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Metabolic engineering of xanthophyll content in tomato fruits

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Abstract Ripe tomato fruits accumulate significant amounts of the linear carotene lycopene, but only trace amounts of xanthophylls (oxygenated carotenoids). We overexpressed the lycopene β-cyclase (*b-Lcy*) and β-carotene hydroxylase (*b-Chy*) genes under the control of the fruit-specific *Pds* promoter. Transgene and protein expression was followed through semi-quantitative reverse transcription-PCR, Western blotting, and enzyme assays. Fruits of the transformants showed a significant increase of β-carotene, β-cryptoxanthin and zeaxanthin. The carotenoid composition of leaves remained unaltered. The transgenes and the phenotype are inherited in a dominant Mendelian fashion. This is the first example of successful metabolic engineering of xanthophyll content in tomato fruits. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: β-Carotene; Zeaxanthin; β-Cryptoxanthin

1. Introduction

Carotenoids are essential components of the photosynthetic membranes in all plants, algae and cyanobacteria [1,2], as well as of the chromoplasts found in many plant fruits and flowers. They play an important role in human nutrition. β-Carotene is the dietary precursor of vitamin A and its deficiency leads to xerophthalmia and child mortality [3]. Xanthophylls (oxygenated carotenoids) are dietary anti-oxidants (reviewed in [4]), and have important preventive effects against degenerative eye diseases [5].

Lycopene, the major carotene in tomato fruits, is synthesized from the first dedicated precursor, phytoene, through a series of desaturation reactions [1,2]. In leaf chloroplasts, lycopene is converted into xanthophylls by β- and ε-cyclases (b-Lcy and e-Lcy) and hydroxylases (b-Chy and e-Chy) (Fig. 1A). Green tomato fruits have a carotenoid content similar to leaves – essentially, β-carotene, lutein and violaxanthin. During fruit ripening, the genes mediating lycopene synthesis are up-regulated and those mediating its cyclization are down-regulated (for a review, see [6]), resulting in the accumulation of this compound in ripe fruits (approximately 90% of total carotenoids, the remainder being composed of β-carotene and traces of lutein). Tomato is one of the most productive crops in terms of carotenoids produced per unit cultivated area. Through metabolic engineering, the large pool of lycopene could be converted into high value-added downstream compounds. Xanthophylls are an important class of target compounds, because of their anti-oxidant properties, their chemical stability and the difficulty of their chemical synthesis.

Several genes that cause the accumulation of specific carotenes in the tomato fruit are known, such as *B* [7] which encodes a chromoplast-specific lycopene β-cyclase and causes β-carotene accumulation. No gene is known causing the accumulation of xanthophylls.

Recently, the metabolic engineering of high levels of lycopene and β-carotene in tomato fruits has been reported [8–10]. The work presented here extends the biochemical characterization of the β-carotene overproducers produced by our laboratory [9], and reports the production, in tomato fruits, of the novel compounds β-cryptoxanthin and zeaxanthin.

2. Materials and methods

Standard molecular biology protocols were followed as described [11]. The *Pds::b-Lcy::Nos* construct has been described previously [9]. The *Pds::b-Chy::Nos* construct was made by cloning the *Capsicum annuum* β-carotene hydroxylase cDNA [12] as a *Bam*HI–*Sac*I insert between the *Pds* promoter and the *Nos* terminator [13]. The two constructs were combined together (*Lcy-Chy* double construct) in the *Agrobacterium*-based vector, pBI101, and introduced into tomato plants according to a published protocol [14]. Plants were grown in the greenhouse under controlled temperatures (15–25°C). Fruits were observed everyday for change of color (breaker stage) and harvested 10 days later. Reverse transcription (RT)-PCR was performed as described [15].

