P1) and 3 (P3) are presented in the left panel. RNA from different tissues of stage 3 flowers is presented in the right panel: S, sepals; P, petals; A, anthers; and C, carpels. Samples Ax3 and Ax1/3 were amplified from anther RNA, 3-fold and one-third of the original concentration, respectively. Bottom panel: Relative levels of *CrtR-b1* and *CrtR-b2* mRNA from roots, leaves, flowers, and mature fruit were determined by real-time RT-PCR using gene-specific primers. Expression data were normalized to the expression of *actin*. Data shown are means + SD ($n = 4$).

expression in tomato flowers of *Lcy-e* (Ronen et al., 1999) channels the carotenoid pathway in tomato flowers to the β -xanthophylls neoxanthin and violaxanthin. In flowers of marigold (*Tagetes*), high level of expression of the genes of *Psy1*, *Pds*, *Zds*, and *Lcy-e* but not *Lcy-b* bring about the accumulation of the α -branch xanthophyll lutein (Moehs et al., 2001). Upregulation of *Psy1*, *Pds*, and *Zds* was reported in flowers of *Gentiana* (Zhu et al., 2002) and of *Psy1* in flowers of *Narcissus pseudonarcissus* (Schledz et al., 1996) and *Cucumis sativus* (Vishnevetsky et al., 1997). Taken together, these data indicate that in flowers, similarly to fruits, gene expression, most probably at the level of transcription, is the major mechanism that determines the flux of carotenoid biosynthesis to the various xanthophylls.

Why Are Flowers of *wf* Nearly Colorless?

Here, we describe the cloning from tomato of the *wf* gene. Surprisingly, lack of carotenoids in flowers of this mutant is caused by a lesion in the enzyme β -carotene hydroxylase, which functions downstream from β -carotene in the pathway leading to the xanthophylls violaxanthin and neoxanthin. It is intriguing that inhibition of β -carotene hydroxylation leads to a reduction of 80% in total carotenoid concentration in the petals of *wf* rather than to the replacement of the xanthophylls by an equivalent amount of β -carotene and upstream carotenoids. Three possible mechanisms may explain the decline in total carotenoids: (1) reduced synthesis due to downregulation of the pathway, possibly at the

transcriptional level; (2) inability of petals to accumulate carotenoid species other than xanthophylls; and (3) degradation of β -carotene, which accumulates as a result of the *wf* mutation.

Because expression of genes encoding the rate-limiting isoprenoid and carotenoid enzymes DXS (Rodríguez-Concepción et al., 2001) and PSY (Romer et al., 2000; Paine et al., 2005), as well as genes for other enzymes in the pathway (GGPS2, PDS, and ZDS), is not altered in *wf* (Figure 8), downregulation of the carotenoid pathway at the transcriptional level as a result of the mutation in *CrtR-b2* can be ruled out. Although carotenoid biosynthesis is regulated mainly at the transcriptional level, one cannot exclude a mechanism of feedback inhibition, at the enzymatic or protein level, for example by β -carotene, of the rate-limiting enzymes DXS or PSY.

Posttranscriptional regulation has been described for PSY2 in tomato fruit (Fraser et al., 1999) and in the MEP pathway enzymes in *Arabidopsis* (Sauret-Gueto et al., 2006), specifically of DXS (Guevara-García et al., 2005).

An alternative explanation that links the white phenotype to a failure in accumulation of carotene species is supported by the mechanisms of sequestration in chromoplasts that enable storage of high concentrations of xanthophylls (Vishnevetsky et al., 1999). Most of the xanthophylls in tomato flowers are esterified with fatty acids. These xanthophyll-esters are necessary for both stabilization and accumulation by association with specific polypeptides. Since β -carotene cannot be esterified, its accumulation in chromoplasts is very limited. This suggestion for limited storage capability is in

