Comprehending crystalline β-carotene accumulation by comparing engineered cell models and natural carotenoid-rich system of citrus

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Supplementary Material

Supplementary data

Table S1. Primers used in this study

Overexpression construct	
CrtB	Forward: 5’-GTAAAGTGCATGGCTGTTGGCTCGAA-3’
	Reverse: 5’-CGCGGATCCCTAAATCGGGCGCTGCCAGAG-3’
tp-rbcS	Forward: 5’-CCGCTCGAGATGGCTTCTATGATATCCTC-3’
	Reverse: 5’-AACAGCCATGCACTTTACTCTTCCAC-3’
tp-rbcS-CrtB	Forward: 5’-CCGCTCGAGATGGCTTCTATGATATCCTC-3’
	Reverse: 5’-CGCGGATCCCTAAATCGGGCGCTGCCAGAG-3’
Probes used in Southern blot analysis	
CrtB-S	Forward: 5’-ATAAACCTGCTTCGCTGTGGC-3’
	Reverse: 5’-ATTCCCAGTGAAGAAGGTCAACAC-3’
Generation of anti-CrtB antibodies	
CrtB-A	Forward: 5’-ATCGAATTCGTGGGTCGTTGTTATCTGC-3’
	Reverse: 5’-ATCCTCGAGTTACGGATGAGGCCGCATC-3’

Table S2. Specific primers used in real-time reverse transcriptase-PCR

Gene detected	Sequence	Accession NO.
DXS	Forward: 5'-CTCTTCCTTCGCCGTTTCC-3'	EY664196
	Reverse: 5'-CAGACCAGCGGCAAAAGTAAC-3'	
DXR	Forward: 5'-CGATTCTGCTACCCTTTTCAAC-3'	EY705355
	Reverse: 5'-ATTGAGTGTATGATAGACTGTGGATG-3'	
HDS	Forward: 5'-GGTCGGATGAAATCTGCTAT-3'	DY268645
	Reverse: 5'-TTCTGGTGGTCCGTAAGTG-3'	
HDR	Forward: 5'-TCTCTTGAAGGTGTGAGGTATTGC-3'	CB293311
	Reverse: 5'-GTTACACAAGAGCGACAAGATGC-3'	
IPI	Forward: 5'-GGTGAGGAATGCTGCACAAAG-3'	EY702410
	Reverse: 5'-AACTCATCAACTGGCACATCTTC-3'	
GGPPS	Forward: 5'-TAGAGTTCCCTCAGTTACGCACAG-3'	AJ243739
	Reverse: 5'-GCCAGTTCTCTTGTCTTTTGTATCC-3'	
PSY	Forward: 5'-CCCGGACTGCTGTGTTTAAT-3'	DQ235260
	Reverse: 5'-GAGCAAGGATGCCTCAAATC-3'	
PDS	Forward: 5'-ATAATTGGCGGACAGGCATA-3'	AJ319760
	Reverse: 5'-CCTCTGTCGTCACTCGATCA-3'	
ZDS	Forward: 5'-ATCAGTGCTCGTTGTATGCTTACTATATT-3'	AJ319762
	Reverse: 5'-CCCTTGAGCATCCGCAAT-3'	
CRTISO	Forward: 5'-TTCTTTCCATTCACATGGGTGTT-3'	AY655751
	Reverse: 5'-TCATCCTCAAGCACAAAATGGT-3'	
LCYE	Forward: 5'-CAACTGGATATTGAGGGCATCA-3'	AY533827
	Reverse: 5'-CAAGGAAACCGTGCCACATC-3'	
LCYB1	Forward: 5'-GGCTATATGGTGGCAAGGACTT-3'	AY679168
	Reverse: 5'-CAGAATTGAGGCTTCGAACGA-3'	
LCYB2	Forward: 5'-TGGCTCAACCAGGATGATCA-3'	FJ516403
	Reverse: 5'-TTGGCCACAACCCATTCC-3'	
HYD	Forward: 5'-TTTGGGATGGCCTACATGTTC-3'	AB114661
	Reverse: 5'-GGCACGTCGGCAATGG-3'	
ZEP	Forward: 5'-GAAGCAATTCTTCGACGTGACA-3'	AB114662
	Reverse: 5'-ACCGAGTCCCCAAGCAAAGT-3'	
NCED2	Forward: 5'-GCCCAACAAATCCTTGAAAGTAGA-3'	AB219171
	Reverse: 5'-GGGTAAGGCTGTTTAGGGAAATG-3'	
NCED3	Forward: 5'-TCTCTTCAAACACCTTCCATTCC-3'	AB219177
	Reverse: 5'-GCTGCAGGTGATGGAGGGTAT-3'	
CCD1	Forward: 5'-GGAGGATGAAGTGGTTCTGATCA-3'	AB219165
	Reverse: 5'-ACAGCCCCATTGACCATGTC-3'	
CCD4a	Forward: 5'-AACCGAACGTGCCCATTT-3'	DQ309330
	Reverse: 5'-TGTCTACGAACACCGCATACTT-3'	
CCD4b	Forward: 5'-AAGTGATGCCGAGATGAAGTGGT-3'	DQ309331
	Reverse: 5'-GATGTAGCATTTGTCGCACTAAGAA-3'	
Actin	Forward: 5'-CCAAGCAGCATGAAGATCAA-3'	GU911361
	Reverse: 5'-ATCTGCTGGAAGGTGCTGAG-3'	