Total RNA was retro-transcribed using oligo-d$(T)_{16}$ and then amplified for 30 cycles with gene-specific oligonucleotides: *At b-Lcy*: TCAGCAGAGAGTTGATCTC and ATGCTTCAGAAATGTATTACC; *Ca b-Chy*: GCTGCTTGCTTTGTGCTG and CCTAGGAACAAGCCATATG; *Ef-1a*: ATTGTGGTCATTGGYCAYGT and CCAATCTTGTAYACATCCTG; *Psy1*: GAGGCATAGGAATTTGGTG and CCAAAGCAGCATTATATACG; *Psy2*: GAACCATGCTAATGACTCC and ATCTCGTCCAGTATCTTGC; *Pds*: GTAGCTGCATGGAAAGATG and TCTCTTTCCAGTCTTCAGG; *Zds*: GGCTACTTCTTCAGCTTATC and AGAGCATATGCAACAGGATC; *Le e-Lcy*: AGGTGATTCATGAGGAATC and AAGAACCTTCTTGTAGCAG; *Le b-Chy1*: GGAGAGGTTTACTTATCTTG and CATTCTATGTTTATGAACAATC; *Le b-Chy2*: GAGGAGAAGAGTTTAGCTG and TCTCCATAGAAAGCTTGTTG; *B*: AGTCTTCAATTGTTTGTGATG and ACCTAGTCATGTTTGAGC, while adjusting the dilution to obtain a non-saturating signal. Each PCR was performed in triplicate and then quantified using a digital camera and the 'NIH Image' software.

The protocol of Lichtenthaler [16] was used for spectrophotometric determination of leaf chlorophyll and carotenoids. For high-performance liquid chromatography (HPLC) analysis, the pigments were extracted successively with acetone and then with chloroform until total discoloration of the tissues. Fruit pigments were analyzed using the HPLC procedure described previously [9]. Leaf pigments were

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analyzed using a Zorbax ODS column (250 mm×4.5 mm i.d.) eluted with 100% (v/v) solvent A (methanol/acetone/water/triethylamine, 90/17/11/0.1, v/v) for 10 min followed by a 5 min linear gradient to 100% solvent B (methanol/acetone, 60/40, v/v). β-Carotene isomers were separated by HPLC using a Vydac C18 column as described previously [17].

SDS–PAGE and Western blot analysis were performed as described [18] using total fruit proteins extracted with hot phenol [19]. In each case, 50 μg of proteins was used. The anti-cyclase and -hydroxylase antibodies were described previously [12,20] and were used at 1/2500 dilution.

Enzyme activities were assayed using acetone powder prepared from tomato chromoplast membranes [21]. For lycopene β-cyclase, the incubation medium (1 ml final volume) contained 15,15′-[^{3}H]lycopene (4.8×10^5 dpm; 25 mCi/mmol), enzyme extract (equivalent to 2 mg proteins), 0.25 M sorbitol, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM dithiothreitol, 2 mM FAD, 1 mM NADP, 5 μg Tween 80, buffered with 50 mM Tris-maleate buffer (pH 6.8). For β-carotene hydroxylase, the incubation medium (250 μl final volume) contained [^{14}C]β-carotene (2×10^5 dpm; 10 mCi/mmol), 100 μg Tween 80, enzyme extract (equivalent to 2 mg proteins), 25 μg of spinach ferredoxin, 0.5 U of spinach ferredoxin-NADP$^+$ oxidoreductase, 0.25 mM NADPH, 50 μM mixture of monogalactosyldiglyceride and digalactosyldiglyceride (1:1), 1 U catalase, 200 μg of bovine serum albumin, buffered with 50 mM potassium phosphate (pH 7.6). The incubations were performed at 26°C for 2 h and the reaction products were extracted with chloroform/methanol (2:1, v/v) and after addition of carrier amounts of β-carotene, β-cryptoxanthin and zeaxanthin the pigment extract was subjected to HPLC analysis [22]. Incorporated radioactivity was monitored by liquid scintillation counting.

3. Results

3.1. Transformation with the Lcy and Lcy+Chy transgenes

In order to achieve fruit-specific expression, we cloned the *Arabidopsis b-Lcy* [23] and the pepper *b-Chy* [12] genes between the tomato fruit-specific *Pds* promoter [13] and the *Nos* terminator. Two constructs were used: *Pds::Lcy::Nos* [9] (referred to heretofore as *Lcy*) and *Pds::Lcy::Nos-Pds::Chy::Nos* (referred to heretofore as *Lcy+Chy*). The two constructs were introduced into tomato (cv. Moneymaker (*MM*)) by *Agrobacterium*-mediated transformation, and transformed plants were checked through PCR (data not shown) and grown to maturity in the greenhouse. The growth habit and appearance of the plants were comparable to the untransformed *MM* plants.