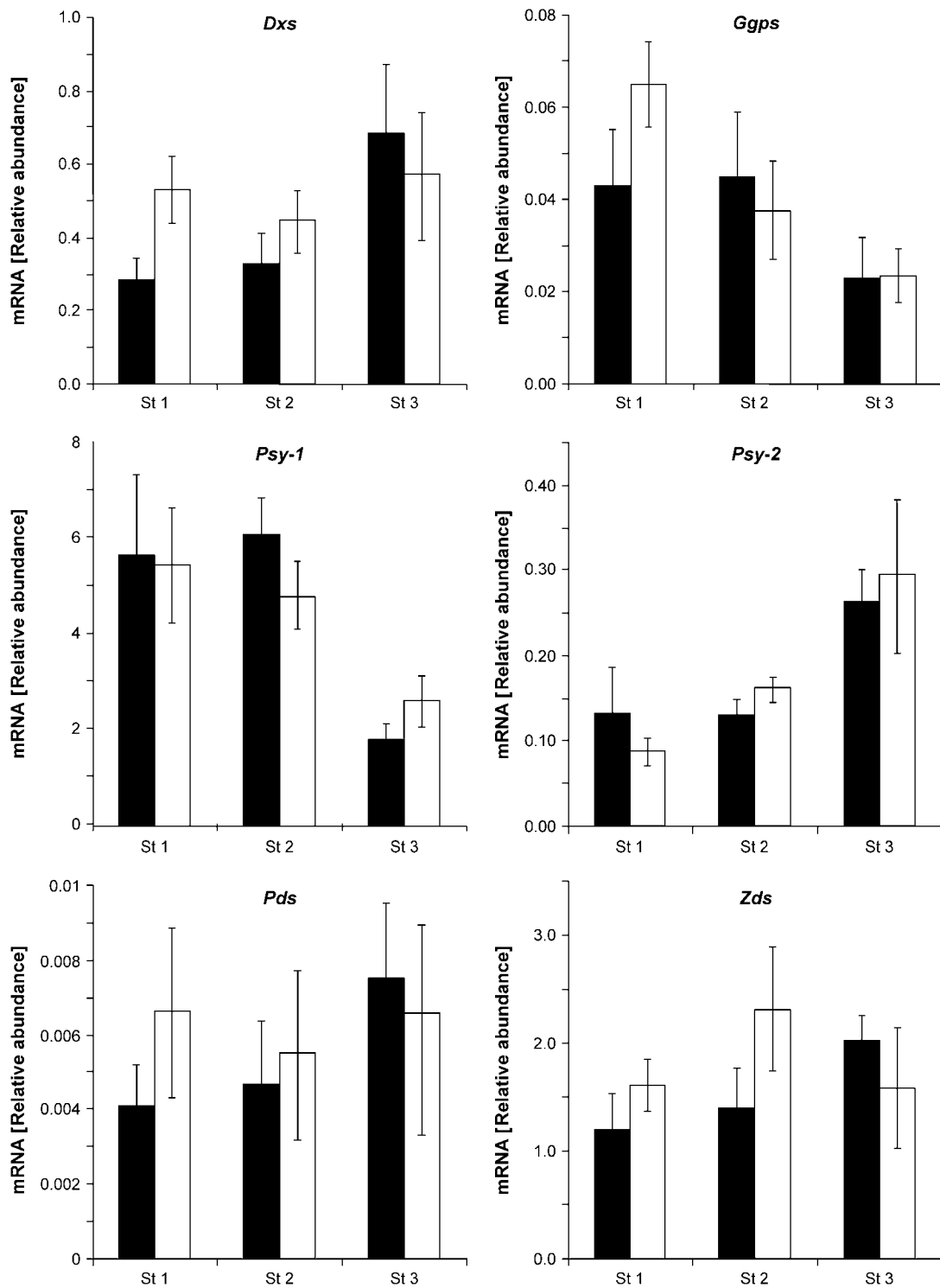


Figure 8. Expression of Isoprenoid and Carotenoid Biosynthesis Genes in Flowers of the Wild Type (M82) and Mutant *wf1-2*.

Total RNA was extracted from flowers at developmental stages 1 to 3 (Figure 1). Levels of mRNA were determined by real-time RT-PCR using gene-specific primers for the following genes: *Dxs*, *Ggps2*, *Psy1*, *Psy2*, *Pds*, and *Zds*. Expression data were normalized to the expression of *actin*. Data shown are means + SD ($n = 5$). Black bars correspond to the wild type (M82) and white bars to *wf1-2*.

agreement with the fact that during early stages of flower development, when carotenoids still exist in low concentrations, the difference in carotenoid content between *wf* and the wild type is relatively small. For example, in flowers at stage 1 (2 d before anthesis; Figure 1), total carotenoid concentration was $345 \pm 48 \mu\text{g}\cdot\text{g}^{-1}$ fresh weight (FW) in the wild type (M82) and $226 \pm 34 \mu\text{g}\cdot\text{g}^{-1}$ FW in *wf1-2*, whereas in fully developed flowers (stage 3), it was $676 \pm 72 \mu\text{g}\cdot\text{g}^{-1}$ FW in the wild type and $147 \pm 14 \mu\text{g}\cdot\text{g}^{-1}$ FW in *wf1-2*.