Table S3. Analysis of carotenoid profiles detected in the wild-type and ECM lines

Peak No.	R_t (min)	λ_{max} (nm)	Peak ratio	Carotenoid type	Wild type				35S::*CrtB*			
1	5.35	416.5, 440.6, 469.7	91	Violaxanthin	a	b	c	d	A	B	C	D
2	6.28	412.9, 437.0, 466.1	96	9-*cis*-violaxanthin	a		c	d	A		C	D
3	6.84	399.6, 422.5, 449.1	99	Luteoxanthin	a	b	c	d	A	B	C	D
4	7.99	412.9, 438.2, 464.9	91	9-*cis*-violaxanthin	a	b	c	d	A	B	C	D
5	8.42	421.3, 449.1, 478.2	98	Antheraxanthin	a	b	c	d	A	B	C	D
6	9.66	394.8, 420.1, 445.5	119	Unknown	a			d	A	B		D
7	10.90	424.3, 446.7, 474.5	63	Lutein	a	b	c	d	A	B	C	D
8	14.50	424.3, 450.3, 479.4	35	Zeaxanthin	a	b		d	A	B	C	D
9	15.73	417.7, 443.1, 470.9	100	Unknown						B		
10	16.80	423.0, 445.5, 474.5	79	Unknown						B	C	
11	18.36	427.0, 447.9, 474.5	14	Unknown					A	B	C	
12	18.90	287.5	NA	All-trans-phytoene	a	b	c	d	A	B	C	D
13	19.20	332.7, 348.3, 365.9	71	Phytofluene a	a	b	c	d	A	B	C	D
14	19.50	426.5, 447.9, 474.5	58	α-cryptoxanthin					A	B	C	D
15	20.83	416.8, 443.1, 466.1	55	Unknown					A	B		
16	21.27	443.1, 468.5	50	Unknown					A	B	C	D
17	21.86	332.7, 349.5, 365.9	92	Phytofluene b	a	b	c	d	A	B	C	D
18	22.52	450.3, 475.8	31	β-cryptoxanthin					A	B	C	D
19	24.70	339.9, 446.7, 472.1	NA	Unknown	a		c	d	A	B	C	D
20	26.30	424.4, 447.9, 475.8	55	α-carotene	a	b	c	d	A	B	C	D
21	28.51	380.4, 402.0, 426.1	121	All-*trans*-ε-carotene					A	B	C	D
22	30.04	428.0, 455.2, 480.6	23	β-carotene	a	b	c	d	A	B	C	D
23	31.26	426.1, 447.9, 473.3	33	*cis*-β-carotene	a	b	c	d	A	B	C	D
24	32.93	438.0, 463.6, 489.1	18	δ-carotene						B	C	D
25	36.75	433.5, 457.6, 489.1	73	δ-carotene	a		c		A	B	C	
26	37.20	437.8, 460.0, 489.1	61	Unknown					A	B		
27	39.35	440.6, 467.3, 497.6	43	γ-carotene					A	B	C	
28	41.09	440.1, 463.6, 494.0	42	*cis*-lycopene	a	b	c		A	B	C	
29	41.67	438.6, 463.6, 494.0	64	*cis*-lycopene					A	B	C	
30	53.14	446.7, 473.3, 504.9	76	Lycopene					A	B		

The peak numbers are the same as those in Figure 3. R_t = retention time. Peak ratio is the percentage Ⅲ/Ⅱ for carotenoids. a/A, Marsh grapefruit (M); b/B, Star Ruby grapefruit (RB); c/C, Cara Cara sweet orange (HQC); d/D, Sunburst mandarin (SBT), they represent the occurrence of each carotenoid type in four varieties. The carotenoids were identified by their characteristic absorption spectra and typical retention time based on the literatures (Fraser *et al.,* 2007; Lee, 2001; Liu *et al*., 2007; Xu *et al*., 2006) and standards of the CaroNature Co. (Bern, Switzerland).