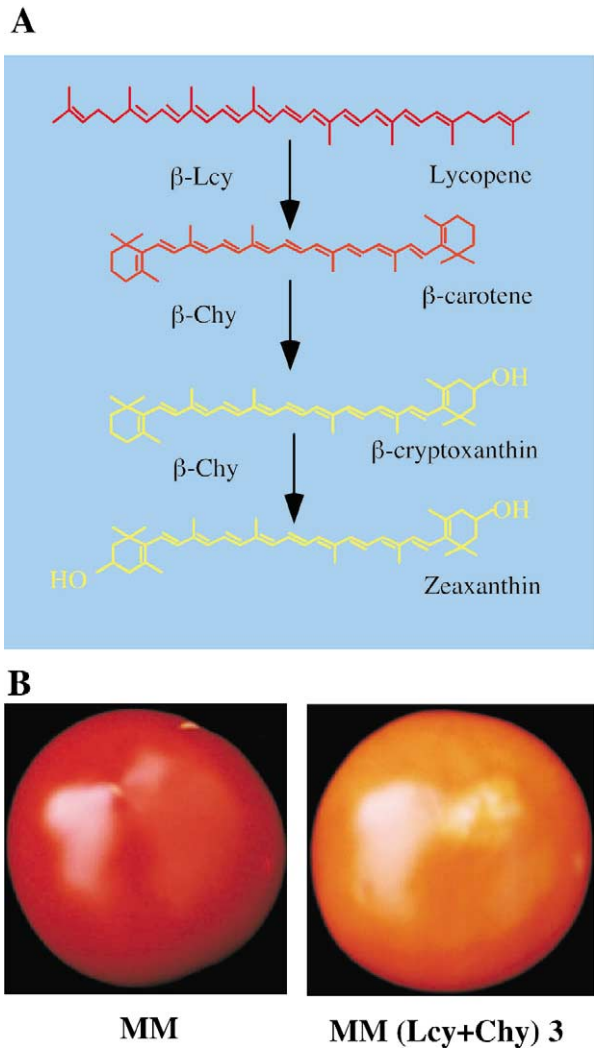

Fig. 1. A: Biosynthesis of zeaxanthin from lycopene. b- and ε-Lcy = lycopene β- and ε-cyclases; b- and e-Chy = β- and ε-hydroxylases. The existence of a separate ε-hydroxylase is hypothetical. B: Phenotypes of fruits (10 days after breaker stage) from Moneymaker and the *MM(Lcy+Chy)3* transformant.

3.2. Carotenoid analysis and transgene expression

The color of the ripe fruits varied from the complete red of wild-type Moneymaker to red–orange to complete orange for several *MM(Lcy)* and *MM(Lcy+Chy)* transformants (Fig. 1B), indicating significant changes in the carotenoid composition. Fruits from three lines for each construct, showing the most distinct color changes, were harvested 10 days after breaker stage and the carotenoid content was determined through HPLC analysis (Table 1). The transformants show up to 12-fold increase in β-carotene content over the parental Moneymaker line. *MM(Lcy+Chy)1* produces as much as 63 μg/g fresh weight β-carotene as compared to 5 μg/g for the *MM* line. There is an increase in the total carotenoid content of the fruits (except in *MM(Lcy)6*) similar to what reported previously [9].

