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

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Abstract

Genetic manipulation of carotenoid biosynthesis has become a recent focus for the alleviation of vitamin A deficiency. However, the genetically modified phenotypes often challenge the expectation, suggesting the incomplete comprehension of carotenogenesis. Here, embryogenic calli were engineered from four citrus genotypes as engineered cell models (ECMs) by over-expressing a bacterial phytoene synthase gene (CrtB). Ripe flavedos (the coloured outer layer of citrus fruits), which exhibit diverse natural carotenoid patterns, were offered as a comparative system to the ECMs. In the ECMs, carotenoid patterns showed diversity depending on the genotypes and produced additional carotenoids, such as lycopene, that were absent from the wild-type lines. Especially in the ECMs from dark-grown culture, there emerged a favoured β,β-pathway characterized by a striking accumulation of β-carotene, which was dramatically different from those in the wild-type calli and ripe flavedos. Unlike flavedos that contained a typical chromoplast development, the ECMs sequestered most carotenoids in the amyloplasts in crystal form, which led the amyloplast morphology to show a chromoplast-like profile. Transcriptional analysis revealed a markedly flavedo-specific expression of the β-carotene hydroxylase gene (HYD), which was suppressed in the calli. Co-expression of CrtB and HYD in the ECMs confirmed that HYD predominantly mediated the preferred carotenoid patterns between the ECMs and flavedos, and also revealed that the carotenoid crystals in the ECMs were mainly composed of β-carotene. In addition, a model is proposed to interpret the common appearance of a favoured β,β-pathway and the likelihood of carotenoid degradation potentially mediated by photo-oxidation and vacuolar phagocytosis in the ECMs is discussed.

Key words: Amyloplast, carotenogenesis, chromoplast, citrus, CrtB, crystal, engineered cell model, flavedo, phytoene synthase.

Introduction

Carotenoids and their derivatives play essential physiological and ecological roles in plant growth, development, and reproduction (Cazzonelli and Pogson, 2010). They are also essential health-protecting compounds involved in human vision, immunity, embryonic development, and reproduction (von Lintig, 2010). Carotenoids in nature originate from phytoene biosynthesis via the condensation of geranylgeranyl pyrophosphate (GGPP), which is the crucial rate-controlling step mediated by phytoene synthase (PSY) (Welsch et al., 2010) (see Supplementary Fig. S1 at JXB online). Enzymatic desaturation and isomerization, as well as a light-mediated photoisomerization, convert non-coloured phytoene into coloured lycopene (Chen et al., 2010). The lycopene flux then branches into two pathways via cyclization reaction. Lycopene β-cyclase (LCYB) adds two β-rings to the ends of lycopene molecule to form β-carotene, while the dual action
of lycopene $\varepsilon$-cyclase (LCYE) and LCYB results in the formation of $\alpha$-carotene with one $\beta$-ring and one $\varepsilon$-ring (Bai et al., 2009). Subsequently, carotenes are converted into various xanthophylls, which is mediated by carotene hydroxylases and epoxidase as well as de-epoxidase (Vallabhani et al., 2009).

Carotenoids are assembled and accumulated in nearly all types of plastids (Howitt and Pogson, 2006), while the deposition of massive carotenoids requires plastid modification for enhancing the storage capacity. Chromoplasts have been postulated to be the most important plastid type for striking carotenoid sequestration in higher plants (Kim et al., 2010). They are generally derived from pre-existing plastids like chloroplasts and amyloplasts during the ripening of carotenoid-containing organs such as fruits and flowers (Egea et al., 2010). For example, amyloplast to chromoplast conversion is observed in developing ornamental tobacco floral nectaries (Horner et al., 2007). Amyloplasts are starch-storing plastids, and are distinguished by the starch-granule-filled profile (Wise, 2006). During amyloplast conversion, starch breakdown begins, the starch granules diminish then disappear, while carotenoid sequestering structures such as plastoglobules and carotenoid crystals form and chromoplasts appear (Horner et al., 2007). Genes including fibrillin, CHRC, and OR have been identified to participate in the regulation of chromoplast development and carotenoid sequestration (Li and Van Eck, 2007).

In recent decades, genetic manipulation has been successfully used to modify carotenogenesis in many plants (Giuliano et al., 2008; Fraser et al., 2009; Farré et al., 2011). In particular, over-expression of PSY leads to a higher carotenoid generation in carotenoid-poor organs, such as canola seeds (Shewmaker et al., 1999), potato tubers (Ducruex et al., 2005), white carrot root (Maass et al., 2009), and cassava root (Welsch et al., 2010). These engineered crops have exhibited an effective approach to human health promotion, especially the alleviation of vitamin A deficiency. However, many physiological and biochemical alterations associated with modification of the carotenoid pathway that occur in genetically engineered plants remain unexplainable. For example, an altered carotenoid profile, particularly an increased $\beta,\beta$-carotene flux, is commonly observed (Ducruex et al., 2005; Paine et al., 2005; Schaub et al., 2005; Zhu et al., 2008; Maass et al., 2009; Diretto et al., 2010). In addition, it is difficult to obtain the desired phenotypes for some important carotenoids, such as lycopene (Schaub et al., 2005). Moreover, engineered plants with enhanced carotenoid biosynthesis require sink formation for effective carotenoid storage (Li and Van Eck, 2007). Morphological changes in plastids have been observed in PSY over-expressed tomato fruits and canola endosperm (Shewmaker et al., 1999; Fraser et al., 2007), which implies the possibility of plastid modification for carotenoid deposition. Over-expression of PSY can also drive crystalline-type carotenoid sequestering in Arabidopsis callus (Maass et al., 2009). However, the crucial mechanism governing carotenoid metabolic sink formation is still unclear, and needs to be further clarified.