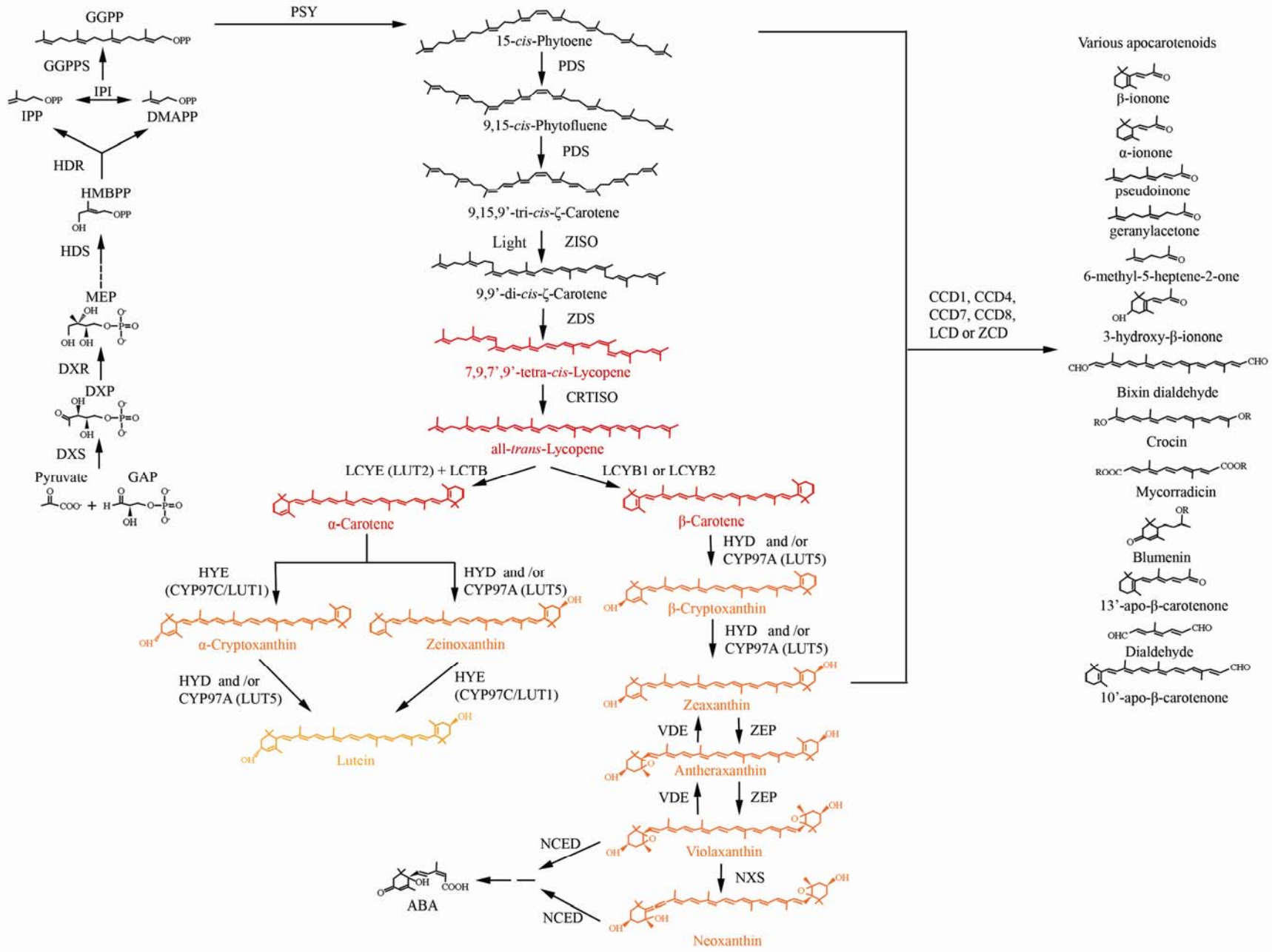
GGPP
GGPPS
IPI
IPP
DMAPP
HDR
HMBPP
HDS
MEP
DXR
DXP
DXS
Pyruvate
GAP
PSY
15-cis-Phytoene
PDS
9,15-cis-Phytofluene
PDS
9,15,9'-tri-cis-ζ-Carotene
Light
ZISO
9,9'-di-cis-ζ-Carotene
ZDS
7,9,7',9'-tetra-cis-Lycopene
CRTISO
all-trans-Lycopene
LCYE (LUT2) + LCTB
LCYB1 or LCYB2
α-Carotene
β-Carotene
HYE (CYP97C/LUT1)
HYD and /or CYP97A (LUT5)
α-Cryptoxanthin
Zeinoxanthin
β-Cryptoxanthin
HYD and /or CYP97A (LUT5)
HYD and /or CYP97A (LUT5)
HYE (CYP97C/LUT1)
Lutein
Zeaxanthin
VDE
ZEP
Antheraxanthin
VDE
ZEP
Violaxanthin
NCED
NXS
ABA
NCED
Neoxanthin
CCD1, CCD4, CCD7, CCD8, LCD or ZCD
Various apocarotenoids
β-ionone
α-ionone
pseudoinone
geranylacetone
6-methyl-5-heptene-2-one
3-hydroxy-β-ionone
Bixin dialdehyde
Crocin
Mycorradicin
Blumenin
13'-apo-β-carotenone
Dialdehyde
10'-apo-β-carotenone

Figure S1. Carotenoid biosynthesis pathway in higher plants.

GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-derythritol 4-phosphate; HMBPP, 4-hydroxy-2-methylbut-2- enyl 1-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; HDR, hydroxymethylbutenyl 4-diphosphate reductase; IPI, isopentenyl diphosphate isomerase; GGPPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; CRTISO, carotene isomerase; ZDS, ζ-carotene desaturase; LCYB1, lycopene β-cyclase 1; LCYB2, lycopene β-cyclase 2; LCYE (LUT2), lycopene ε-cyclase; HYD, β-carotene hydroxylase (non-heme di-iron group); CPY97A (LUT5), β-carotene hydroxylase (cytochrome P450 monoxygenase group); HYE (CPY97C/LUT1), carotene ε-ring hydroxylase (cytochrome P450 monoxygenase group); ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; CCD1, -4, -7 and -8, carotenoid cleavage dioxygenase 1, 4, 7, and 8; ZCD, zeaxanthin cleavage dioxygenase; LCD, lycopene cleavage dioxygenase; NCED, 9-cis-epoxycarotenoid dioxygenase; NXS, neoxanthin synthsase. This carotenoid biosynthesis pathway in higher plants was made based on the literatures (Cazzonelli and Pogson, 2010; Chen *et al*., 2010; Farré *et al*., 2011; Vallabhaneni *et al*., 2010; Yu *et al*., 2011; Zhu *et al*., 2010).

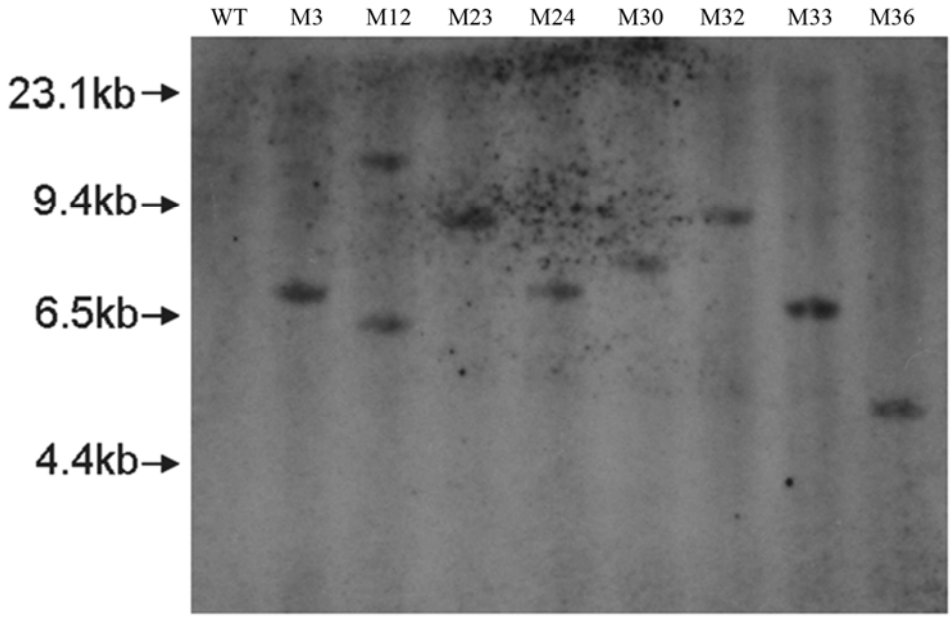

Figure S2. A DNA gel blot analysis indicating inserted patterns of T-DNA in the representative lines. Extensive single-copy insertion confirms the occurrence of credible independent transformation events in each tested line.