Table 1
Fruit pigment composition (μg/g fresh weight)

Line	Lyco	Beta	Crypto	Zea	Lut	Viola+Neo
MM	58 ± 6	5 ± 3	ND[a]	ND[a]	1.9 ± 0.2	1.4 ± 0.1
MM(Lcy)3	49 ± 25	57 ± 23	ND[a]	ND[a]	1.7 ± 0.9	1.0 ± 0.6
MM(Lcy)5	58 ± 23	50 ± 8	ND[a]	ND[a]	2.0 ± 1.0	2.0 ± 1.5
MM(Lcy)6	5 ± 2	28 ± 9	ND[a]	ND[a]	1.0 ± 0.3	0.6 ± 0.3
MM(Lcy+Chy)1	11 ± 0.8	63 ± 8	11 ± 1	13 ± 4	1.8 ± 0.3	0.9 ± 0.1
MM(Lcy+Chy)2	36 ± 8	46 ± 3	5 ± 0.5	9 ± 2	1.4 ± 0.1	1.0 ± 0.1
MM(Lcy+Chy)3	28 ± 6	48 ± 6	5 ± 0.6	8 ± 2	1.5 ± 0.1	1.0 ± 0.1

[a]Not detectable.

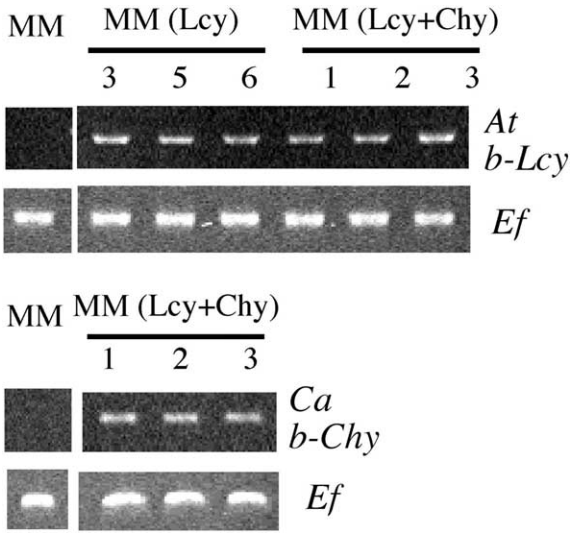

Fig. 2. RT-PCR analysis of the expression of *Arabidopsis b-Lcy* and *Capsicum b-Chy* in transformed fruits (10 days after breaker stage). The *Ef-1a* gene is shown as an internal standard.

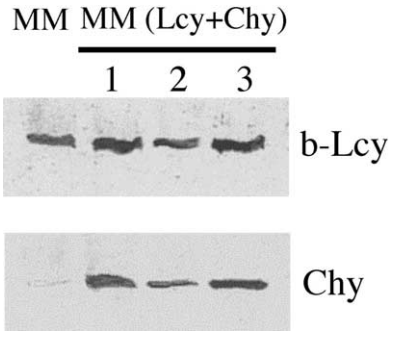

Fig. 3. Western analysis of b-Lcy and b-Chy proteins in transformed fruits (10 days after breaker stage).

Besides β-carotene, the *MM(Lcy+Chy)* transformants also accumulate significant amounts of β-cryptoxanthin and zeaxanthin (up to 24 μg/g fresh weight in transformant 1), which are not detectable in the *MM* parental line or in the *MM(Lcy)* transformants (Table 1). It is difficult to estimate the increase in the amount of these two xanthophylls, since in untransformed fruits they are below the detection power of our analysis, but we estimate that it is more than 100-fold in all three (*Lcy+Chy*) transformants. The increase in total xanthophylls is as much as 10-fold in *MM(Lcy+Chy)1*.

The expression of the transgenes, *Arabidopsis b-Lcy* and *Capsicum b-Chy*, was detected by RT-PCR (Fig. 2) and compared to that of the housekeeping gene, *EF-1a* [24].

The relative content of β-carotene isomers was analyzed by HPLC (Table 2) in fruits from *MM(Lcy)* transformants, and compared to that found in the *B* genotype, a 'natural' accumulator of β-carotene [7]. The major isomer in all the lines was all-*trans* β-carotene, with transformant no. 5 also accumulating significant levels of the 9-*cis* and 13-*cis* isomers.

The carotenoid and chlorophyll composition in leaves was analyzed spectrophotometrically and by HPLC. Table 3 shows that the composition is essentially unchanged in all the transformants.