Genetic manipulation of carotenogenesis in higher plants has primarily been focused on fruits (tomato), seeds (rice, maize, and canola), and roots or tubers (potato, carrot, and cassava). However, inherent deficiencies in these systems, including time-consuming cultivation and management due to long growth cycles and poorly controlled growth conditions, challenge the further study on carotenogenesis. Plant cell culture offers an attractive option for producing natural active compounds. Despite the fact that plant cells in vitro often exhibit an inconsistent yield of natural products owing to the dedifferentiation process, it could be circumvented partially through selection of the genotype and explant source for deriving cell lines (Engelmann et al., 2010; Lee et al., 2010), as well as through metabolic engineering (Hellwig et al., 2004). Interestingly, the carotenoid accumulation patterns in OR mutant cauliflower curd (Li et al., 2007), engineered potato tuber (Diretto et al., 2007, 2010), and carrot root (Jayaraj et al., 2008) are similar to those in the homologous cell lines in vitro. Besides, maize callus provides an efficient model for identifying the most productive PSY gene from different plant species (Paine et al., 2005). Arabidopsis callus has been used as a non-green system in investigating carotenoid metabolic sink formation driven by the over-expression of AtPSY (Maass et al., 2009). Thus, it is particularly important to construct a plant cell model cultured in vitro that could facilitate the study on the molecular, biochemical and cytological processes related to carotenoid accumulation.

Citrus is one of the most important and widely grown fruit crops with great economic significance and value for humans (Talon and Gmitter, 2008). Citrus fruits have diverse carotenoid patterns, highly regulated by the co-ordinated expression of carotenogenic genes (Kato et al., 2004). Over the years, citrus fruits have served as a unique system to provide new insights into the understanding of carotenogenesis. A novel chromoplast-specific LCYB gene has been found to be associated with lycopene accumulation in the fruits of Star Ruby grapefruit (C. paradisi Macf.) (Alquezar et al., 2009). Recent studies using comparative ‘omics’ revealed some processes, such as post-transcriptional regulation, partial impairment of lycopene downstream flux, enhanced photosynthesis, and miRNA-directed molecular process, in conferring the red-fleshed phenotype in the ‘Honganliu’ sweet orange (Pan et al., 2009; Xu et al., 2009, 2010). In addition, an in vitro culture system of citrus juice sacs provides a new approach to the comprehension of carotenogenesis in response to controlled environmental stimuli (Zhang et al., 2012).

By applying genetic manipulation of carotenoid accumulation as well as using carotenoid-rich citrus fruit, it is possible to understand the basis of carotenoid biosynthesis further. Therefore, engineered call models (ECMs) were established by over-expressing CrtB (phytoene synthase from Erwinia herbicola) or co-expressing CrtB and DSM2 (a non-haem-diiron $\beta$-carotene hydroxylase from rice) in various non-coloured citrus embryogenic callus cells. To compare with the ECMS, the ripe flavedos from four genotypes were offered as natural carotenoid accumulation
systems. Further analysis using comparative biochemistry, molecular biology, and cytology contributed to a better understanding of crystalline β-carotene accumulation in the amyloplasts of the ECM cells.

Materials and methods

Plant materials

Abortion ovule embryogenic calli from four citrus genotypes were used in this study. They were derived from Star Ruby grapefruit (C. paradise Macf.), Pink Marsh grapefruit (C. paradise Macf.), Cara Cara navel orange (C. sinensis (L.) Osb.), and Sunburst mandarin (Citrus reticulata Blanco×(C. paradisi Macf.×C. reticulata)), designated as RB, M, HQC, and SBT, respectively. The specimens were supplied by the National Key Laboratory of Crop Genetic Improvement at Huazhong Agricultural University (HIZAU), Wuhan, China.

Fruits were obtained from citrus trees grown at the National Center for Citrus Breeding, Huazhong Agricultural University. Marsh grapefruit (M) (C. paradise Macf.) was selected instead of Pink Marsh grapefruit because Pink Marsh grapefruit is a periclinal chimera whose abortion ovules and flavedos are identical to those of the Marsh grapefruit in terms of their genotype (Cameron et al. 1964). Ripe fruits were collected at the same time in November. Flavedos of the fruits were separated with scalpels. A part of flavedos tissues were used for cytology, and others were immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. The samples used for carotenoid extraction were lyophilized at −55 °C in a Lyolab 3000 (Heto, Denmark) and then stored at −80 °C.

Plasmid construction and transformation

The CrtB gene (a bacterial phytoene synthase gene, GenBank No. M90698) from Erwinia herbicola was PCR-amplified from plasmid vector PSL525, which was kindly provided by Professor Shih-Tung Liu (Taiwan, China). Pea rbcS trans peptide cDNA (GenBank No. X00806) was amplified by RT-PCR from the pea leaf pool. To create the chimeric gene (tp-rbcS-CrtB) construct, the PCR products were gel-purified, digested with Xhol/BamHI, and ligated into the corresponding sites in the PMV vector with a kanamycin resistance marker and the CaMV 35S promoter (modified pBI121) (Zhang et al., 2009). The primer pairs used were as listed in Supplementary Table S1 at JXB online. The plasmid pCB2004H with DSM2 [a non-haem-diiron β-carotene hydroxylase gene isolated from rice (Du et al., 2010)] over-expression construct was kindly provided by Professor Lijin Xiong, Huazhong Agricultural University, China. These constructs and empty vectors were electroporated into an Agrobacterium tumefaciens strain (EHA105).

Explant preparation and transformation were performed according to Duan et al. (2007). To reduce the occurrence of chimeric calli, a very small piece of each recovered callus, as an independent cell line, was transferred onto selective culture media and subcultured for several cycles. To generate CrtB/DMS2 double transgenic lines, the DSM2 over-expression construct was introduced into a stable CrtB transformed callus line (M-33) and the transgenic calli were selected with hygromycin (50 mg l−1). Each independent line was propagated in the dark or under 35 μmol m−2 s−1 illumination for 16 h daily and kept at 25±1 °C.

Twenty-day-old calli were harvested for cellular analysis or stored at −80 °C for molecular analysis. The calli used for carotenoids extraction were lyophilized and stored at −80 °C until use.

DNA analysis

Genomic DNA for PCR and Southern blot analysis was extracted from stored calli according to Cheng et al. (2003). A PCR-derived CrtB fragment (see Supplementary Table S1 at JXB online) was labelled with a Dig-DNA labelling kit and used as a probe (Roche Diagnostics GmbH). Prehybridization, hybridization, and membrane washing and detection were conducted according to the manufacturer’s instructions (Roche Diagnostics GmbH).

