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ORIGINAL PAPER

Induced mutation in *β-CAROTENE HYDROXYLASE* results in accumulation of β-carotene and conversion of red to orange color in pepper fruit

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Abstract Pepper fruit is typically red, but green, orange and yellow cultivars are gaining consumer acceptance. This color variation is mainly due to variations in carotenoid composition. Orange color in pepper can result from a number of carotenoid profiles, but its genetic basis is only partly known. We identified an EMS-induced orange-fruited mutant using the wild-type blocky red-fruited cultivar 'Maor' as progenitor. This mutant accumulates mainly β-carotene in its fruit, instead of the complex pattern of red and yellow carotenoids in 'Maor'. We identified an A^{709} to G transition in the cDNA of *β-CAROTENE HYDROXYLASE2* in the orange pepper and complete co-segregation of this single-nucleotide polymorphism with the mutated phenotype. We therefore hypothesized that *β-CAROTENE HYDROXYLASE2* controls the orange mutation in pepper. Interestingly, the expression of *β-CAROTENE HYDROXYLASE2* and additional carotenogenesis genes was elevated in the orange fruit compared with the red fruit, indicating possible feedback regulation of genes in the pathway. Because carotenoids serve as precursors for volatile compounds, we compared the volatile profiles of the two parents. The orange pepper contained more volatile compounds than 'Maor', with predominant elevation of norisoprenoids derived from β-carotene degradation, while sesquiterpenes predominated in the red fruit. Because of the importance of β-carotene as a provitamin A precursor in the human diet, the orange-fruited mutant might serve as a natural source for pepper fruit biofortification. Moreover, the change in volatile profile may result in a fruit flavor that differs from other pepper cultivars.

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Introduction

Several biochemical pathways synthesize the pigments that contribute to pepper fruit color. These include carotenoids, which determine the mature fruit color, and anthocyanins and chlorophyll, which determine the immature fruit color. Carotenoids are C40 isoprenoid polyene compounds. They are synthesized in plastids where they are involved in leaf photosynthesis. They also provide protection against photo-oxidation and attract pollinators and seed-dispersing animals by coloring flowers and fruits. The inheritance of mature fruit color in pepper is controlled by three independent loci: *Y*, *C1* and *C2* (Hernandez and Smith 1985). Segregation of these loci produces color variation ranging from white-cream to red in mature fruits. A dominant allele at *Y* is required for the production of capsorubin and capsanthin, the unique carotenoids that determine the red color in pepper fruit. A recessive allele at this locus results in yellow fruit, in which violaxanthin and antheraxanthin, the precursors of capsorubin and capsanthin, respectively, are accumulated. Mutation in *C2* results in orange fruit, whereas a triple recessive genotype produces white-cream fruit (Hernandez and Smith 1985). *C1* is a postulated modifier of *Y* and *C2*.

Carotenoid biosynthesis is a well-characterized biochemical pathway in plants, and most of its enzymes and genes have been identified (reviewed by Kopsell and Kopsell 2006). Thus, carotenoid biosynthetic genes have

been tested as candidates for association with fruit-color mutations. This candidate gene approach enabled identification of the gene coding for CAPSANTHIN–CAPSORUBIN SYNTHASE (CCS) as a candidate for *Y* (Lefebvre et al. 1998; Popovsky and Paran 2000; Lang et al. 2004). Similarly, the gene coding for PHYTOENE SYNTHASE (PSY), which catalyzes the formation of phytoene in the early steps of carotenoid biosynthesis, has been found as a candidate for *C2* (Thorup et al. 2000; Huh et al. 2001). The identity of the gene associated with *C1* is currently unknown.

Mutation in *PSY* has been associated with the orange fruit color of Habanero pepper (Huh et al. 2001). Whereas the profile of carotenoid types in the orange Habanero fruit was the same as in red pepper, their quantity was six times lower in the orange compared with red fruit (Huh et al. 2001). Alternatively, orange fruit may result from the accumulation of yellow and orange carotenoids, such as zeaxanthin and β-carotene (Lang et al. 2004; Guzman et al. 2010). Some orange peppers accumulating yellow and orange carotenoids are associated with a mutation in *CCS* (Popovsky and Paran 2000; Lang et al. 2004). Because mutation in *CCS* can also result in a yellow fruit color (Lefebvre et al. 1998; Ha et al. 2007), an additional as-yet unknown gene must exist that controls the difference between yellow and orange fruits carrying the *CCS* mutation. Evidence for an additional gene, likely with a regulatory function, which controls pepper fruit color was obtained by Rodriguez-Uribe et al. (2012), who crossed two orange cultivars to obtain progeny that segregated for orange and yellow fruits. Whereas *CCS* was transcribed in both parents, *CCS* transcript was not detected in the yellow progeny. Therefore, an unknown gene was postulated to repress *CCS* expression in the yellow, but not in orange fruit. The complexity of the genetic control of orange color formation in pepper is further demonstrated in the orange-fruited cultivar 'Canary', in which expression of wild-type *CCS* was evident but no protein or red pigments were observed (Rodriguez-Uribe et al. 2012).