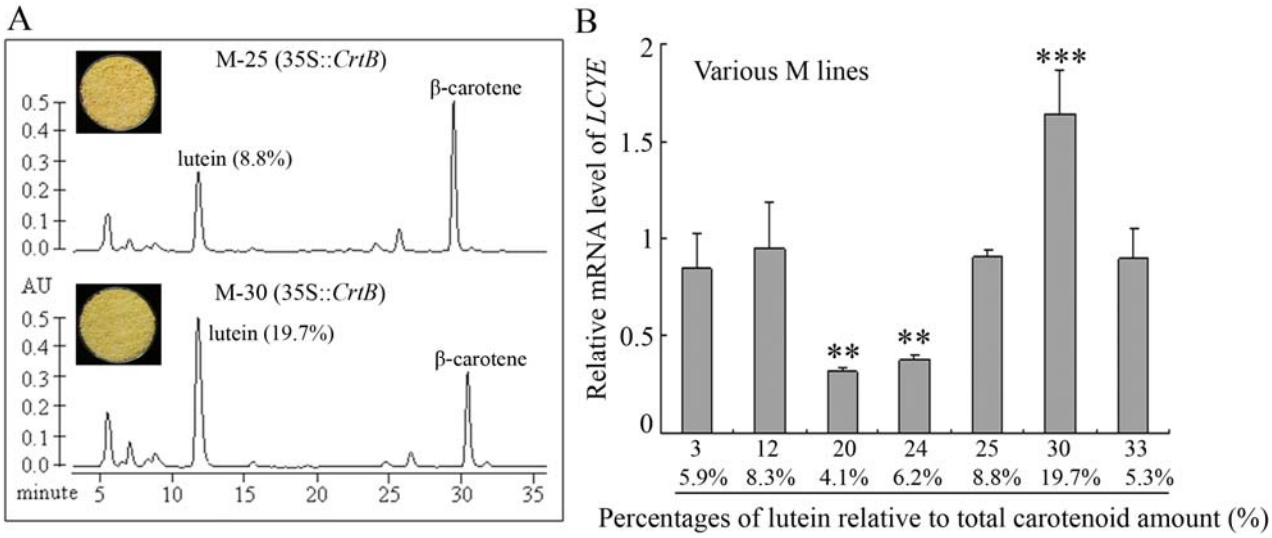

Figure S3. A novel 35S::*CrtB* ECM (M-30) containing a high proportion of lutein due to the highest expression of *LCYE* gene. The transcript level is expressed relative to wild type. Repeated experiments were performed. Values are means ± SD from three technical replicates of a representative experiment. ** and *** indicate the values are significantly different at significance level of $P < 0.01$ and $P < 0.001$, respectively.

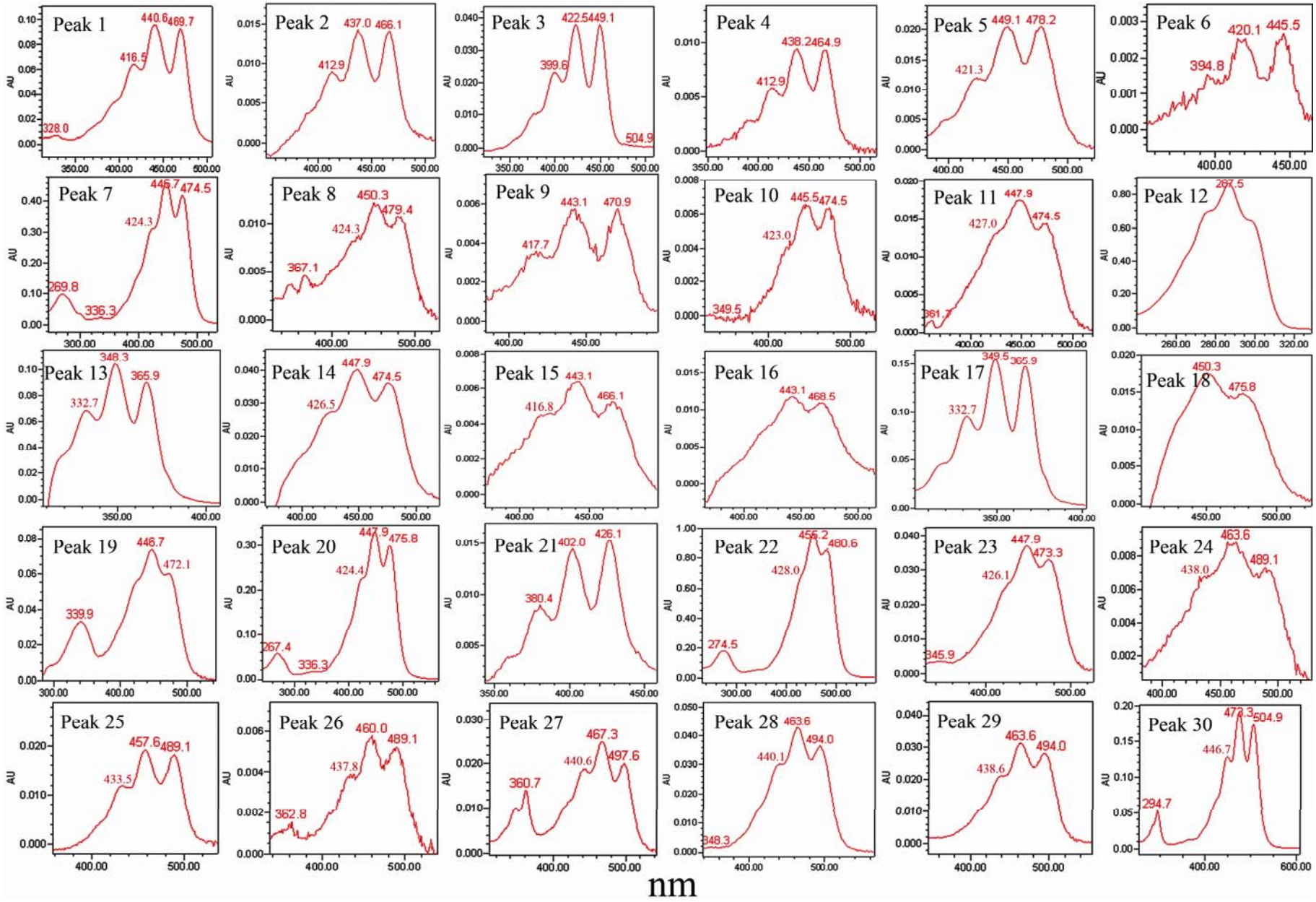

Figure S4. Absorbance spectra of the peaks detected in the ECMs (peak identification in Table S3).

