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Metabolic engineering of tomato for high-yield production of astaxanthin

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ABSTRACT

Dietary carotenoids have been shown to be beneficial to health by decreasing the risk of many diseases. Attempts to enhance carotenoids in food crops have been successful although higher plants appear to resist big changes of carotenoid biosynthesis by metabolic engineering. Here we report the generation of a more nutritious tomato by modifying the intrinsic carotenes to astaxanthin, a high-value ketocarotenoid rarely found in plants. This was achieved by co-expression of the algal β-carotene ketolase from *Chlamydomonas reinhardtii* and β-carotene hydroxylase from *Haematococcus pluvialis*, a unique pair of enzymes identified to co-operate perfectly in converting β-carotene to astaxanthin by functional complementation in *Escherichia coli*. Expression of the two enzymes in tomato up-regulated most intrinsic carotenogenic genes, and efficiently directed carbon flux into carotenoids, leading to massive accumulations of mostly free astaxanthin in leaves (3.12 mg/g) but esterified astaxanthin in fruits (16.1 mg/g) and a 16-fold increase of total carotenoid capacity therein without affecting the plant normal growth and development. This study opened up the possibility of employing crop plants as green factories for economical production of astaxanthin.

1. Introduction

Astaxanthin (3,3′-dihydroxy-β-carotene-4,4′-dione) is a unique red carotenoid responsible for the pigmentation of many marine animals which acquire the red pigment from their diets not only for coloring but also for improving survival and growth of juveniles (Chien and Shiau, 2005). Growing evidence also shows that astaxanthin provides beneficial effects on human health, including the enhancement of general well-being and the immune system, protection against lipid-membrane peroxidation and DNA damage (Yuan et al., 2011). While chemically synthetic astaxanthin is restricted in use as a feed additive for salmon and trout farming, the demand for natural astaxanthin is expected to grow rapidly in the nutraceutical, cosmeceutical and pharmaceutical markets. However, the bioresources of astaxanthin are limited and thus expensive, which has attracted scientists to develop heterologous biosynthesis hosts for the sustainable production of natural astaxanthin (Misawa, 2009; Zhu et al., 2009).

Typically the biosynthesis of astaxanthin from β-carotene requires a ketolase (BKT) and a hydroxylase (BHY) to add a carbonyl and a hydroxyl at positions 4 and 3 of each terminal β-ionone ring, respectively (Fig. 1). BHYs are ubiquitous in all plants and organisms with oxygenic photosynthesis. In contrast, BKTs (assigned as *CrtW* in prokaryotes) are present only in some bacteria and a few green algae (Kajiwara et al., 1995; Lotan and Hirschberg, 1995; Misawa et al., 1995; Fernández-González et al., 1997). These genes have been used for genetic engineering of astaxanthin synthesis in plants (Mann et al., 2000; Hasunuma et al., 2008; Jayaraj et al., 2008). The highest astaxanthin content achieved so far is 5.44 mg/g (dry weight) by transplastomic tobacco (Hasunuma et al., 2008), which is about one tenth of that in the green microalga *Haematococcus pluvialis* (Boussiba et al., 1999). In contrast to *H. pluvialis* which possesses an efficient mechanism for sequestrating astaxanthin in esterified forms, plant vegetative leaves, bacteria and yeasts lack a storage mechanism for depositing astaxanthin in the same way as the alga. Certain plant tissues, such as fruits or flowers, do have the ability to store carotenoids (e.g. xanthophylls) in ester forms at very high levels (Piccaglia et al., 1998), suggesting the possibility of the tissues to accumulate much higher amounts of astaxanthin than that in vegetative leaves.

Tomato can serve as an excellent producer for astaxanthin because sufficient precursors (lycopene and β-carotene) are available and the storage capacity for lipophilic carotenoids is high (Fraser et al., 2007). Some successes in enhancing the contents of endogenous carotenoids, especially β-carotene (pre-vitamin A) in tomato fruit have been achieved (Fraser et al., 2009). However, tomato seems to resist engineered changes of intrinsic carotenes to non-native astaxanthin as demonstrated by transgenic tomato co-expressing the *crtW* and *crtZ* from *Parococcus* (Ralley et al., 2004). This might be due to the poor co-operation of the bacterial

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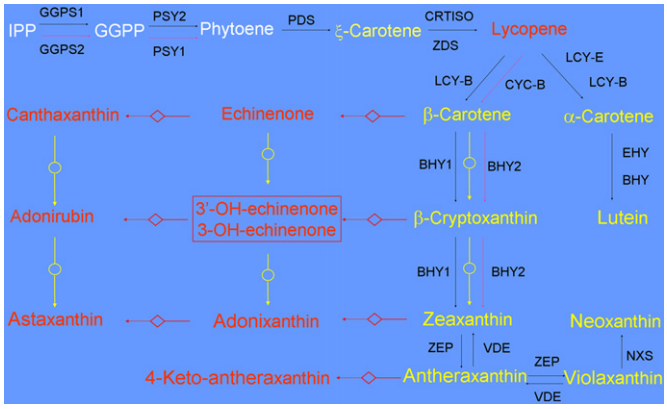