In a survey of orange pepper carotenoid composition, large variation in the quantity of β-carotene was observed among cultivars, but the genetic basis of this variation was not revealed (Guzman et al. 2010). To gain a better understanding of the genetic control of fruit color variation in pepper, we screened an EMS-mutagenized population of red pepper for alterations in fruit color. In this paper, we describe an orange-fruited pepper mutant that accumulates β-carotene as the predominant carotenoid. We further show that the mutation likely causing the change in fruit color is in the gene (*CHY2*) coding for the fruit-specific β-CAROTENE HYDROXYLASE2.

Materials and methods

Plant material

An EMS-mutagenized population of the *Capsicum annuum* blocky cultivar 'Maor' (Paran et al. 2007) was screened for changes in mature fruit color from red to orange: 3,000 M_2 families (10 plants per family) were planted in the open field during the summers of 2005–2007. The mutant E-172-3 was detected as having orange-colored mature fruit. The mutant plants were crossed to 'Maor' and F_3 plants homozygous for the mutation were used in the present study. For the experiments described in this work, plants were grown in 5-l pots in a heated greenhouse during the fall–winter season at maximum and minimum temperatures of 30 and 16 °C, respectively, under natural light.

Sequencing of *CHY2*

To determine the sequence of *CHY2* in 'Maor' and in the orange mutant, RNA was extracted from mature fruits and cDNA was synthesized using gene-specific primers CH2-F and CH2-R based on GenBank accession no. Y09225 (Table 1). The cDNAs were sequenced by Hylabs Ltd. (Rehovot, Israel). The sequence of *CHY2* from 'Maor' was deposited in NCBI GenBank with accession no. JQ031264.

Development of dCAPS marker

To generate dCAPS marker (Michaels and Amasino 1998), we used a 28-bp forward primer, CH2 CAP-F, from exon 5 and reverse primer CH2 CAP-R from intron 5 of *CHY2* (Table 1). These primers amplified a 244-bp fragment from both parents using genomic DNA as the template for PCR. The introduced C at position 27 instead of T in the CH2 CAP-F primer (in italic in Table 1) created a mismatch that generated a F*sp*BI restriction site in the amplification product using E-172-3 DNA, but not 'Maor' DNA. Digestion of the amplified product of E-172-3 with F*sp*BI (Fermentas) resulted in a 214-bp fragment, which was distinguished by agarose gel electrophoresis from that of 'Maor'. The validity of the marker was tested for cosegregation with the color phenotype in an F_2 population (94 individuals) of 'Maor' × E-172-3.

Carotenoid analyses

HPLC analysis of carotenoids in ripe fruits of E-172-3 and 'Maor' (three biological repeats for each genotype, each sample contained a bulk of three fruits) was performed as described by Kolotilin et al. (2007) with saponification. Absorbance spectra and retention times of the eluted peaks

Table 1 List of primers used in this study

Primer name	Use	Primer sequence (5′→3′)
CH2-F	Cloning	GTACCGTACATGGCTGCTGAA
CH2-R		TGGGCTAAAGCCTAAATACAAT
CH2 CAP-F	dCAPS	CGGCGCTGGATTAGGGATTACAGTATCT
CH2 CAP-R		CACAAAGGACCAGTCAACTTA
PSY-RT-F	RT-PCR	CCAGAAGAGGAAGAGTCTATTTGCC
PSY-RT-R		GAAGATTCTCCATTTATCGGTCACTC
CHY2-RT-F	RT-PCR	GGATGGCCTACATGTTTGTTCAC
CHY2-RT-R		TCCGAGTGATGAAGCTGATGTG
VDE-RT-F	RT-PCR	GCCCTGAACCACCCTTTGTT
VDE-RT-R		CCCTCACCTTCTCAACTTGTTCT
CCS-RT-F	RT-PCR	TGTTATGGCTATTGGTGGGACTTC
CCS-RT-R		GCCACAAACCATTCCAAACTCT
UBIQUITIN–F	RT-PCR	AATAAGGATGCAGGCTTCAAGGGC
UBIQUITIN–R		TGATGTCACGGGACCGAAGAAGAT

were compared with those of a commercially available β-carotene (Sigma) and capsanthin (ExtraSynthese) standards. Quantification of β-carotene was performed based on a calibration curve prepared using the corresponding standard. Quantification of total carotenoids was based on the sum of peak areas in the chromatograms at a wavelength of 450 nm.