Quantitative analysis of gene expression

First-strand cDNA was synthesized from 1 μg of total RNA isolated from calli and flavedos using the RevertAid M-MuLV KIT (MBI, Lithuania) as previously described by Liu et al. (2006). The primer pairs used were as listed (Liu et al., 2007) or designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) ((see Supplementary Table S2 at JXB online). Actin was used as an endogenous control to normalize expression in different samples (Liu et al., 2007). Quantitative real-time PCR was performed using ABI 7500 Real Time System (PE Applied Biosystems; Foster City, CA, USA) according to Liu et al. (2007).

Generation of anti-CrtB antibodies and western blot analysis

The sequence of CrtB encoding 117 amino acids in the C-termius was amplified, cloned into pGEX-4T, and transformed into E. coli strain BL21 (see Supplementary Table S1 at JXB online). After induction and purification, inclusion bodies were dissolved in SDS sample buffer and separated by SDS-PAGE. Gel slices containing the peptide were used to immunize rabbits to generate anti-CrtB antibodies.

Total callus and flavedos proteins were prepared as described previously (Isaacson et al., 2006; Pan et al., 2009), and quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Membranes were blocked with a TBST solution, containing 5% (w/v) non-fat dry milk powder overnight at 4 °C and were incubated with anti-CrtB antibodies (1:2500) in the same solution for 2 h at room temperature. After rinsing five times for 10 min each with TBST solution, the membranes were incubated with secondary antibodies (peroxidase-conjugated immunoperoxidase goat anti-rabbit IgG [H+L], Pierce) at 1:20 000 in TBST solution, containing 5% (w/v) non-fat dry milk powder and rinsed six times for 10 min each with TBST solution. The signal was detected using SuperSignal West Pico system (Thermo Scientific, USA) according to the manufacturer’s instructions. Two rabbit polyclonal antibodies (Agrisera®, Sweden) against the Rubisco large subunit (RubEL, 53 kDa) and PsbA protein (D1 protein of Photosystem II, 32 kDa) were used to detect the plastid marker proteins.

Carotenoid profile analysis

Carotenoid extraction and analysis using reversed-phase high-performance liquid chromatography (RP-HPLC) was conducted as previously described (Paine et al., 2005; Liu et al., 2007). For the calli, a 0.2 g homogeneous lyophilized sample was extracted. Because of the abundant carotenoid esters in citrus fruits, the extracts from 0.3 g of lyophilized flavedos were saponified with 15% (w/v) KOH/methanol. The RP-HPLC analysis was performed in a Waters liquid chromatography system equipped with a model 600E solvent delivery system, a model 2996 photodiode array detection (PDA) system, a model 717 plus autosampler and an Empower chromatography manager. A C30 carotenoid column (150×4.6 mm; YMC, Japan) was used to elute the carotenoids. The carotenoids were identified by their characteristic absorbance spectra and typical retention time based on the literature and standards from CaroNature Co. (Bern, Switzerland). The quantification of the carotenoids was achieved using calibration curves for standards including violaxanthin, lutein, phytolene, α-carotene, β-carotene, β-cryptoxanthin, and lycopene; phytoflouene was quantified as phytolene, and α-cryptoxanthin was quantified as β-cryptoxanthin.
**Microscopy analysis**

Protoplasts from the calli were isolated as described by Groser and Gmitter (1990), after which protoplast suspensions were plated onto microscope slides to observe modes of carotenoid deposition. Microscopic and light microscopy of fresh flavedo cells were performed using a frozen sectioning technique with a Leica CM1900 (Leica, Germany). More detailed inspections of the carotenoid accumulative structures **in vitro** were performed on a protocol for isolating chromoplasts from fresh tissues (Lopez et al., 2008). An optical microscope (BX61, Olympus) equipped with a DP70 camera was used in tandem with differential interference contrast (DIC) technique.

The same callus and flavedo samples were also selected for transmission electron microscopy (TEM) analysis. Samples were prepared using a normal fixation process with 2.5% glutaraldehyde adjusted to pH 7.4, and 0.1 M phosphate buffer with 2% OsO4. They were dehydrated and embedded in epoxy resin (Lufit, 1961) and SPI-812, respectively. Ultrathin sections obtained with a Leica UC6 ultramicrotome were stained with uranyl acetate and subsequently with lead citrate. Recording of the images was performed with a HITACHI H-7650 transmission electron microscope at 80 kV and a Gatan 832 CCD camera. Images were obtained at various magnifications along with a corresponding waffle-patterndiffraction grafting replica with line spacing being 0.463 µm (Product No. 607, Ted Pella, Inc., Redding, CA).

**Statistical analysis**

The data presented in this study were analysed using SAS statistical software. Analysis of variance (ANOVA) was used to determine Pearson correlation (r) and to compare the statistical differences based on Student–Newman–Keuls’ multiple range test at the significance levels of P <0.05 (*), P <0.01 (**), and P <0.001 (***) respectively.

**Results**

**Construction of carotenoid engineered call models (ECMs)**

CrtB was first transformed into embryogenic calli from four citrus genotypes. Positive transformants of 58 independent lines were recovered, including five lines from Star Ruby grapefruit (RB), five lines from Sunburst mandarin (SBT), six lines from Cara Cara navel orange (HQC), and 32 lines from Pink Marsh grapefruit (M). Most of the transgenic lines had a yellow or orange-red colour that was not observed in the empty-vector controls and wild types. Coloured transgenic callus lines were selected as engineered cell models (ECMs). Four engineered genotypes presented distinctive colour characteristics where RB ECMs appeared red and the others orange or yellow (Fig. 1A).

Southern blot analysis confirmed the independent transformation events in the representative M ECMs (see Supplementary Fig. S2 at JXB online). However, no correlation was observed between transgene copy number and carotenoid content (data not shown). Western blot analysis using anti-CrtB antibodies indicated the presence of CrtB proteins in the representative ECMs but an absence in the wild type and negative control line (Fig. 2A, B). Furthermore, correlation between CrtB protein levels and carotenoid contents was observed in seven M ECMs (Fig. 2B). The ECMs showed normal growth, and their pigment expression was found to be stable in subcultures for at least three years.

**Carotenoid accumulation patterns in the ECMs**

The carotenoid profile was investigated in various callus lines from dark-grown culture using HPLC. Low amount of carotenoids was observed in wild-type embryogenic calli (Table 1). They mainly consisted of violaxanthin, lutein, β-carotene, and phytoene, and their accumulation patterns were markedly diverse among the genotypes (Fig. 3). In particular, RB contained predominantly lutein, and SBT had significantly suppressed lutein biosynthesis. Other important carotenoids, such as β-cryptoxanthin, lycopene, and esterified xanthophylls, were all at negligible levels or undetected in these calli.