Enzymatic cleavage of carotenoids may reduce the carotenoid level, as demonstrated in the mutant *ccd1-1* of *Arabidopsis* (Auldrige et al., 2006). Analysis by gas chromatography–mass spectrometry of volatiles produced by tomato flowers revealed that in *wf*, the levels of β -ionone and β -cyclocytral, both produced by an oxidative cleavage of β -carotene, was 24- and 7-fold, respectively, higher in *wf1-2* than in M82 (E. Lewinsohn, unpublished data). This result indicates that cleavage of β -carotene does take place in the petals; therefore, reduced total carotenoids in *wf* could be due to this degradation.

Two Non-Heme β -Carotene Hydroxylase Enzymes in Tomato

Two *CrtR-b* genes that encode β -carotene hydroxylase exist in tomato. The products of both are capable of proper hydroxylation of the β -rings of β -carotene in *E. coli* cells to give β -cryptoxanthin and zeaxanthin, but they fail to hydroxylate the ϵ -ring in δ -carotene. Comparison of the deduced amino acid sequence of CRTR-B from different plants identified a conserved His-rich domain that is found in other non-heme di-iron monooxygenases (Bouvier et al., 1998). This SUR2-type (syringomycin response protein 2) domain occurs in enzymes such as sterol oxidase, sterol desaturase, and fatty acid hydroxylase that use iron-activated oxygen for catalyzing oxygenation or desaturation of hydrophobic molecules. Biochemical characterization in bell pepper (*Capsicum annuum*) has determined that iron, ferredoxin, and ferredoxin oxidoreductase are needed for the enzymatic activity of β -hydroxylation (Bouvier et al., 1998).

Two bona fide β -carotene hydroxylase enzymes exist also in *Arabidopsis* and pepper. A phylogenetic analysis based on amino acid sequences of β -carotene hydroxylases that was conducted using MEGA version 3.1 (Kumar et al., 2004) shows that tomato CRTR-B1 is more similar to one of the *CrtR-b* genes of bell pepper (CRTR-B2) than to its homolog in tomato *CrtR-b2* (Figure 9). Likewise, CRTR-B2 of tomato is more similar to pepper CRTR-B1 than to the tomato CRTR-B1. The pattern of expression of the *CrtR-b* genes described here indicates that in tomato, one enzyme, CRTR-B1, functions in carotenoid biosynthesis in photosynthetic tissues and the second enzyme, CRTR-B2, operates in flowers in the accumulation of carotenoids as secondary metabolites for pigmentation.

In pepper, expression of *CrtR-b1* is upregulated in fruit chromoplasts where massive accumulation of xanthophylls takes place (Bouvier et al., 1998). Thus, both tomato *CrtR-b2* and pepper *CrtR-b1* are upregulated in xanthophyll-accumulating tissues in flowers and fruits, respectively. The similarity between the tomato *CrtR-b2* and pepper *CrtR-b1* genes extends also to their chromosomal location in the genome as they are mapped to chromosome 6 in both pepper and tomato. Similarly, tomato *CrtR-b2* and pepper *CrtR-b1* are both mapped to chromosome 3 (Figure 3; Thorup et al., 2000). These similarities in *CrtR-b* between pepper and tomato suggest that the *CrtR-b* gene duplication preceded the separation of tomato and pepper during evolution. Moreover, the gene structure is identical in all hydroxylases of tomato, pepper, and *Arabidopsis*, and the splicing sites of the six introns are completely conserved. Altogether, these data can be explained if all *CrtR-b* genes in these species evolved from the same ancestral gene.

In *Arabidopsis*, which does not produce carotenoids in the petals, both genes are expressed in photosynthetic tissues under normal growth conditions. However, the mRNA level of β -carotene hydroxylase 1 is 10- to 50-fold higher than β -carotene hydroxylase 2 (Tian and DellaPenna, 2001). There is no colored chromoplast-containing tissue in *Arabidopsis*, and the reason for the existence of three β -hydroxylase genes, two non-heme β -carotene