Volatile extraction

The volatile constituents of the two pepper lines were compared using solid-phase microextraction (SPME) and solvent extraction (SE). For SPME, frozen fruit powder (2.5 g) was homogenized with 7 ml of a 20 % (w/v) NaCl solution, and 1 g solid NaCl was added to the slurry to inhibit enzymatic reactions. The mixture was poured into a 20-ml vial followed by addition of 0.7 µg 2-heptanone as an internal standard. The vial was sealed and kept at 4 °C until analysis. The volatiles were adsorbed to a 65-µm PDMS/DVB fiber (polydimethylsiloxane/divinylbenzene; Supelco, PA) for 25 min in an automatic HS-SPME MPS2 (Gerstel, Mülheim, Germany) equipped with a heating block set at 50^{o}C. The fiber was inserted into the injection port of the GC–MS for 5 min to desorb the volatiles in splitless mode (Davidovich-Rikanati et al. 2008).

For SE, 50 g frozen fruit tissue was ground in liquid N_2 to a fine powder. The volatile components were extracted with 100 ml of methyl *tert*-butyl ether (MTBE) by vigorous shaking on a shaker apparatus overnight at room temperature, following the addition of 20 µg *iso*-butyl benzene as an internal standard. The upper MTBE layer was separated and dried with sodium sulfate, and a 1-µl aliquot was injected into the GC–MS in splitless mode for analysis (Davidovich-Rikanati et al. 2008).

GC–MS analyses

A 1-µl aliquot of the liquid sample or SPME fiber was injected into an Agilent GC 6890 system, coupled to a quadrupole MS detector 5973 N (Santa Clara, CA). The instrument was equipped with an Rxi-5sil MS column (30 m length × 0.25 mm i.d., 0.25 µm film thickness harboring a stationary phase of 95 % dimethyl and 5 % diphenyl polysiloxane). Helium (15.03 psi) was used as the carrier gas with splitless injection. The injector temperature was 250 °C, and the detector temperature was 280 °C. The following conditions were used for the runs: initial temperature 50 °C for 1 min, followed by a ramp of 5–180 °C/min, and 10 up to 280 °C/min (5 min). A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 41–350 m/z. A mixture of straight-chain alkanes (C7–C23) was injected into the column under the above conditions for determination of retention indices. The identification of the volatiles was assigned by comparison of their retention indices with those in the literature and by comparison of spectral data with standard or Nist 98 and QuadLib 2205 GC–MS libraries. Component amounts in each sample were calculated as (peak area × internal standard response factor)/(response factor × internal standard peak area). Results are the average of three biological replicates and presented as ng compound/g FW tissue.

Expression analysis

Quantitative RT-PCR analysis was performed according to Jeifetz et al. (2011). RNA was extracted from fruits at the breaker stage. Four biological replicates were used for each genotype, and each cDNA sample was run in triplicate.

Specific gene expression was normalized to the internal control gene *UBIQUITIN* (SGN-U198046) and the gene-expression value of the wild type was used as a control, set at 1.0. Primer pairs were synthesized for *PSY*, *CHY2*, *VIOLAXANTHIN DE-EPOXIDASE* (*VDE*) and *CCS* (Table 1).

Results

We identified an orange-fruited mutant, E-172-3, in an EMS-mutagenized population of the red-fruited cv. 'Maor' (Fig. 1). The mutation was inherited as a single recessive trait (2 mutant plants in an M_2 family of 10 plants and F_1 with 'Maor' had red fruit). The mutant was crossed with other orange-fruited cultivars such as 'Narobi', bearing a mutation in *CCS* (De Ruiter Seeds; Popovsky and Paran 2000) and 'NuMex Sunset', bearing a mutation in *PSY* (New Mexico State University; Y. Borovsky and I. Paran unpublished). The F_1 plants from these crosses had red fruits, indicating that the gene controlling the orange color in E-172-3 is different from that in the latter orange cultivars.