By contrast, the levels of total carotenoids markedly increased in all ECMs compared with the wild types (Table 1 shows the carotenoid profiles in four representative ECMs). Phenotypes with a darker orange colour in the RB ECMs were associated with a larger amount of carotenoids, ranging from 3052 to 4880 µg g⁻¹ dry weight. Although ECMs’ carotenoid patterns were diverse among genotypes, up-regulation of the β,β-pathway was favoured (Table 1; Fig. 3). For example, in wild-type RB and M, the proportion of β-carotene in the total carotenoids was only 1.51% and 24.48%, respectively, and lutein accounted for 50.99% and 22.45%; but in the corresponding ECMs, β-carotene rose to 55.98% and 71.78% while lutein decreased to 1.84% and 5.21%, respectively (Fig. 3). It was also noted that violaxanthin increased slightly, so its proportion decreased in the ECMs. To characterize the altered balance of carotenoid patterns in the ECMs further, lutein levels were analysed in 32 M callus lines, including the wild type, empty-vector control, negative control, and 29 ECMs (35S::CrtB). Statistical analysis showed that the content/proportion change of lutein relative to lycopene flux followed a power function (Fig. 4). Herein, lycopene flux represented the total content of violaxanthin, lutein, α-carotene, β-carotene, and lycopene. This power relationship showed the dynamic change of branch flux, suggesting that the β,ε-branch was less competitive when the upstream flux become strong. Noticeably, a novel ECM (M-30) was found not to correlate well with the power function (Fig. 4). M-30 showed a dark yellow colour and contained a higher lutein proportion (19.7%) in relation to other ECMs (see Supplementary Fig. S3A at JXB online).

In addition, a large number of carotenoids under the detection threshold in wild types were detected in the ECMs (Fig. 3; see Supplementary Fig. S4 and Table S3 at JXB online). In particular, a low level of lycopene was observed in the ECMs of RB and M. Furthermore, identical HPLC chromatography profiles for saponified and unsaponified ECM samples demonstrated that non-esterified carotenoids were predominant in the ECMs (see Supplementary Fig. S5A at JXB online).

Effect of illumination on carotenoid accumulation in the ECMs

To determine the carotenoid accumulation in the ECMs from light-grown culture, illumination (35 µmol m⁻² s⁻¹
were both yellow instead of dark orange. HPLC analysis showed that the levels of carotenoids except lutein decreased under illumination. This was especially the case for β-carotene, which showed a 56% and an 85% reduction in M-33 and RB-4, respectively.

**Carotenoid accumulation patterns in the ripe flavedos from four genotypes**

To compare the ECMs with the natural carotenoid-rich system, carotenoid profiles in the ripe flavedos from four corresponding genotypes were characterized (Fig. 1C; Table 1). The flavedos of SBT and HQC chiefly contained violaxanthin, cryptoxanthin, phytoene, and phytofluene. RB accumulated high level of lycopene as well as β-carotene, phytoene, and phytofluene in the flavedo. There was a low level of carotenoids consisting predominantly of violaxanthin, phytoene, and phytofluene in the flavedo of Marsh grapefruit (M). In addition, carotenoid esterification is a typical character of carotenogenesis in the ripe flavedos (see Supplementary Fig. S5B at JXB online).

**Light microscopic observations of carotenoid storage structures**

Significant carotenoid accumulation is accommodated by chromoplast development. To examine the formation of carotenoid sequestering structures in the ECMs, light microscopy of the protoplasts was performed. Unlike wild-type controls, visible red-orange carotenoid sequestering structures were found in the ECM cells (Fig. 5A, B, E, F), and polarization microscopy confirmed their crystal nature (Fig. 5C, D, G, H). Our further observation showed that the crystals were formed in a circular pattern localizing to the periphery of the amyloplasts (Fig. 5I). Some orange conglomerates, which were undergoing Brownian motion, were observed inside the vacuoles of the ECM cells (Fig. 5J). The conglomerates exhibited birefringence (Fig. 5K), indicating their crystal nature and the likelihood of containing
Table 1. Carotenoid content and composition in wild-type callus lines, representative ECMs and flavedos