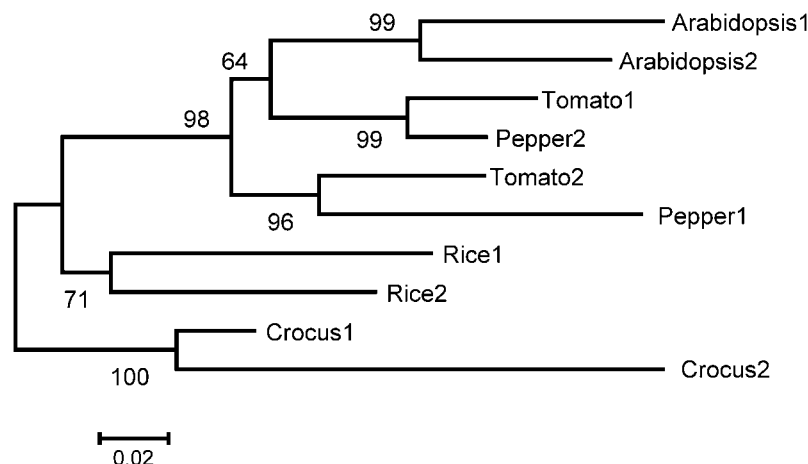


Figure 9. Phylogenetic Tree Based on Amino Acid Sequences of β -Carotene Hydroxylases in Higher Plants.

The following sequences have been analyzed: *Arabidopsis* 1, *Arabidopsis* 2, *Crocus* 1, *Crocus* 2, Pepper 1 (CRTR-B2), Pepper 2 (CRTR-B1), Rice 1, Rice 2, Tomato 1, and Tomato 2. Alignment of the amino acid sequences of these polypeptides is presented in Supplemental Figure 1 online.

hydroxylases (Tian and DellaPenna, 2001) and a p450 oxygenase (Kim and DellaPenna, 2006), has remained unexplained.

Interestingly, we have observed that *CrtR-b2* is expressed in tomato roots, although at a level that is <1% of that in flowers (Figure 7). Expression of *CrtR-b1* in roots is at least 15-fold lower than *CrtR-b2*. Nevertheless, root architecture in *wf*, as well as overall plant morphology, do not show any phenotype (data not shown), implying that abscisic acid is not significantly altered. Since abscisic acid is produced from β -xanthophylls, it is likely that a third β -carotene hydroxylase, possibly a P450-type enzyme (Kim and DellaPenna, 2006), is active in tomato roots.

The formation of a limited amount of xanthophylls in flowers of *wf* indicates the existence of low-level β -ring hydroxylation

activity that can be provided by CRTR-B1, which is expressed at a low level, or by a third β -carotene hydroxylase. Another possible source of β -carotene hydroxylation in *wf* flowers is a residual β -carotene hydroxylation activity by the ϵ -ring hydroxylase that has been suggested (Tian et al., 2004).

Gene Duplication and the Evolution of a Chromoplast-Specific Carotenoid Biosynthesis Pathway

In tomato, four carotenoid biosynthesis enzymes, geranylgeranyl diphosphate synthase (*Ggps*; TC120732 and TC127610) (Tomato Expression Database, Cornell; <http://ted.bti.cornell.edu/>), phytoene synthase (*Psy1* and *Psy2*), lycopene β -cyclase (*Lcy-b* and