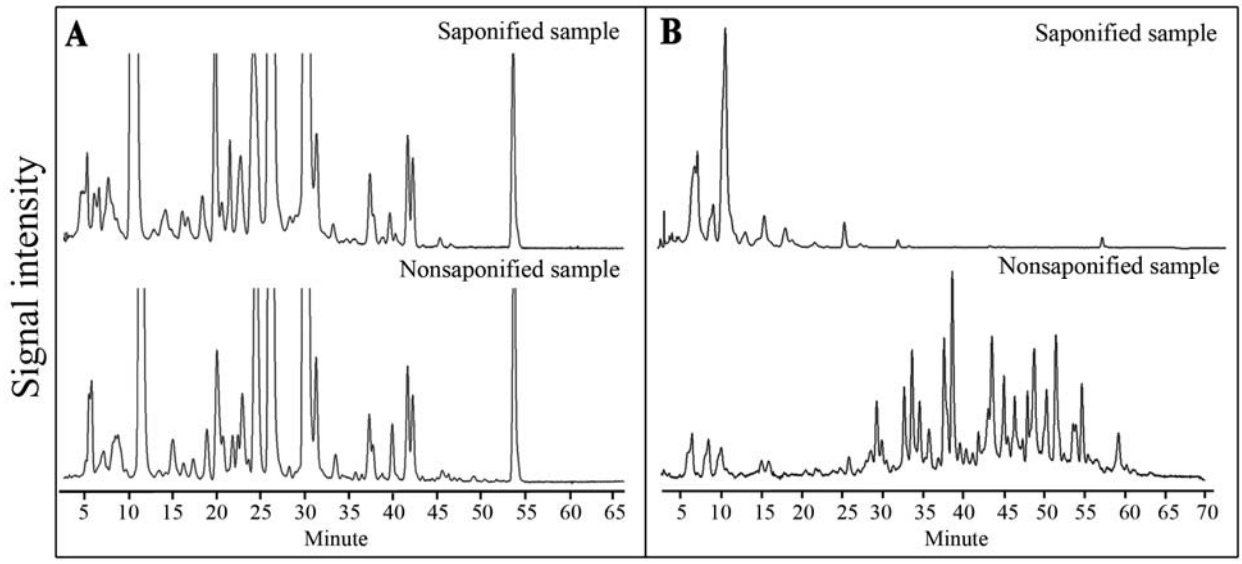

Figure S5. HPLC chromatogram of carotenoids extracted from the RB ECM (A) and HQC flavedo (B) at 450 nm. Comparative analysis of saponified and non-saponified samples shows that free-type carotenoids are the major components of the ECMs, while the esterified forms are predominant in the flavedo.

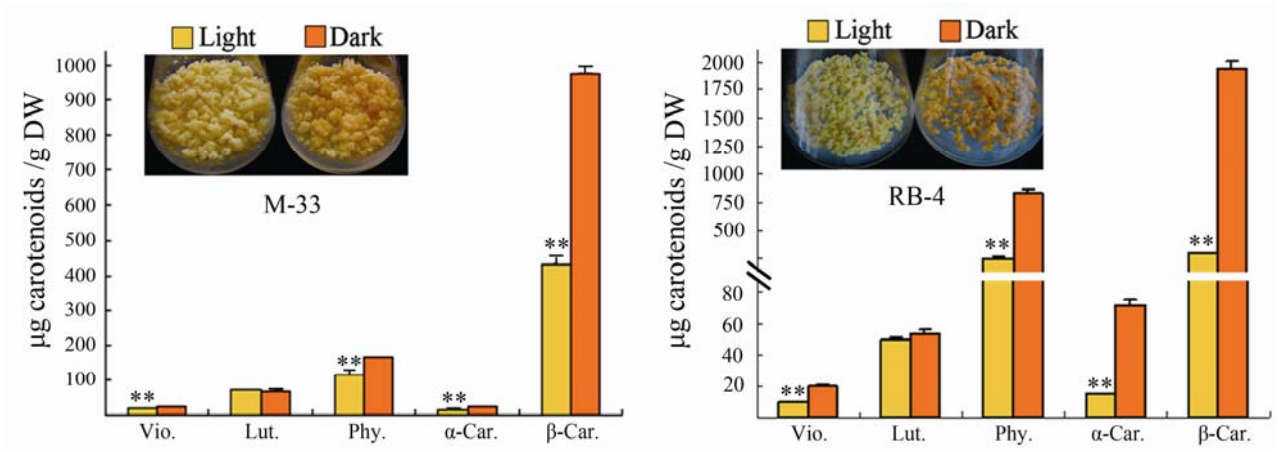

Figure S6. Comparative analysis of carotenoids contents in light/dark grown engineered lines. Via. violaxanthin; Lut. lutein; Phy. phytoene; α-car. α-carotene; β-car. β-carotene. Columns and bars represent the means and ± SD (n = 3 replicate experiments for M and n = 2 replicate experiments for RB), respectively. ** indicates that the values are significantly different compared with dark-grown lines at the significant levels of $P < 0.01$.