| Event  | Vio | Lutein | α-Cry | β-Cry | Phy | Phytof | α-Car | β-Car | Lyc | Total | β/α ratio | Xan./Tol. |
|--------|-----|--------|-------|-------|-----|--------|-------|-------|-----|-------|-----------|----------|
| Wild-type callus lines from dark-grown culture (non-saponified samples) |      |        |       |       |     |        |       |       |     |       |           |          |
| M      | 13.78±0.46 | 12.05±0.56 | –     | –     | 9.4±0.89 | 4.32±0.48 | 0.98±0.042 | 13.14±1.26 | –   | 53.68±3.13 | 2.07±0.086 | 0.48±0.019 |
| RB     | 2.16±0.14  | 9.17±0.45  | –     | –     | 3.98±0.82 | 1.52±0.17 | 0.88±0.008 | 0.27±0.17  | 17.98±0.94 | 0.24±0.015 | 0.63±0.03  |
| HQC    | 3.48±0.14  | 2.63±0.19  | –     | –     | 9.8±0.57 | 5.41±0.45 | 0.99±0.079 | 5.06±2.26  | 27.37±2.18 | 2.36±0.55  | 0.22±0.024 |
| SBT    | 7.75±0.45  | 9.14±0.94  | –     | –     | 3.26±0.18 | 0.88±0.007 | 5.73±0.31  | –     | 27.09±0.39 | 15.32±0.47 | 0.29±0.016 |
| ECMs from dark-grown culture (non-saponified samples) |      |        |       |       |     |        |       |       |     |       |           |          |
| M-33   | 31.97±0.89 | 65.14±0.73 | 3.49±0.027 | 3.13±0.14 | 138.51±2.16 | 70.79±0.95 | 26.76±0.32 | 897.82±16.2 | 19.73±0.54 | 1257.3±19.64 | 9.78±0.056 | 0.083±0.001 |
| RB-4   | 14.84±1.81 | 89.73±5.63 | 15.34±1.48 | 9.51±0.70 | 1204.39±72.54 | 644.66±37.75 | 132.53±6.97 | 273.07±7.820 | 62.22±4.47 | 4935.3±204.85 | 11.60±0.39 | 0.026±0.0009 |
| HQC-2  | 14.29±0.59 | 11.27±0.44 | 5.69±0.79 | 3.89±0.72 | 1108.63±32.79 | 523.00±8.98 | 15.26±0.37 | 1.179.28±31.11 | –     | 2861.30±73.02 | 37.16±1.09 | 0.012±0.002 |
| SBT-6  | 18.10±0.98 | 2.59±0.21  | 2.26±0.46 | 3.94±0.44 | 298.47±15.03 | 118.21±6.24 | 2.75±0.18 | 472.10±29.39 | –     | 918.42±51.55 | 65.02±4.96  | 0.029±0.001 |
| Flavedos (saponified samples) |      |        |       |       |     |        |       |       |     |       |           |          |
| M      | 10.37±1.29 | 1.64±0.22  | –     | –     | 0.76±0.074 | 8.77±1.06 | 6.12±0.68 | 0.32±0.01 | 2.31±0.38 | –       | 30.28±3.62 | 6.89±0.23  | 0.42±0.009 |
| RB     | 9.86±0.78  | 2.19±0.37  | 1.62±0.036 | –     | 167.75±7.92 | 177.38±9.09 | 2.23±0.13 | 177.19±13.32 | 225.71±13.86 | 763.92±45.22 | 30.99±0.63 | 0.018±0.0005 |
| HQC    | 9.49±5.91  | –        | –     | –     | 3.66±0.17 | 72.23±5.03 | 25.87±1.35 | –     | 0.60±0.11 | –       | 199.86±10.09 | –       | 0.5±0.017 |
| SBT    | 163.46±13.03 | 24.20±2.38 | 79.95±7.85 | 101.78±6.89 | –     | 7.04±1.02 | –     | –     | –       | –       | 376.44±28.77 | –       | 0.5±0.011 |

Comparative microscopy indicated distinctive plastid forms in flavedos and calli. To characterize the plastids (Fig. 6A, B). Without the use of TEM, each mutant group and cell type showed the same proportion of plastids in all cell types. By contrast, the TEM showed less diversity among the plastid types observed in all callus lines, including four wild types and four representative ECMs (35S::CaArabidopsis thaliana, 2008). Abundant carotenoid crystals were observed in the RB-4, SBT-6, and SBT-16 samples (Wang et al., 2010). The crystals were spherical or ellipsoidal, with diameters ranging from 1 to 5 μm. Further investigation using TEM revealed that the crystals contained carotenoids, including xanthophylls and carotenoids (Fig. 7A, B). The crystals were found to be enclosed within a membrane envelope, indicating their origin from the crystalline chromoplast. Interestingly, the crystalline chromoplasts were also observed in the flavedo cells of the representative ECMs (35S::CaArabidopsis thaliana, 2010). The crystals were spherical or ellipsoidal, with diameters ranging from 1 to 5 μm. Further investigation using TEM revealed that the crystals contained carotenoids, including xanthophylls and carotenoids (Fig. 7A, B). The crystals were found to be enclosed within a membrane envelope, indicating their origin from the crystalline chromoplast.
Peak No. | Carotenoids | M–wild type | M–35S::CrB | RB–wild type | RB–35::CrB | HQC–wild type | HQC–35::CrB | SBT–wild type | SBT–35::CrB
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 1         | Violaxanthin | 25.6 ± 0.96 | 21.46 ± 0.99 | 25.2 ± 0.10 | 21.46 ± 0.99 | 25.8 ± 0.09 | 21.46 ± 0.99 | 25.16 ± 0.09 | 21.46 ± 0.99 |
| 2         | 9-cis-violaxanthin | 17.0 ± 0.10 | 17.0 ± 0.10 | 11.4 ± 0.03 | 11.4 ± 0.03 | 17.0 ± 0.10 | 11.4 ± 0.03 | 11.4 ± 0.03 | 11.4 ± 0.03 |
| 3         | Luteoxanthin | 8.0 ± 0.07 | 4.8 ± 0.03 | 6.8 ± 0.02 | 4.8 ± 0.03 | 8.0 ± 0.07 | 4.8 ± 0.03 | 4.8 ± 0.03 | 4.8 ± 0.03 |
| 4         | 9-cis-violaxanthin | 6.0 ± 0.03 | 6.0 ± 0.03 | 5.0 ± 0.02 | 5.0 ± 0.02 | 6.0 ± 0.03 | 5.0 ± 0.02 | 5.0 ± 0.02 | 5.0 ± 0.02 |
| 5         | Anthexanthin | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 6         | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 7         | Lutein | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 8         | Zeaxanthin | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 9         | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 10        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 11        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 12        | α-cryptoxanthin | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 13        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 14        | β-cryptoxanthin | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 15        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 16        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 17        | β-carotene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 18        | cis-β-carotene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 19        | Δ-carotene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 20        | Δ-carotene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 21        | Unknown | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 22        | γ-carotene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 23        | cis-lycopene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 24        | cis-lycopene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |
| 25        | Lycopene | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 | 0 ± 0.01 |

**Fig. 3.** HPLC chromatograms of carotenoids (non-saponified) in the wild types and representative ECMs (35S::CrB) monitored at 450 nm. The peaks are numbered according to the elution time as detailed in Supplementary Table S3 at JXB online. Inserted tables show the proportion (%) and ±SD (n=3 replicate experiments) of the representative carotenoids relative to total carotenoid amount.

Further, Western blot analysis was performed to detect two plastid proteins, a Rubisco large subunit and PsbA/D1, both of which are photosynthetic proteins and typical of chloroplasts, and are also detected in the chromoplasts arising from chloroplasts (Barsan et al., 2010). The result revealed that both proteins were missing in all calli, both light-grown and dark-grown cultures, although they were expressed actively in all flavedos (Fig. 7).