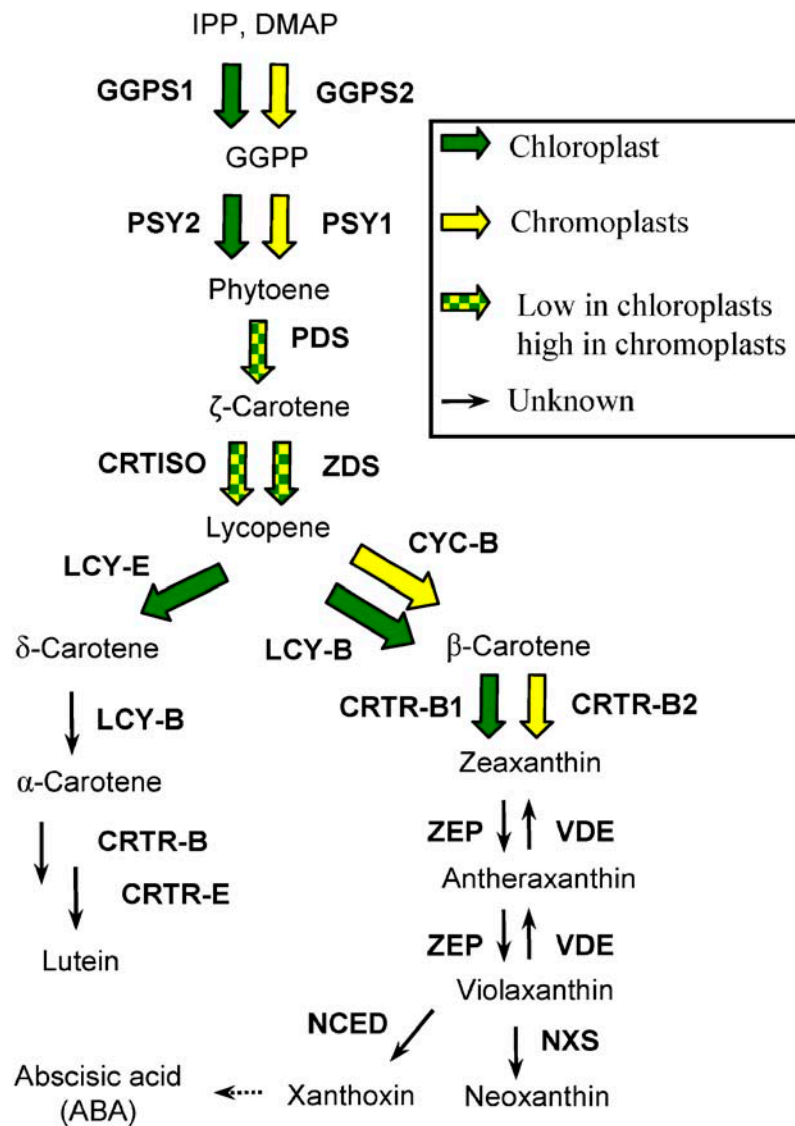


Figure 10. Carotenoid Biosynthesis Pathway in Tomato.

Cyc-b, chromoplast-specific lycopene β -cyclase; *Ggps*, geranylgeranyl diphosphate synthase; *Lcy-b*, lycopene β -cyclase; *Lcy-e*, lycopene ϵ -cyclase; *CrtR-b*, β -ring hydroxylase; *CrtR-e*, ϵ -ring hydroxylase; *Nxs*, neoxanthin synthase; *Pds*, phytoene desaturase; *Psy*, phytoene synthase; *Vde*, violaxanthin de-epoxidase; *Nced*, 9-*cis*-epoxycarotenoid dioxygenase; *Zds*, ζ -carotene desaturase; *Zep*, zeaxanthin epoxidase.

Cyc-B), and β -carotene hydroxylase (*CrtR-b1* and *Crtr-b2*), are each encoded by at least two genes. In each of these enzymes, one isoform is constitutively expressed in leaves, whereas the other is specific for chromoplasts in flowers and/or fruits (Figure 10). The phytoene synthase encoded by *Psy2* is constitutively expressed in photosynthetic tissues (Bartley and Scolnik, 1993; Fraser et al., 1999), and the one encoded by *Psy1* is expressed in flowers and fruits only (Bartley et al., 1992; Fraser et al., 1999). A null mutation in *Psy1* in the *r* mutant eliminates most of the carotenoids in fruits (Fray and Grierson, 1993) but not in leaves. The lycopene β -cyclase encoded by *Lcy-b* is expressed in leaves and at a relatively low level in flowers, and the β -cyclase encoded by *Cyc-B* is expressed exclusively in flowers and also at low levels in fruits (Pecker et al., 1996; Ronen et al., 2000). A null mutation in the *Cyc-B* gene, named *old-gold*, causes accumulation of low levels of the red pigment lycopene in the flowers and eliminates β -carotene in fruits but has no effect on carotenoids in leaves (Ronen et al., 2000). The mutation *wf* that revealed a flower-specific *CrtR-b* emphasizes gene duplication as a major mechanism underlying the establishment of a parallel carotenoid biosynthesis pathway that is activated in chromoplasts for high accumulation of pigments.

It is interesting to note that other enzymes in the carotenoid biosynthesis pathway, phytoene desaturase (*Pds*), ζ -carotene desaturase (*Zds*), *CrtISO*, zeaxanthin epoxidase (*Zep*), and violaxanthin de-epoxidase (*Vde*), are encoded by single genes in the tomato genome (Liu et al., 2003). The reason why these genes have not been duplicated in evolution is unknown. The duplicated carotenoid genes encode rate-controlling biosynthetic enzymes. None of the enzymes *Pds*, *Zds*, and *CrtISO* is a bottleneck in the biosynthetic flux of carotenoids in fruits and flowers of tomato, since almost no carotenoid intermediates accumulate in these tissues. Therefore, their duplication for the enhancement of carotenoid synthesis in chromoplasts may not be necessary. Alternatively, duplication of genes for these enzymes is less likely to evolve if their mode of action is in the form of a large heteromeric complex. In such a case, parallel duplication of all three genes would be required to duplicate a whole function to be selected in evolution.