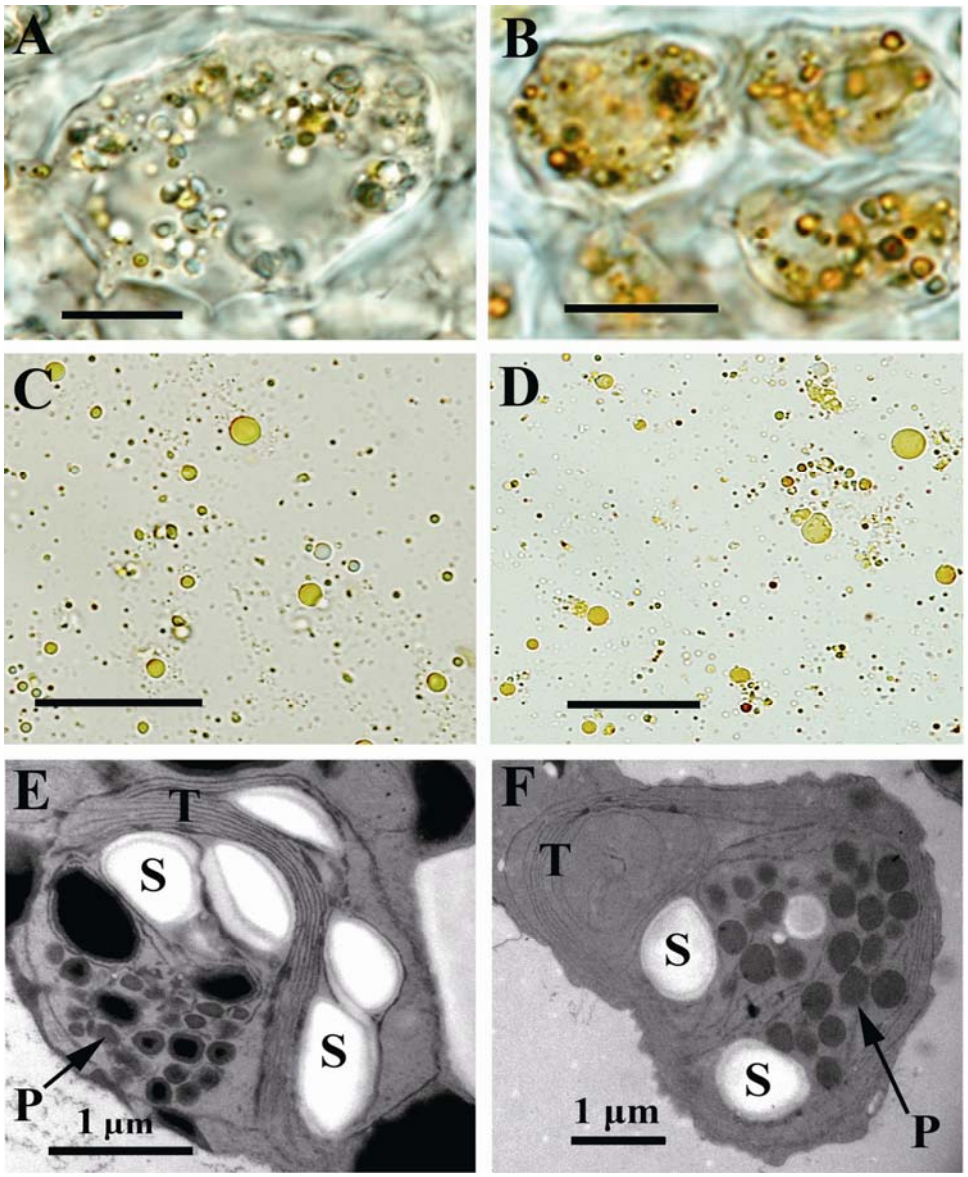

Figure S7. Additional photos of the visual inspections of the flavedo cells and plastid isolation. A, Cara Cara navel orange (HQC); B, Sunburst mandarin orange (SBT); C and D, Globular chromoplasts obtained using a protocol for isolating the plastids from the fresh flavedo cells of HQC and SBT, respectively; E and F, Ultrastructure of the chromoplast in the flavedo cells of HQC and SBT, respectively. The bar in A, B, C, D represents 10 μm. S, starch granule; P, plastoglobule; T, remnant thylakoids.