**Transcript profiles of isoprenoid and carotenoid metabolism in the ECMs and flavedos**

Expression data showed that most genes encoding enzymes for isoprenoid and carotenoid metabolism were expressed at a similar level in all callus lines, including four wild types and four representative ECMs (M-33, RB-4, SBT-6, HQC-2) (Fig. 8; see Supplementary Fig. S8 at JXB online). However,
some exceptions were observed. The gene encoding phytoene desaturase (PDS) was expressed approximately 6-fold higher in HQC and RB compared to M. LCYE was expressed 7-fold higher and approximately 15-fold lower in RB and SBT, respectively, compared with M. Transcriptional analysis of all tested M callus lines showed that M-30 exhibited the highest transcript level of LCYE gene (P < 0.001) (see Supplementary Fig. S3B at JXB online).

Furthermore, comparison of the expression patterns of the calli and the corresponding flavedos, it was possible to group these genes into three categories. The first group exhibited a flavado-specific expression pattern, including the genes encoding DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), HYD (hydroxymethylbutenyl 4-diphosphate synthase), HDR (hydroxymethylbutenyl 4-diphosphate reductase), PSY, LCYB2 (lycopene β-cyclase 2), HYD (β-carotene hydroxylase), NCEDs (9-cis-epoxycarotenoid dioxygenases), and CCDS (carotenoid cleavage dioxygenases). In particular, HYD was the most flavado-specific gene, reflected by the striking up-regulation of its expression in the flavado of almost all four genotypes whereas suppression in all calli (see Supplementary Fig. S8B at JXB online). The second group showed a constitutive expression pattern in the calli and corresponding flavados, including the genes encoding IPI (isopentenyl diphosphate isomerase), GGPPS (geranylgeranyl diphosphate synthase), PDS, and CRTISO (carotenoid isomerase). Other genes were assigned to the third category due to their unique expression patterns. In particular, DXS (encoding 1-deoxy-D-xylulose 5-phosphate synthase) and ZDS (encoding ζ-carotene desaturase) were expressed at higher levels in the flavados of RB as compared to other lines, while LCYE was expressed at unusually low levels only in the callus of SBT.

Over-expression of CrtB in combination with DSM2 in callus tissue

Transcriptional analysis suggested HYD plays a key role in regulating the preferred carotenoid patterns between the ECMs and flavados. To prove this, one non-haem-diiron β-carotene hydroxylase gene (DMS2, also named as HYD3) from rice (Du et al., 2010) was introduced into an ECM of M. This co-transformation system was also expected to be used to examine whether β-carotene was the major component of the crystals in the ECMs.

As shown in Fig. 9, co-transformed lines showed an altered phenotype from orange to gold. HPLC analysis revealed an 80.4% reduction of β-carotene on average and a 67.7% reduction of α-carotene in the co-transformed lines. As expected, xanthophyll content was noticeably increased in the co-transformed lines, particularly violaxanthin content, which exhibited a 2.4-fold increase. However, unlike flavados, the co-transformed lines accumulated unesterified xanthophylls. Furthermore, carotenoid crystals became smaller in size, as shown by small dots attaching to the amyloplasts of co-transformed cells, or even being invisible (Fig. 9A, B). Subsequently, the ultrastructural inspection of the amyloplasts in the co-transformed cells showed an increased number of plastoglobules (Fig. 9C).

Discussion

Comparative analysis of various ECMs with the natural carotenoid-rich system (flavedo) provides a powerful tool for gaining insight into the key regulatory patterns and metabolic sink formation, particularly those with regard to β-carotene accumulation. More importantly, the ECMs from dark-grown culture were found to be excellent accumulators of carotenoids consisting predominantly of β-carotene. In a representative RB ECM, the carotenoid content, with about 56% β-carotene, was even higher than those of carotenoid-rich crops, such as carrot roots (2000–4000 μg g⁻¹ dry weight) and tomatoes (2000 μg g⁻¹ dry weight) (Maass et al., 2009). Therefore, an improved understanding of the mechanisms underlying β-carotene accumulation in the ECMs could promote effective carotenoid engineering in non-green tissues, which represent major food sources for humans (Howitt and Pogson, 2006).

ECMs possess a favoured β,β-pathway

CrtB created a considerable carotenogenic flux in the callus tissues of various genotypes, including a red-fruit variety (RB) which accumulated lycopene in ripe fruits. However,
was expressed at unusually low levels only in the callus of the flavedos of RB as compared to other lines, while their unique expression patterns. In particular, ase). Other genes were assigned to the third category due to diphosphate synthase), PDS, and CRTISO (carotene isomer-

showed a constitutive expression pattern in the calli and the corresponding flavedos, including the genes encoding IPI group these genes into three categories. The first group the calli and the corresponding flavedos, it was possible to

regulating the preferred carotenoid patterns between the Transcriptional analysis suggested HYD plays a key role in callus tissue

Over-expression of CrtB in combination with DSM2 in almost all four genotypes whereas suppression in all calli (see

Supplementary Fig. S8B at online). The second group

Supplementary Fig. S3B at online). The second group

gene (\(P\)) was expressed \(<0.001\) (see

Statistical significance (\(P\)) are determined using SAS statistical software. The arrows show a significantly deviating line (M-30).

Furthermore, comparison of the expression patterns of those of carotenoid-rich crops, such as carrot roots (2000–

content, with about 56% \(b\)-carotene. In a representative RB ECM, the carotenoid accumulation in the ECMs could promote effective carot-

understanding of the mechanisms underlying

Comparative analysis of various ECMs with the natural

metabolic sink formation, particularly those with regard to

As expected, xanthophyll content was noticeably increased in the co-transformed lines, particularly violaxanthin con-

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This co-transformation system was also expected to be used to examine whether

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from rice (Du et al., 2010) was introduced into an ECM of M.