Carotenoid profiles in E-172-3 compared with 'Maor'

To determine what changes in the carotenoid profile had occurred in E-172-3 compared with 'Maor', carotenoids were extracted from ripe fruits and analyzed by HPLC. Whereas 'Maor' had a complex pattern of carotenoids typical to red peppers (Schweiggert et al. 2005), E-172-3 had a predominant peak eluting at 26 min that corresponded to β-carotene and a small peak corresponding to capsanthin eluting at 4.5 min as well as a small unidentified peak eluting at 24 min (Fig. 2). E-172-3 had 18.4 ± 1.2 μg g^{-1} FW β-carotene compared with 'Maor' which had 5.8 ± 0.8 μg g^{-1} FW β-carotene, a 3.2-fold increase in β-carotene content (Fig. 3). However, in 'Maor', β-carotene constituted only 7.4 % of the total carotenoids, whereas in E-172-3, its content was 55.7 % of total carotenoids. This significant increase in β-carotene content in E-172-3 was accompanied by a 5.5-fold decrease in the content of the major red pigment capsanthin compared with 'Maor' (Fig. 2) and significant decrease in total carotenoid content. The fruit of 'Maor' had total carotenoids of 79.2 ± 8.9 μg g^{-1} FW, compared with 33 ± 1.4 μg g^{-1} FW in E-172-3, a 2.4-fold decrease in the mutant.

Fig. 1 Fruits of 'Maor' (*red*) and E-172-3 (*orange*). *Bar* 5 cm

β-CAROTENE HYDROXYLASE is disrupted in E-172-3

The accumulation of β-carotene as the major carotenoid in E-172-3 led us to hypothesize that the cause for this change is a lesion in the gene coding for β-CAROTENE HYDROXYLASE, which catalyzes hydroxylation of the β ring of carotene to produce zeaxanthin (Paran and van der Knaap 2007). To test this hypothesis, we compared the sequences of *CHY2* cDNA extracted from 'Maor' and E-172-3 fruit. This comparison revealed a single nucleotide change from G in the wild-type allele to A in the mutant allele at nucleotide 709 of the open reading frame. This single-nucleotide polymorphism (SNP) led to an amino-

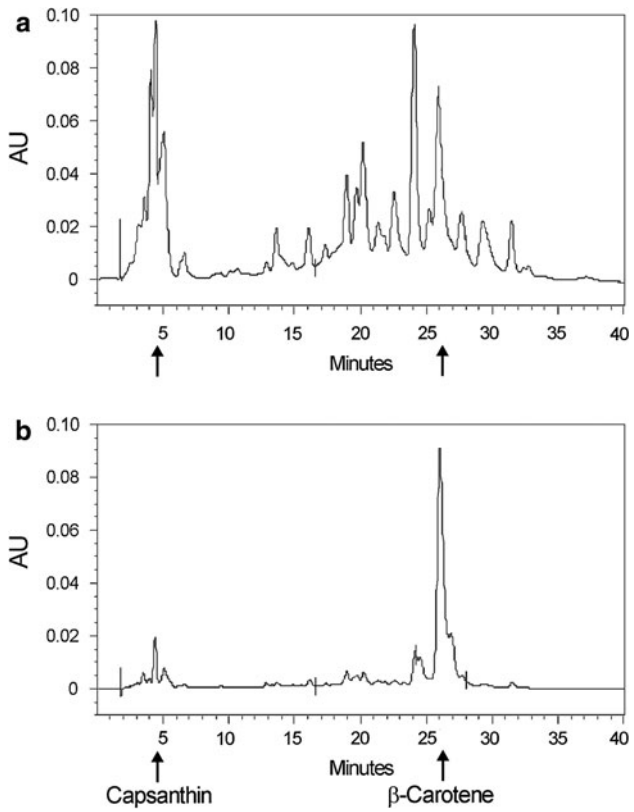

Fig. 2 HPLC chromatograms of carotenoids in ripe pepper fruits. **a** 'Maor'. **b** E-172-3. *Arrows* indicate β-carotene and capsanthin peaks