3.3. Enzyme expression and activity, expression of endogenous genes, and inheritance

To show that the change in carotenoid composition was due to the expression of the transgene-encoded enzymes, we performed Western blotting on fruit protein extracts (10 days after breaker stage), using affinity-purified antibodies to the *Capsicum* b-Lcy and b-Chy proteins (Fig. 3). The anti-b-Lcy antibody detects an approximately 50 kDa protein in all transgenic and non-transgenic lines. We believe this is the *B* gene product, whose mRNA is expressed in ripening fruits, and that has significant sequence homology to β-cyclase [7]. Due to the presence of this endogenous band, it is difficult to determine the levels of expression of the *Arabidopsis* b-Lcy protein (which has a similar molecular weight). The anti-b-Chy antibody detects a protein with an apparent molecular weight of 20 kDa (corresponding to that of b-Chy) in all the *MM(Lcy+Chy)* transformants, but not in the *MM* parental line or the *MM(Lcy)* transformants.

We assayed the β-cyclase and β-hydroxylase activities in fruit extracts, using radiolabelled lycopene and β-carotene as substrates. The results (Table 4) show high levels of lycopene β-cyclase activity in all the transformants overexpressing b-Lcy. The β-hydroxylase activity is high in the *MM(Lcy+Chy)* transformants, but not in the *MM* parental line or the *MM(Lcy)* transformants. Taken together, these data confirm that the introduced b-Lcy and b-Chy enzymes are expressed and active in the transgenic fruits.

The expression of the endogenous genes for the whole carotenoid pathway from GGPP to zeaxanthin was measured by semi-quantitative RT-PCR on fruit RNA. Apart from minor differences, the expression of all the carotenogenic genes was similar in all the lines (Fig. 4), with the exception of the *B* gene, which was overexpressed in *B* fruits [7].

The carotenoid content of fruits from T1 plants was analyzed by HPLC. Approximately 3/4 of the plants had elevated levels of fruit β-carotene (*MM(Lcy)* transformants) or of β-carotene/β-cryptoxanthin/zeaxanthin (*MM(Lcy+Chy)* transformants). The overproducing plants contained the transgene(s), while the segregating azygous plants had a normal fruit carotenoid composition (data not shown). Therefore, the xanthophyll overproduction trait is inherited as a single Mendelian dominant character. A more detailed analysis will be presented elsewhere.

Table 2
Isomeric composition of fruit β-carotene (%)

Line	*trans*-β-Carotene	13-*cis*-β-Carotene	9-*cis*-β-Carotene
B	100	ND[a]	ND[a]
MM(Lcy)3	97	ND[a]	ND[a]
MM(Lcy)5	46	29	25
MM(Lcy)6	99	ND[a]	ND[a]

[a]Not detectable.

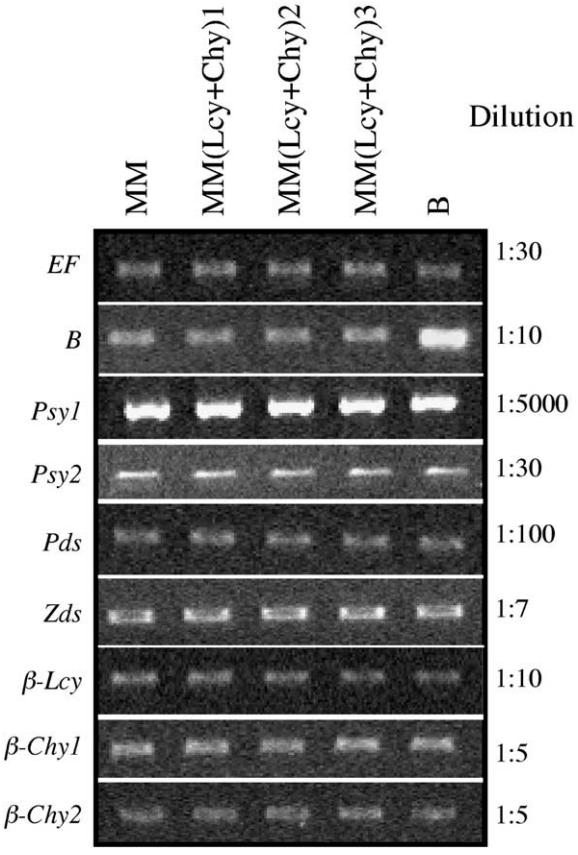

Fig. 4. RT-PCR analysis of the expression of endogenous carotenoid genes in fruits from the *MM(Lcy+Chy)* transformants. Different cDNA dilutions (shown on the right) were amplified for 30 cycles (see Section 2) and the amplification products were separated on an agarose gel. The *Ef-1a* gene is shown as an internal standard.