Comprehending crystalline \(\beta\)-carotene accumulation

| Fig. 5. Light microscopic inspection. (A) Protoplasts from wild-type RB under normal light field microscope. (B) Protoplasts from wild-

type M under normal light field microscope. (C, D) Polarization micrographs for (A) and (B), respectively. (E, F) Protoplasts exhibiting the orange-coloured structures in the ECM cells of RB and M, respectively. (G, H) Carotenoid crystals confirmed by polarization microscopy in the ECM cells of RB and M, respectively. (I) Crystals located around the amyloplasts. (J) Coloured conglomerate (shown with arrows) observed in the vacuole of the ECM cells. (K) Conglomerate in the vacuole containing carotenoid crystals. (L) Flattened sheet (carotenoid crystals) in the lysate sample of the ECM cells. (M) Coloured plastid structure containing plastoglobules peripherally. (N) Coloured plastid structures from the wild-type cells (a) and ECM cells (b). (O) Inspection of the flavedo cells from Marsh; arrows show the chromoplasts. (P) Inspection of the flavedo cells from Ruby Star; arrows show the chromoplasts. (Q) Globular chromoplast (shown with arrows) in the lysate samples of the flavedo cells from Marsh. (R) Polarization microscopy for (Q). (S) Chromoplasts in the lysate samples of the flavedo cells from RB. (T) Polarization microscopy for (S), confirming the carotenoid crystals inside the chromoplasts of RB. The bar in each figure represents 10 \(\mu\)m. |
Thus, the balance of carotenoid accumulation was altered as calli, while lutein took up a substantial proportion (51.1%). Only accounted for 1.5% of total carotenoids in wild-type RB confirmed by the result of our experiment in which predominant in wild-type tissues (or organs), this is also observations in other engineered plants, such as canola \(^{2b}\), consistent with the \(^{3b}\) effective bi-cyclic cyclase, in the absence of which, lycopene substrate. This proposed system suggested a feedback\(^{2e}\) proportion of the substrate preference by cyclase, which may be acting to lead to an altered carotenoid composition. This is leading to a carotenoid metabolic sink. By comparison, almost all the \(^3e\)-carotene accumulated mainly in plant tissues, whereas LCYE is \(^{1e}\) generally the mono-cyclic cyclase, and less lycopene accumu-
ates when LCYE is absent (Pogson \(^{1e}\), 1996; Alquezar \(^{1e}\), 2009). A previous study of tobacco floral nectaries has clearly \(^{1e}\) suggested by the multiplication of plastoglobules observed crystalline \(^\text{plastoglobules}\) inside, starch granules were still the most predominant in wild-type tissues (or organs), this is also confirmed by the result of our experiment in which \(^c\)-carotene only accounted for 1.5% of total carotenoids in wild-type RB calli, while lutein took up a substantial proportion (51.1%). Thus, the balance of carotenoid accumulation was altered as a result of genetic manipulation. It is noted that, in the present study, a suppressed transcript level for LCYE could explain the scarce accumulation of lutein in SBT callus, and M-30, which probably contained additional variation, exhibited increased LCYE transcription, yielding high lutein accumulation (see Supplementary Fig. S3 at JXB online).

In the ECMs, no abundant lycopene accumulation was found, except for a low level in that of RB and M (Table 1; Fig. 3). Supposedly, lycopene accumulation in fruits is a tissue-specific developmental process that is difficult to assess from non-fruit tissues, conforming to the report that a unique chromoplast-specific lycopene \(^c\)-cyclase gene (LCYR2) determines the lycopene accumulation in the RB fruits (Alquezar \(^\text{et al.}^{1}\), 2009). The ECMs contained a substantial favoured \(^c\)-\(^c\)-branch pathway, consistent with the observations in other engineered plants, such as canola seeds (Shewmaker \(^\text{et al.}^{1}\), 1999), golden rice (Schaub \(^\text{et al.}^{1}\), 2005), and golden potatoes (Diretto \(^\text{et al.}^{1}\), 2010).

However, in most cases, the \(^c\)-\(^c\)-branch pathway is not predominant in wild-type tissues (or organs), this is also confirmed by the result of our experiment in which \(^c\)-carotene was sequestered in amyloplasts without crystallization, which was suggested by the multiplication of plastoglobules observed in potato tubers (Lopez \(^\text{et al.}^{1}\), 2000). In this complex, it is speculated that crystalline \(^\text{plastoglobules}\) inside, starch granules were still the most predominant in wild-type tissues (or organs), this is also confirmed by the result of our experiment in which \(^c\)-carotene accumulated mainly in plant tissues, whereas LCYE is gener-
ally the mono-cyclic cyclase, and less lycopene accumu-
lates when LCYE is absent (Pogson \(^{1e}\), 1996; Alquezar \(^\text{et al.}^{1}\), 2009; Bai \(^\text{et al.}^{1}\), 2009). Accordingly, in the ECMs abundant lycopene molecules that were not captured by LCYE diffused to LCYB and were converted to \(^c\)-carotene. Subsequently, \(^c\)-carotene was predominantly accumulated, but less leaked to xanthophylls, which implicated the low carotenoid hydroxylase activity in the ECMs.

Therefore, this model suggests that LCYE has a bottleneck role in the presence of abundant lycopene substrates leading to an altered carotenoid composition. This is
Supported by the reports that diverse LCYE enzymatic activity determines alternative β-carotene and lutein compositions in maize and wheat (Harjes et al., 2008; Howitt et al., 2009), as well as by the fact that over-expression of CrtI, a bacterial phytoene desaturase that converts phytoene to lycopene, leads to substantially increased β,β-carotene accumulation in tobacco leaves (Misawa et al., 1993) and tomato fruits (Romer et al., 2000).

In addition, a β- and ε-cyclase complex has once been proposed to explain the branch control of carotenoid pathway (Bai et al., 2009). In this complex, it is speculated that there is competition for substrate between LCYB and LCYE, probably relying on their different cyclase affinity to substrate. This proposed system suggested a feedback regulation mechanism, mediated by the altered lycopene substrate preference by cyclase, which may be acting to influence the proportion of β,β-carotenoids and β,ε-carotenoids (Cazzonelli and Pogson, 2010).

**Amyloplasts serve as crystalline-type β-carotene sequestering sink in the ECMs**

As revealed by cellular inspection and Western blot analysis, plastid types differed in the callus and flavedo cells. In flavedo cells, chromoplasts transformed from chloroplasts served as a carotenoid metabolic sink. By comparison, almost all the observed carotenoids in the ECM cells were sequestered within amyloplasts; despite the fact that amyloplasts showed chromoplast-like profiles owing to visible crystals and plastoglobules inside, starch granules were still the most predominant (Fig. 6A). β-Carotene content was correlated with crystal development in CrtB/DSM2 co-transformed lines, which reveals that the carotenoid crystals predominantly contained β-carotene. Other carotenoids, such as xanthophylls, might be deposited in amyloplasts without crystallization, which was suggested by the multiplication of plastoglobules observed in the amyloplasts (Figs 6A, 9C), and also by the coloured plastid structures (Fig. 5M), which were probably the broken amyloplasts losing most starch granules, as previously observed in potato tubers (Lopez et al., 2008). In agreement with the previous description of crystal formation in plastids driven by over-expression of PSY (Maass et al., 2009), the observations in the present study demonstrate that carotenoid sequestering sink can also be formed in the amyloplasts.