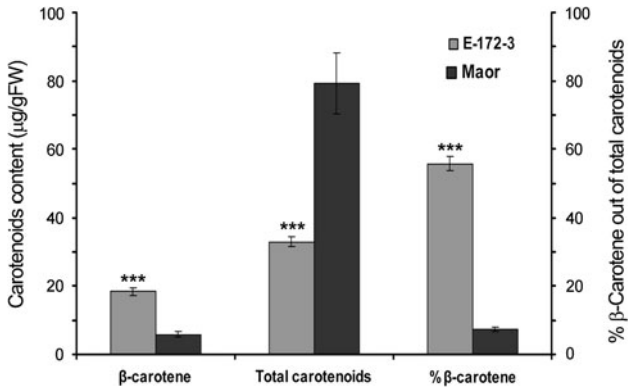

Fig. 3 Carotenoid content and percentage in ripe fruits of 'Maor' and E-172-3. *Asterisks* indicate significant difference (***$P < 0.001$) between 'Maor' and E-172-3 by Student's *t* test. Data for each line are means of three biological replicates ± SE

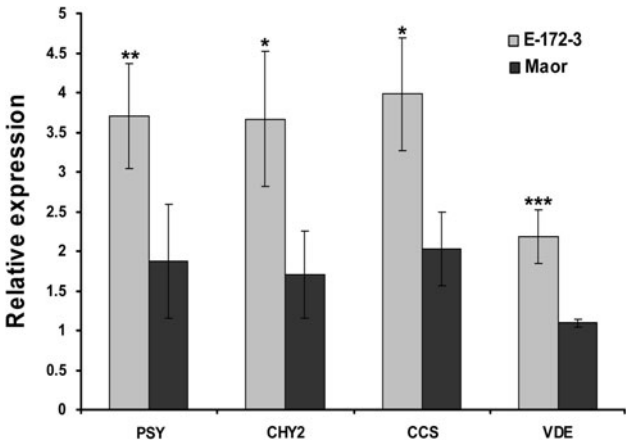

Fig. 5 Real-time RT-PCR analysis of *PSY*, *CHY2*, *CCS* and *VDE* in 'Maor' and E-172-3 fruits at the breaker stage. *UBIQUITN* expression values were used to normalize the expression level of the four genes. *Asterisks* indicate significant difference (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$) between 'Maor' and E-172-3 by Student's *t* test. Data for each line are means of four biological replicates ± SE

acid substitution of glycine[237] (small amino acid) to arginine (basic amino acid). GC to AT transition is the most common mutation induced by EMS (Tadmor et al. 2007). Based on this SNP, we developed a PCR-based marker that enables differentiation between the wild-type and mutant alleles and following the mutation in a segregating population (Fig. 4). Complete co-segregation between the marker and the phenotype in an F_2 population from a cross of 'Maor' and E-172-3 confirmed the association of the SNP with the fruit-color change between the wild type and the mutant.

Expression pattern of genes in the carotenoid biosynthesis pathway

To determine the effect of the mutation in E-172-3 on transcript levels of *CHY2* and other genes in the carotenoid biosynthetic pathway, we compared the expression of four genes in 'Maor' and E-172-3 by quantitative real-time RT-PCR. In addition to *CHY2*, the genes included *PSY* in the early stage of the pathway and *VDE* and *CCS* in the late stages of the pathway (Giuliano et al. 2008). Despite the reduction in carotenoid content in E-172-3 compared with 'Maor', the transcript levels of *PSY*, *CHY2*, *VDE* and *CCS* were significantly higher in E-172-3 than in 'Maor' (Fig. 5).

Volatile analysis in E-172-3 and 'Maor'

Because carotenoids serve as precursors for volatile compounds (Lewinsohn et al. 2005a, b; Klee 2010), we examined whether the change in carotenoid profile between 'Maor' and E-172-3 concomitantly results in a change in volatile profile between the lines. The total volatile content (Table 2) was higher in E-172-3 than in the control wild type. By SE, total volatile content was 13.3 and 8.1 µg g FW^{-1} for E-172-3 and 'Maor', respectively, a 1.63-fold difference between the lines. By SPME, the total volatile content was 1.2 and 0.6 µg g FW^{-1} for E-172-3 and 'Maor', respectively, a twofold difference between the lines. Similar patterns of volatiles (with respect to the major components) were obtained with both extraction techniques. Norisoprenoid derivatives were the major group of volatiles present in E-172-3. These included dihydroactinidiolide, *β*-cyclocitral, *β*-ionone and *β*-ionone-epoxide, which are *β*-carotene derivatives (Lewinsohn et al. 2005a, b). Other norisoprenoids included 6-methyl-5-hepten-2-one and geranyl acetone which arise from non-cyclic carotenoid moieties. The fatty acid-related