4. Discussion

Overproduction of β-carotene (provitamin A), or of the xanthophylls zeaxanthin or astaxanthin has been one of the goals of metabolic engineering [25]. Astaxanthin overproduction has been obtained in the nectary tissue of tobacco [26]. However, the challenge for metabolic engineering of xanthophylls is their overproduction in high-yielding organs like the tomato fruit. Fruit chromoplasts of some species, like bell pepper, accumulate xanthophylls. However, since their biochemistry and ultrastructure are different from that of tomato fruit chromoplasts [27] (which normally accumulate lycopene), it is difficult to predict if successful xanthophyll accumulation can be achieved in the latter. In fact, we and others have failed to produce xanthophylls through the overexpression of xanthophyll-synthesizing genes in fruits of the *B* genotype [7], which contain a large pool of β-carotene ([28]; P. Bramley, personal communication; J. Hirschberg, personal communication).

Table 4
b-Lcy and and b-Chy enzyme activities in fruits

	Activities (dpm/mg protein)	
	b-Lcy	b-Chy
MM	3 650	Trace
MM(Lcy+Chy)1	35 000	4 632
MM(Lcy+Chy)2	22 700	2 839
MM(Lcy+Chy)3	43 400	7 032

We have shown previously that overexpression of *b-Lcy*, in 'antisense' or 'sense' orientation, under the control of the fruit-specific *Pds* promoter [13] causes the overproduction of lycopene or β-carotene in ripe tomato fruits [9]. In this paper, we prove that the β-carotene pool in the 'sense' overexpressors can be converted into xanthophylls through the overexpression of *b-Chy* from pepper. Leaf carotenoid levels remain, again, unaltered.

The *b-Lcy* transformants predominantly accumulate all-*trans* β-carotene, similar to the *B* line [7], indicating that the cyclization pathways mediated by the 'chloroplast-specific' *Arabidopsis* β-cyclase and the 'chromoplast-specific' cyclase encoded by *B* are mechanistically similar.

High levels of a protein immunologically related to b-Lcy, with an apparent molecular weight of approximately 50 kDa (indistinguishable from b-Lcy), are present in all the transformed and untransformed lines. It probably represents the chromoplast-specific B cyclase [7], because its amount is increased in *B* fruits (data not shown). The *b-Lcy* transformed lines show a dramatic increase in β-carotene and cyclase activity (Tables 1 and 4), compared to only a minor increase in immunodetectable protein (Fig. 3). This suggests that the endogenous, cross-reactive protein has a poor cyclase activity, compared to the transgenically introduced b-Lcy.

High levels of hydroxylated β-carotene derivatives (Table 1), of immunodetectable β-Chy protein (Fig. 3) and of β-hydroxylase activity (Table 4) are found only when b-Chy is expressed in conjunction with b-Lcy. The transformed lines showed unaltered levels of expression of the endogenous carotenoid biosynthesis genes, suggesting that xanthophyll production is the result of the expression of the introduced transgenes rather than of a de-regulation of endogenous genes.

Tomato is a important crop in most areas of the globe. This work proves the feasibility of synthesizing anti-oxidant xanthophylls in tomato fruits through the genetic engineering of appropriate genes. A similar approach may be suitable for transforming the tomato fruit into a 'cell factory' for carot-

Table 3
Leaf pigment composition (μg/g fresh weight)

Line	Total chlorophyll	Total carotenoids	Beta (%)	Lut+Zea (%)	Viola (%)	Neo (%)
MM	116 ± 10	13 ± 1	27.25	42.44	16.76	13.55
MM(Lcy)3	132 ± 6	16 ± 1	30.45	44.44	15.81	9.3
MM(Lcy)5	132 ± 7	19 ± 3	29.37	49.63	14.92	6.08
MM(Lcy)6	118 ± 5	18 ± 2	30.28	46.27	15.4	8.05
MM(Lcy+Chy)1	116 ± 11	13 ± 3	29.6	45.44	14.16	10.8
MM(Lcy+Chy)2	111 ± 17	14 ± 3	31.65	44.15	13.05	11.15
MM(Lcy+Chy)3	110 ± 4	17 ± 1	31.7	43.03	13.87	11.4

enoid metabolites, including water-soluble oxidation products, such as crocetin, or volatile molecules involved in flavor.