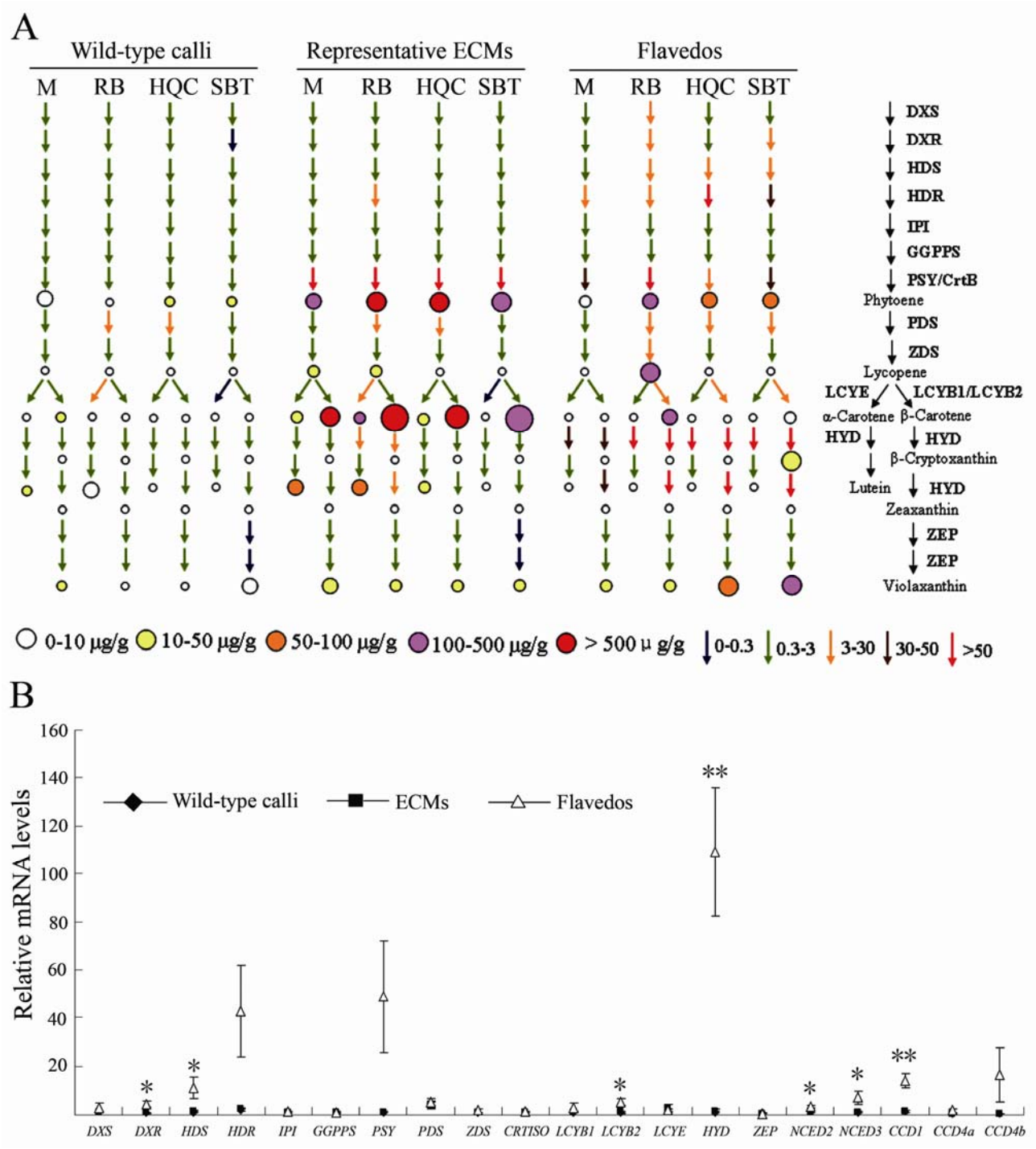

Figure S8. Comparative transcriptional analysis of genes encoding enzymes for isoprenoid and carotenoid metabolism among wild-type calli, ECMs and flavedos. (A) A brief schematic representation shows the relationship between transcript levels and carotenoid metabolites. The pathway related carotenoid biosynthesis (right) just shows the tested nodes in the present study. The transcript levels in wild-type M callus are assigned a value of 1 for reference. Carotenoid content is symbolized by a circle, and the size and color of the circle are proportional to the quantity of each carotenoid. The

transcript levels are represented by arrows, and variation in the transcript levels is indicated by the different colors of the arrows. For each gene, the transcript level is expressed relative to that of wild-type M callus. (B) Integrated expression patterns of isoprenoid and carotenoid biosynthetic genes in wild-type calli, ECMs and flavedos. Transcript levels are expressed relative to the wild-type M line. All data are presented as means ± SE from four genotypes. The ECMs include four representative lines (M-33, RB-4, SBT-6, HQC-2). * and ** indicate that the values of flvedos are significantly different compared with the corresponding callus lines at the significant levels of $P < 0.05$ and $P < 0.01$, respectively.

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