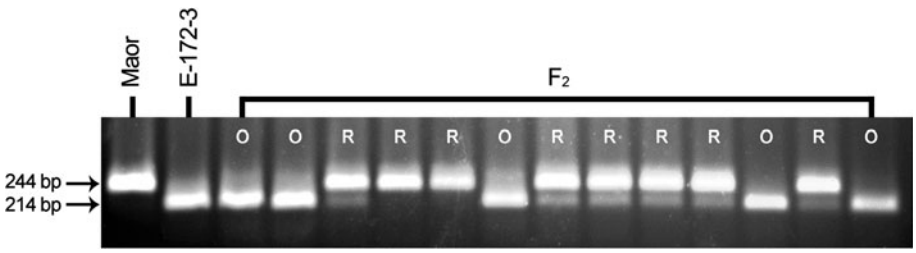

Fig. 4 dCAPS marker for the orange-fruited mutation. PCR products of the parents and F_2 individuals were amplified using genomic DNA as a template with *CHY2* primers and digested with F*sp*BI. O-orange fruit, R-red fruit. Cosegregation of the marker and fruit color is observed

Table 2 List of volatiles present in pepper. Values represent mean ± SE (μg g FW^{-1}) of three biological replications

Group	No.	Compound	RT[a]	Solvent extraction		SPME	
				E-172-3	'Maor'	E-172-3	'Maor'
Fatty acid- related	1	Hexanal	2.97	nd	nd	59.1 ± 14.3**	4.2 ± 3.2
	2	(*E*)-2-Hexenal	3.89	nd	nd	197.1 ± 52.8**	27.3 ± 4.1
	3	(Z)-2-Hexenol	4.02	nd	nd	5.6 ± 3.8**	41.3 ± 9.1
	4	Hexanol	4.1	nd	nd	1.4 ± 0.7	8.1 ± 3.2
	5	(E)-2-Hexenoic acid	7.99	nd	nd	56.2 ± 8.6**	24.8 ± 1.3
	6	Pentadecane	28.82	394.3 ± 162.1	nd	27.3 ± 5.8**	2.1 ± 0.2
	7	Isopropyl tetradecanoate	29.01	1147.1 ± 731.7	nd	nd	nd
Aromatic amino acid-related	8	Benzene acetaldehyde	8.4	nd	nd	6.2 ± 0.2*	2.7 ± 1.0
	9	Methyl salicylate	12.89	63.7 ± 32.8**	685.3 ± 243.0	2.2 ± 0.8*	38.4 ± 12.3
	10	2-Methoxy-4-vinylphenol	16.2	2528.1 ± 1055.4	889.2 ± 388.9	nd	nd
Monoterpene	11	α-Pinene	5.6	nd	nd	4.4 ± 2.0	8.9 ± 5.1
	12	*p*-2,4 (8)-Menthadiene	7.17	nd	nd	5.7 ± 0.9	nd
	13	(*E*)-*β*-Ocimene	8.6	nd	nd	1.5 ± 0.1***	6.6 ± 0.4
Sesquiterpene	14	(+)-Cyclosativene	17.99	nd	1155.1 ± 69.0	28.5 ± 6.2**	168.7 ± 25.2
	15	α-Copaene	18.18	259.5 ± 114.5***	1320.5 ± 30.2	31.9 ± 8.6**	184.1 ± 27.4
	16	*β*-Elemene	18.56	nd	34.3 ± 18	nd	2.5 ± 0.4
	17	(+)-Sativene	18.6	nd	nd	nd	1.6 ± 0.2
	18	*β*-Cubebene	19.6	nd	28.2 ± 3.9	nd	2.9 ± 0.5
	19	α-(E)-Bergamotene	19.77	18.5 ± 18.5	nd	3.5 ± 0.6*	1.4 ± 0.7
	20	g-Muurolene	20.82	nd	nd	0.4 ± 0.3**	3.5 ± 0.6
Norisoprenoid	21	6-Methyl-5-hepten-2-one	6.87	nd	nd	30.4 ± 5.4**	12.9 ± 1.3
	22	*β*-Cyclocitral	13.68	381.1 ± 122.9	nd	169.3 ± 16.3***	0.3 ± 0.3
	23	2,6,6-Trimethyl-1-cyclohexen	14.74	390.8 ± 46	nd	5.1 ± 0.7	nd
	24	Theaspirane A	16	nd	nd	1.8 ± 0.4	nd
	25	Theaspirane B	16.4	nd	nd	1.6 ± 0.4	nd
	26	α-Ionene	17.54	1258.1 ± 352.6*	274.0 ± 44.8	nd	nd
	27	*β*- Ionone dihydro	19.68	nd	nd	7.5 ± 2.6	nd
	28	Geranyl acetone	20.06	59.9 ± 21.4*	7.7 ± 4.9	25.5 ± 4.05	19.9 ± 8.5
	29	(*E*)-*β*- Ionone	20.88	723.7 ± 175.6**	35.4 ± 4.9	42.7 ± 5.8***	0.8 ± 0.1
	30	(*E*)-*β*- Ionone -5,6-epoxide	20.95	341.4 ± 80**	8.3 ± 0.7	38 ± 5.4***	0.6 ± 0.1
	31	Dihydroactinidiolide	22.08	3567.2 ± 505.4**	6.5 ± 6.5	116.6 ± 5.4***	0.6 ± 0.1
Nitrogen-containing	32	2-Methoxy-3-(2-methylpropyl) pyrazine	12.4	18.3 ± 8.6	11.3 ± 0.4	18.5 ± 1.8	14.7 ± 0.1
Unknown/others	33	3-(Methylthio) propanal	4.9	nd	nd	10.2 ± 1.9*	6 ± 0.4
	34	Ion (57, 132, 98)	6.36	nd	nd	3.1 ± 0.8**	20.4 ± 3.2
	35	2,2,6-Cyclohexanone, trimethyl	8.23	64.0 ± 8.0	nd	36.6 ± 4.2***	0.8 ± 0
	36	3,5,5-Trimethyl-2-cyclohexenone	8.9	19.1 ± 3.4	nd	17.9 ± 1.6	nd
	37	4-Terpineol	10.19	nd	nd	19.2 ± 3.6	nd
	38	Ion 135 (150, 121, 91,79)	10.26	51.9 ± 11.9	nd	29.8 ± 2.0	nd
	39	Ion 71 (95, 110, 128, 43)	10.44	558.5 ± 158.1**	14.0 ± 0.9	20.4 ± 3.5	nd
	40	Ion (98, 111, 82,69, 125, 55)	11.8	52.7 ± 14.8	nd	124.1 ± 13.6	nd
	41	3,5-Dimethyl, cyclohexanol	14.16	nd	nd	13.2 ± 2.6	nd
	42	Ion 166 (137, 123, 109)	16.1	nd	nd	3.8 ± 0.6	nd
	43	Ion 177	16.9	nd	nd	5.9 ± 0.7	nd
	44	2-Methyl- tetradecane	20.54	495.5 ± 217.6	nd	32.7 ± 6.6	nd
	45	Ion 69 (81, 93, 41)	23.3	295.1 ± 149.8	165.8 ± 51.3	13.0 ± 1.7*	6.6 ± 2.6
	46	Ion 83 (101, 111, 125, 208)	27.64	302.6 ± 145.9***	1286.4 ± 115.7	nd	nd
	47	Vomifoliol	28.28	330.9 ± 78.2***	2228.5 ± 181.0	nd	nd