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References

[1] Bartley, G.E., Scolnik, P.A. and Giuliano, G. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 287–301.
[2] Cunningham, F.X. and Gantt, E. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 557–583.
[3] McLaren, D.S. (1999) J. Indian Med. Assoc. 97, 320–323.
[4] Sies, H., Stahl, W. and Sundquist, A.R. (1992) Ann. N.Y. Acad. Sci. 669, 7–20.
[5] Moeller, S.M., Jacques, P.F. and Blumberg, J.B. (2000) J. Am. Coll. Nutr. 19, 522S–527S.
[6] Hirschberg, J. (2001) Curr. Opin. Plant Biol. 4, 210–218.
[7] Ronen, G., Carmel-Goren, L., Zamir, D. and Hirschberg, J. (2000) Proc. Natl. Acad. Sci. USA 97, 11102–11107.
[8] Romer, S., Fraser, P.D., Kiano, J.W., Shipton, C.A., Misawa, N., Schuch, W. and Bramley, P.M. (2000) Nat. Biotechnol. 18, 666–669.
[9] Rosati, C. et al. (2000) Plant J. 24, 413–420.
[10] Fraser, P.D. et al. (2002) Proc. Natl. Acad. Sci. USA 99, 1092–1097.
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[12] Bouvier, F., Keller, Y., d'Harlingue, A. and Camara, B. (1998) Biochim. Biophys. Acta 1391, 320–328.
[13] Corona, V., Aracri, B., Kosturkova, G., Bartley, G.E., Pitto, L., Giorgetti, L., Scolnik, P.A. and Giuliano, G. (1996) Plant J. 9, 505–512.
[14] van Roekel, J.S.C., Damm, B., Melchers, L.S. and Hoekema, A. (1993) Plant Cell Rep. 12, 644–647.
[15] Giuliano, G., Bartley, G.E. and Scolnik, P.A. (1993) Plant Cell 5, 379–387.
[16] Lichtenthaler, H. (1987) Methods Enzymol. 148, 350–382.
[17] Quackenbush, F.W. (1987) J. Liq. Chromatogr. 10, 643–653.
[18] Suire, C., Bouvier, F., Backhaus, R.A., Begu, D., Bonneu, M. and Camara, B. (2000) Plant Physiol. 124, 971–978.
[19] Van Etten, J.L., Freer, S.N. and McCune, B.K. (1979) J. Bacteriol. 138, 650–652.
[20] Bouvier, F., d'Harlingue, A. and Camara, B. (1997) Arch. Biochem. Biophys. 346, 53–64.
[21] Camara, B. and Dogbo, O. (1986) Plant Physiol. 80, 172–174.
[22] Camara, B. (1985) Methods Enzymol. 110, 244–253.
[23] Scolnik, P.A. and Bartley, G.E. (1995) Plant Physiol. 108, 1343.
[24] Mahe, A., Grisvard, J. and Dron, M. (1992) Mol. Plant–Microbe Interact. 5, 242–248.
[25] Giuliano, G., Aquilani, R. and Dharmapuri, S. (2000) Trends Plant Sci. 5, 406–409.
[26] Mann, V., Harker, M., Pecker, I. and Hirschberg, J. (2000) Nat. Biotechnol. 18, 888–992.
[27] Camara, B., Hugueney, P., Bouvier, F., Kuntz, M. and Moneger, R. (1995) Int. Rev. Cytol. 163, 175–247.
[28] Giuliano, G., Dharmapuri, S. and Camara, B. (2001) Italian patent no. RM2001A000670, filed 9/11/01.