Chromoplast development requires the controlled changes of the pre-existing plastids, such as the internal membrane remodelling (Egea et al., 2010), which may be mediated by some crucial factors. For example, OR functions in directing the transition from non-coloured plastids to chromoplasts, over-expression of OR drives β-carotene crystal formation in potato tubers and cauliflowers (Lopez et al., 2008). In addition, metabolite-induced plastid transition is found in the engineered tomato fruits over-expressing PSY (Fraser et al., 2007). A previous study of tobacco floral nectaries has clearly
shown that amyloplasts could be converted into chromoplasts (Horner et al., 2007). However, amyloplasts are sustained in the ECMs, whether cultured in the light or in the dark. Unlike Arabidopsis, citrus has a chromoplast developmental programme (Alquezar et al., 2009; Maass et al., 2009). Probably, such a programme in the cell models is suppressed by the predominant starch accumulation, or the cell models lack some factors that initiate this progress. Further investigation is needed to find out the causes repressing the transition of amyloplasts to chromoplasts, particularly the impact of starch metabolism on chromoplast development and carotenogenesis.

Hypothetical carotenoid degradation in the ECMs

In the present study, abundant carotenoid crystals without surrounding membranes were found in the isotonic buffer (Fig. 5L), suggesting that the crystals were not firmly attached to the amyloplast membranes. As previously discussed by Maass et al. (2009), carotenoid crystals are free from the lipophilic phase of the plastid membrane, and the crystalline form may protect carotenoids from enzymatic catabolism. However, crystallization seems not to protect β-carotene from photo-oxidative degradation in the ECMs. It is noted that the ECMs under irradiation showed a marked decrease in β-carotene level (see Supplementary Fig. S6 at JXB online). This result is not due to a conversion of β-carotene to β,β-xanthophylls as in the cyanobacterium (Schäfer et al., 2006), because violaxanthin levels decreased during sucrose starvation through vacuolar phagocytosis (Chen et al., 1994). This provides evidence of an additional site for carotenoids and implies that there is an underlying catabolism mechanism that governs carotenoids in the vacuoles. Further studies are required to discover new pathways related to carotenoids degradation.

HYD transcription predominantly determines the variation of carotenoid accumulation in calli and flavedos

Transcriptional analysis confirmed the crucial rate-controlling role of PSY in the ECMs, given an over-expressed CrtB driving the abundant accumulation of carotenoid. However,
the major accumulation pattern of β-carotene in the ECMs is very different from that of esterified β,β-xanthophylls in common fruits (Table 1). In the ECMs, the proportion of violaxanthin was lower than in the wild types (Fig. 3). This phenomenon seemed like a result of feedback inhibition from striking metabolic flux. Furthermore, in the flavedos of the present study, a co-ordinated transcriptional up-regulation of most genes encoding enzymes for isoprenoid and carotenoid metabolism, especially the HYD gene, reflects a fruit-specific regulation of carotenogenesis (Fig. 8; see Supplementary Fig. S8 at JXB online) (Kato et al., 2004). As reported previously, HYD is associated with β-carotene accumulation in the endosperm of maize (Vallabhaneni et al., 2009; Yan et al., 2010). Therefore, the over-expression of an HYD gene from rice was performed in the ECM, which resulted in a significantly decreased β-carotene content and increased violaxanthin and other xanthophylls (Fig. 9). This study identifies the crucial role of HYD in determining the diversified carotenoid accumulation patterns between the ECMs and flavedos, and also suggests that HYD can be a key target for β-carotene metabolic engineering in citrus fruits. However, it is noteworthy that the β,β-xanthophylls in CrtB/DSM2 co-transformed lines were all non-esterified forms, which is presumed to be associated with an inactive fatty acid synthesis in the amyloplasts of the ECM cells. In general, esterification of xanthophylls with fatty acids is a tissue-specific behaviour which is more likely to occur in ripe fruits (Hornero-Mendez and Minguez-Mosquera, 2000; D’Ambrosio et al., 2011).

**Conclusion**

In summary, this paper reports diverse ECMs depending on the genotypes of citrus used for the construction. However, the carotenoid pattern in the ECMs of one genotype seems not to correlate with that in its flavedo. Comparative analysis of various ECMs with flavedos probes into the β-carotene accumulation mechanism with new insights. The present study correlates striking β-carotene accumulation with the suppressed HYD transcription and a channelling control of alternative lycopene flux in the ECMs. It also shows that, in the ECM cells, β-carotene is synthesized and sequestered in the amyloplasts in crystalline form. Furthermore, carotenoid crystals consisting mainly of β-carotene are confirmed. The likelihood of carotenoid degradation mediated by photo-oxidation and vacuolar phagocytosis in the ECMs is also proposed. Utilization of the ECMs is not limited to comprehension of β-carotene accumulation. The ECMs may facilitate the investigations into more potential gene targets associated with carotenogenesis and into the regulation metabolism of carotenoid accumulation in response to various environmental stimuli.

**Supplementary data**

Supplementary data can be found at JXB online.

**Supplementary Table S1.** Primers used in this study.

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

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

**Supplementary Fig. S1.** Carotenoid biosynthesis pathway in higher plants.

**Supplementary Fig. S2.** A DNA gel blot analysis indicating inserted patterns of T-DNA in the representative lines.

**Supplementary Fig. S3.** A novel 35S::CrtB ECM (M-30) containing a high proportion of lutein due to the highest expression of the LCYE gene.

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

**Supplementary Fig. S5.** HPLC chromatograms of carotenoids extracted from the RB ECM (A) and HQC flavedo (B) at 450 nm.

**Supplementary Fig. S6.** Comparative analysis of carotenoids contents in light/dark-grown engineered lines.

**Supplementary Fig. S7.** Additional photographs of the visual inspection of the flavedo cells and plastid isolation.

**Supplementary Fig. S8.** Comparative transcriptional analysis of genes encoding enzymes for isoprenoid and carotenoid metabolism among wild-type calli, ECMs, and flavedos.

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