[a] RT, retention time; nd, not detected

Asterisks indicate significant difference (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$) between 'Maor' and E-172-3 by Student's *t* test

compounds (hexanal, pentadecane, (*E*)-2 -hexenal and isopropyl tetradecanoate) were also prominent in E-172-3. Unexpectedly, sesquiterpene levels of α-copaene and other unidentified compounds were much higher in the wild-type 'Maor' than in E-172-3. Similarly, the aromatic amino acid-related methyl salicylate was higher in 'Maor' than in

the mutant. The nitrogen-containing compound 2-methoxy-3-(2-methylpropyl) pyrazine was similar in both lines.

Discussion

Orange fruit color in pepper can result from different carotenoid profiles in which the relative quantities of red and yellow pigments are variable (Huh et al. 2001; Lang et al. 2004; Guzman et al. 2010). To-date, orange pepper fruit color was shown to be controlled by the carotenoids biosynthetic genes *PSY* and *CCS* (Huh et al. 2001; Lang et al. 2004). In this paper we present a new monogenic recessive orange bell-type pepper mutant, in which the predominant carotenoid in the ripe fruit is β-carotene. The carotenoid profile, sequence analysis and co-segregation of the mutation with the color phenotype, led us to conclude that the orange fruit mutation is likely controlled by *CHY2*.