Plants of *wf* show no evidence of any phenotypic alteration other than in the color of the flowers. The carotenoid composition in their leaves and fruits is similar to the wild type. This is in compliance with the finding that *CrtR-b2* is expressed exclusively in flowers. Among the known eight wild species of tomato only two, *Solanum pimpinellifolium* and *Solanum cheesmanii*, have red- or orange-colored fruits. The rest have green to pale yellow fruits. However, the color of flowers in all wild species of tomato is a strong yellow as a result of accumulation of xanthophylls, mainly neoxanthin (data not shown). We therefore conclude that the duplication and preservation of a second carotene hydroxylase gene in tomato that is dedicated to the generation of yellow pigments in flowers has been under strong selection pressure. Genetic evidence derived from ILs indicates the existence of *CrtR-b2* that is also responsible for flower coloration in the green-fruited species *Solanum pennellii* and *Solanum habrochaites*. We suggest that the generation by gene duplication of a chromoplast pathway of carotenoid biosynthesis had occurred initially to enhance carotenogenesis in flowers, and only later in evolution were these genes recruited to increase pigmentation in fruit

chromoplasts in the colored-fruited species *S. cheesmanii*, *S. pimpinellifolium*, and *S. lycopersicum*.

METHODS

Plants and Bacteria

Tomato (*Solanum lycopersicum*) varieties VF-36 and M82 were used as the wild type for pigment analysis and RNA measurement. For genetic mapping IL3-1 and IL3-2, derived from crosses between *S. lycopersicum* cv M82 and the wild species *Solanum pennellii* (LA716) (Eshed and Zamir, 1995) were used. Seeds for *wf* mutants LA2370 and LA3575 and for the wild species LA1245 (*Solanum pimpinellifolium*), LA1305 (*Solanum peruvianum*), LA0361 (*Solanum habrochaites*; formerly *Solanum hirsutum*), LA1306 (*Solanum chmielewskii*), LA1340 (*S. pennellii*), LA1406 (*Solanum cheesmanii*), and LA1329 (*Solanum parviflorum*) were obtained from the Tomato Genetics Resource Center (University of California, Davis). Mutant lines e1827 (*wf1-2*) and n5681 (*wf1-1*) carrying *wf* mutations were isolated from M82 following mutagenesis and screening (Menda et al., 2004; <http://zamor.sgn.cornell.edu/mutants/>). *Escherichia coli* strain XL1-Blue grown on Luria-Bertani (LB) medium was used in all experiments that are described in this work.

Extraction of DNA from Tomato and RFLP Mapping

Genomic DNA was prepared from 15 mg of leaf tissue as described (Eshed and Zamir, 1995). RFLP on the tomato genomic DNA was done using the DNA markers TG479, TG585, TG517, CT22, and CT90 (Tanksley et al., 1992) and cDNA clones pSICrtR-b1, pSICrtR-b2 (this work), and *Psy1*.

Tomato Genomic and cDNA Libraries

The BAC genomic library of *S. lycopersicum* cv Heinz 1706 (<http://www.clemson.edu>) (Budiman et al., 2000) was screened to identify the genomic sequences of *CrtR-b1* and *CrtR-b2*. Following screening one-third of the library with each of these sequences as probes, five BAC clones were identified.

Tomato (cv VF-36) leaf cDNA library in λ gt10 vector was screened using *CrtR-b1* cDNA from *Arabidopsis thaliana* as a probe. Altogether 900,000 phage plaques were screened. Nineteen positive phages were isolated from the library. For *CrtR-b2* cDNA, which lacked the 5' end, the full putative open reading frame was amplified using reverse transcription with an oligo16T primer followed by PCR with the primers 5'-ACTGAATTGGATCCTCC-ACC-3' (forward) and 5'-CTACAGCCTAAATACAATCGC-3' (reverse). Sequence analysis was done as previously described (Ronen et al., 1999).

Expression of *CrtR-b1* and *CrtR-b2* in *E. coli*

For expression in *E. coli* of carotenoid biosynthesis genes, plasmid pBCAR-T, carrying *CrtE*, *CrtB*, and *CrtI* from *Erwinia uredovora* and *Lcy-b* from tomato, and plasmid pDCAR, carrying the same genes but *Lcy-e* from tomato replaced *Lcy-b*, were used (Ronen et al., 1999). To express *CrtR-b1* in *E. coli*, the 5' end of the cDNA sequences was truncated (nucleotides 1 to 205), and the remaining sequence from nucleotide 206 to the 3' end was cloned in the *EcoRV* site of the plasmid vector pBluescript KS-. Similarly, the cDNA of *CrtR-b2* (Y14810) from nucleotide 16 to the 3' end was inserted in the *EcoRV* site of pBluescript KS-. The recombinant plasmids were designated pTCrtR-b1 and pTCrtR-b2. Inserts were sequenced to identify possible PCR-derived mutations. To enhance the expression of *CrtR-b1* and *CrtR-b2*, isopropylthio- β -galactoside (24 mg/L) was added to the LB medium. *E. coli* cells were grown overnight at 37°C followed by 5 d of shaking at room temperature for pigment accumulation.