The reduced total carotenoid content in the orange fruit of E-172-3 is typical to yellow and orange-fruited peppers (Ha et al. 2007; Guzman et al. 2010). Similar relations between increasing β-carotene and decreasing total carotenoid contents have been observed in transgenic tomatoes expressing bacterial *PHYTOENE DESATURASE* (Romer et al. 2000), suggesting possible regulation of the carotenoid pathway by feedback inhibition. We cannot exclude, however, the possibility of differential carotenoid degradation, as has been shown in peach cultivars with different flesh colors (Brandi et al. 2011). The increase in expression of *PSY*, *CHY2*, *VDE* and *CCS* in E-172-3 compared with 'Maor' may be explained by feedback regulation of genes in the carotenoid biosynthesis pathway as proposed by Rodríguez-Concepción et al. (2001). In this latter study, treatment of tomato fruits with Fosmidomycin, an inhibitor of DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase) in the MEP (2-*C*-methyl-D-erythritol 4-phosphate) pathway, which supplies precursors to the carotenoid biosynthesis pathway, resulted in inhibition of carotenoid accumulation during ripening despite an increase in transcript expression of DXR and genes upstream and downstream of DXR. Presently, we do not provide supporting data for the hypothesis that feedback regulation may account for the elevated expression pattern of the carotenoid biosynthetic genes in E-172-3. However, there are substantial evidences to support such regulation in different steps of the pathway (reviewed by Cazzonelli and Pogson 2010).

The appearance of a small peak corresponding to capsanthin in E-172-3 (Fig. 2) indicates that the change in the amino acid in CHY2 did not completely abolish the enzyme's activity and some precursors are available for the activity of down-stream enzymes including CCS. Future enzyme activity and protein expression studies will allow determining the biochemical properties of the mutated enzyme. The inclusion of small amount of red pigment in the orange fruit of E-172-3 contributes to its strong orange color with red hue. The small amount of red pigments in E-172-3 despite high level of *CCS* expression is likely due to lack of sufficient level of precursors for this enzyme or other mechanisms of translational control, as was suggested for the orange-fruited pepper cultivar 'Canary', in which expression of wild-type *CCS* was detected but no protein or red pigments were observed (Rodriguez-Uribe et al. 2012).

Associations between variations in carotenoid and volatile profiles have been reported in tomato, melon and watermelon (Lewinsohn et al. 2005a, b; Ibdah et al. 2006), and thus the increase in norisoprenoid β-carotene-cleavage aroma compounds in E-172-3 fruit was expected. However, the mechanism underlying the changes in other volatile compounds is still unknown. Because volatiles contribute to fruit flavor, it remains to be determined whether the changes in volatile profile lead to a perceptible change in the flavor of E-172-3 fruit compared with 'Maor'.

The different colors of pepper fruits are due to different carotenoid profiles. Among the most important carotenoids for human nutrition present in pepper fruit are the pro-vitamin A compounds β-carotene and β-cryptoxanthin, as well as xanthophylls such as lutein, capsanthin and zeaxanthin which are considered powerful antioxidants (Rao and Rao 2007; Wahyuni et al. 2011). Typically, red fruit contains capsanthin and capsorubin as the main red-pigmented carotenoids and zeaxanthin and β-carotene as the main yellow/orange-pigmented ones; however, the level of β-carotene in red fruit normally does not exceed 10 % of total carotenoids. The accumulation of β-carotene as the predominant carotenoid in the E-172-3 mutant opens the way to breeding pepper enriched with this beneficial compound.

Because of the importance of β-carotene as a pro-vitamin A precursor in the human diet (Romer et al. 2000), attempts have been made to increase its content in various plant species, by means of metabolic engineering (reviewed by Giuliano et al. 2008) and by exploiting natural variation, e.g., Beta in tomato, Or in cauliflower and lycopene epsilon cyclase in maize (Ronen et al. 2000; Lu et al. 2006; Harjes et al. 2008). In pepper, an induced orange-fruited recessive mutant generated by X-ray irradiation was reported to contain a twofold increase in β-carotene content compared with its wild-type control; however, the molecular nature of the mutation was not described (Chalukova et al. 1993). The orange mutant E-172-3 described in the present study can be used as a source to elevate β-carotene content in pepper fruit and may improve its nutritional quality. However, the increase in the β-carotene fraction of the orange fruit is

compensated for by a decrease in total carotenoid content, thus diminishing its value as a rich source of carotenoids. To overcome this limitation to utilizing the E-172-3 mutation in pepper breeding, the mutation should be transferred into accessions with high total carotenoid content. Such accessions have been reported in several studies of variations in carotenoid content (Hornero-Mendez et al. 2000; Wall et al. 2001; Wahyuni et al. 2011).

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