Pigment Extraction and Analysis

Extraction of carotenoids from bacteria and plants followed previously described protocols (Ronen et al., 1999). Saponification of flower pigments was done in ethanol:KOH (60% [w/v]) 9:1 overnight at 4°C. The carotenoids were separated with di-ethyl-ether following addition of NaCl to a final concentration of 1.2%. Analysis by HPLC was previously described (Ronen et al., 1999). Carotenoids were identified by their characteristic absorption spectra and typical retention time, which corresponded to standard compounds of lycopene, β -carotene, and zeaxanthin. Carotenoid quantification was performed by integrating the peak areas using Millennium chromatography software (Waters).

DNA Sequence Analysis

DNA and RNA were extracted from 200 mg of leaf and flower tissue according to previously described protocols (Ronen et al., 1999). PCR amplifications were performed with the Hot Star Taq Master kit (Qiagen). Thermocycling conditions were 95°C for 10 min followed by 36 cycles of 94°C for 15 s, 60°C for 45 s, and 72°C for 1 to 2 min. The purified PCR products were separated on 1.3% agarose gel and stained with ethidium bromide to verify that a single product in the expected length was amplified. PCR products were sequenced as described by Ronen et al. (1999).

The following gene-specific primers were used for the PCR and sequencing reactions: for *CrtR-b1*, 5'-CCATCCTTCCACCTTTCTCC-3' (forward) and 5'-TTCTTATCAATGAAGAAGGGTGA-3' (reverse); for *CrtR-b2*, 5'-ATTTAGAAGGGAGTGAGACT-3' (forward) and 5'-GCGATTGTATTTAGGCTGTAG-3' (reverse).

Sequence Data Analysis

Sequences of β -carotene hydroxylases in different plants species were examined by comparing the presumed mature polypeptides after cleavage of the transit peptides (see Supplemental Figure 1 online). The following sequences were analyzed: *Arabidopsis* 1 (locus NP-200070) amino acid residues 89 to 303, *Arabidopsis* 2 (AAC49443) residues 91 to 310, *Crocus* 1 (CAC95130) residues 81 to 305, *Crocus* 2 (AAT84408) residues 72 to 291, pepper 1 (CRTR-B2; locus CAA70427) residues 105 to 315, pepper 2 (CRTR-B1; locus CAA70888) residues 106 to 316, rice 1 (AAP54790) residues 76 to 292, rice 2 (XP-473611) residues 102 to 303, tomato 1 (CRTR-B1; locus CAB55625) residues 99 to 309, and tomato 2 (CRTR-B2; locus CAB55626) residues 103 to 314. Alignment of these amino acid sequences was done with the ClustalW program followed by manual adjustments (Vector NTI Suite 5.5). Phylogenetic analyses based on amino acid sequences were calculated with MEGA software version 3.1 (Kumar et al., 2004). A phylogeny tree was calculated by maximum parsimony with bootstrap resampling of 1000 replicates.

Measurement of mRNA by RT-PCR

Protocols for RNA extraction and quantification by RT-PCR were previously described (Ronen et al., 1999). Total RNA was isolated from fruit, flowers (sepals, petals, anthers, and carpels), and leaves using TRI-REAGENT according to the manufacturer's protocol (Molecular Research Center). The amplification procedure by PCR consisted of 17 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. For the purpose of quantification, 5 μ Ci of 32 P-dCTP (specific activity 3000 Ci mmole⁻¹) was included in the PCR reaction mixture. Various initial concentrations of mRNA, ranging over a 9-fold difference, were used to demonstrate a linear ratio between concentration of the template cDNA (corresponding to the mRNA) and the final PCR products.

To rule out the possibility that genomic DNA contaminated the RNA samples, the primers were designed so as to span intron sequences.

The following primers were used for the PCR amplification: for *Pds*, 5'-TTGTGTTTGCCGCTCCAGTGGATAT-3' (forward) and 5'-GCGCCTTC-CATTGAAGCCAAGTAT-3' (reverse); for *CrtR-b1*, 5'-GATCTTAGCTAC-TCGCTTGG-3' (forward) and 5'-CACCTTTCCTCATTGATAAGA-3' (reverse); and for *CrtR-b2*, 5'-AATCCGCCTCAACCGCCC-3' (forward) and 5'-CTA-CAGCCTAAATACAATCGC-3' (reverse).

Products of the PCR amplification were separated by electrophoresis in 7% polyacrylamide gels. The gels were dried and autoradiographed. The amount of DNA was determined by measuring the radioactivity using a phosphor imager (MacBAS V2.5; Fujix) and exposure to x-ray film.

Measurement of mRNA by Real-Time RT-PCR

Samples of leaves, roots, sepals, petals, anthers, and fruits from *wf1-2* and its isogenic line M82 were harvested and frozen immediately in liquid nitrogen. RNA was extracted from 200 mg of tissue as previously described by Ronen et al. (1999). Amplification of the RNA samples with intron-exon junction primers confirmed that RNA samples were not contaminated by genomic DNA.

The real-time PCR reactions consisted of 6 μ L cDNA, 1.5 μ L 3 μ M primers, 7.5 μ L Failsafe real-time PCR PreMix selection kit, and 0.3 μ L Failsafe real-time PCR system DNA polymerase (Epicentre), and the reaction was performed on a Rotor-Gene 2000 thermocycler (Corbett Research).

Thermocycling conditions were 95°C for 2 min followed by 40 cycles of 95°C for 15 s, 60°C for 10 s, 72°C for 15 s, and fluorescence acquisition at 79°C.

Melting curve analysis and sequencing verified PCR product specificity. For quantification, calibration curves were run simultaneously with experimental samples, and C_t calculations were performed by the Rotor-Gene 5.0 software (Corbett Research). The *actin* gene served as reference for normalization.

The following primers for the PCR amplifications were used: for *Dxs*, 5'-AGCTTCCGGCTGGAACAAA-3' (forward) and 5'-CTAGCACAAATAG-CAGCATCC-3' (reverse); for *Ggps2*, 5'-GTACCTCGCTACCCTACA-3' (forward) and 5'-TAATCCCACATTAGGGTTACC-3' (reverse); for *Psy1*, 5'-AACTGTTGATGGCCAAAC-3' (forward) and 5'-CTGTATCGGACA-AAGCACA-3' (reverse); for *Psy2*, 5'-AGTCTGCTAGTAGATGGCC-3' (forward) and 5'-GGGCACTAGAGATCTTGCAT-3' (reverse); for *Pds*, 5'-GCAGAAGGCGTCCAGTTAG-3' (forward) and 5'-TGCACACAAGTG-CTCAACA-3' (reverse); for *Zds*, 5'-CATGTCAAAGGCCACTCAGA-3' (forward) and 5'-ACGGTAACAACAGGCACTCC-3' (reverse); for *CrtR-b1*, 5'-TTGGTGCTGCTGTAGGAATG-3' (forward) and 5'-GCAATGAGGC-CCTTATGGAA-3' (reverse); for *CrtR-b2*, 5'-ACATGTTCCGTTACCGA-TGGA-3' (forward) and 5'-TTGTCCGAGTGATGAAGCTG-3' (reverse); and for *actin*, 5'-TTGCTGACCGTATGAGCAAG-3' (forward) and 5'-GGA-CAATGGATGGACCAGAC-3' (reverse).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers. cDNA: *Arabidopsis* *CrtR-b*, U58919; tomato *actin*, BT013524; tomato *CrtR-b1*, Y14809; tomato *CrtR-b2*, Y14810; tomato *Dxs*, AF143812; tomato *Ggps2*, SGN-U223568; tomato *Pds*, X59948; tomato *Psy1*, Y00521; tomato *Psy2*, L23424; tomato *Zds*, AF195507. Genomic sequences: *Arabidopsis* *CrtR-b1*, U58919; *Arabidopsis* *CrtR-2*, NM_124636; rice *CrtR-b2*, NM_197521; tomato *CrtR-b2*, DQ650804. Polypeptides: *Arabidopsis* CRTR-B1, NP-200070; *Arabidopsis* CRTR-B2, AAC49443; *Crocus* CRTR-B1, CAC95130; *Crocus* CRTR-B2, AAT84408; pepper CRTR-B2, CAA70427; pepper CRTR-B1, CAA70888; CRTR-B1, AAP54790; rice CRTR-B2, XP-473611; tomato CRTR-B1, CAB55625; tomato CRTR-B2, CAB55626.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Alignment of Amino Acid Sequences of β -Carotene Hydroxylases in Higher Plants Used for Calculating the Phylogenetic Tree.

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