Fig. 1. Carotenoid biosynthetic pathways in tomato. Pink arrows indicate carotenogenesis in chromoplasts. Arrows with square or cycle in middle indicate ketolating or hydroxylating reactions conferred by transgenes. GGPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCY-B, lycopene β-cyclase; CYC-B, an isoenzyme of LCY-B responsible for the high β-carotene accumulation in B-type tomato fruit; LCY-E, lycopene ε-cyclase; BHY, β-ring hydroxylase; EHY, ε-ring hydroxylase; NXS, neoxanthin synthase; VDE, violaxanthin de-epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; ZEP, zeaxanthin epoxidase. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

crtW with plant carotenogenic enzymes. We have recently demonstrated that the *BKT* from *Chlamydomonas reinhardtii* confers efficient astaxanthin biosynthesis to the model plants Arabidopsis (Zhong et al., 2011) and tobacco (Huang et al., 2012). This special algal ketolase suits to efficiently convert tomato carotenes to astaxanthin, a xanthophyll that could possibly be sequestrated in esterified forms at high levels in the fruit.

The present study reported the engineering of tomato for high-yield production of astaxanthin by expressing a specific pair of algal *BKT* and *BHY* genes that were identified as the best combination for astaxanthin production from β-carotene in an *Escherichia coli* system. The resulting tomato accumulated high levels of non-native astaxanthin not only in vegetative leaves but also in the fruit of a tomato variety with high synthesis capacity for β-carotene. Moreover, the fruit accumulated 5-fold more astaxanthin than the leaves, reaching attractive levels for commercial production of natural astaxanthin.

2. Materials and methods

2.1. Constructs for E. coli transformation

The coding sequence of tomato *BHY*1 (Ronen et al., 2000) was cloned between the *Hind*III and *Xba*I sites of the plasmid pUC19. Similarly, the *BHY* coding sequences from *C. zofingiensis*, *H. pluvialis*, *Pantoea ananatis*, and Arabidopsis were cloned between the same sites of pUC19. For co-expression of *CrBKT* with different *BHYs* in *E. coli*, polycistronic plasmids were constructed by adding a Shine–Dalgarno (SD) sequence (AGGAGGACAGCC) upstream of the first ATG of *CrBKT* (Zhong et al., 2011) which was then inserted between the *Xba*I and *Sac*I sites of the *BHY*-harboring PUC19 plasmids resulting in polycistronic plasmids. *E. coli* JM109 carrying pACCAR16ΔcrtX was used as the host for functional analysis of the cooperation of *CrBKT* with various *BHYs* and tomato *BHY*1, respectively.

2.2. Constructs for plant transformation

pBI-SlTpCrBKT was derived from pBI121-CRBKT (Zhong et al., 2011) by replacing the transit peptide sequence of Arabidopsis *RBCS* with tomato one (GenBank No. M15236) using PCR-based cloning techniques. The *CrBKT* of pBI-SlTpCrBKT was replaced by *HpBHY* coding sequence (GenBank No. BD250390) to create pBI-SlTpHpBHY. The *CaMV35S*::*SlTpHpBHY*::*nos* cassette was amplified and inserted in the pBI121-SlTpCRBKT at the *Cla*I site resulting in pBI-SlTpHpBHY-SlTpCrBKT.

2.3. Tomato transformation

The generation of transgenic tomato plants was performed according to the protocol as described (van Roekel et al., 1993). Wild-type (cv. UC82B) and *Beta* mutant LA0316 tomato (*Solanum lycopersicum* Mill.) seeds were provided by TGRC (http://www.tgrc.ucdavis.edu/index.aspx). The presence and expression of the transgenes was verified by RT-PCR using *CrBKT* and *HpBHY* specific primers. The kanamycin-resistant putative transformed plants were grown in a green house.

2.4. Pigment extraction and analysis

Caroenoids were extracted and analyzed as described (Huang et al., 2006; Zhong et al., 2011). Pigments were identified on the basis of absorption spectra and retention times relative to standard compounds. Pigments were finally quantified by integrating peak areas that were converted to concentrations by comparison with authentic standards. Saponification of astaxanthin esters were performed according to Yuan and Chen (1999) and allotments of the extract solutions were analyzed by HPLC. Pigments were identified by comparing retention times and spectra against known standards and were finally quantified by integrating peak areas and converted them to concentrations by comparison with authentic standards that purchased from Sigma and Wako. Astaxanthin was measured at 480 nm and other carotenoids were measured at 450 nm.

2.5. Gene expression analysis

Total RNA samples were extracted from young tomato leaves or fruit harvested at the break stage. Real-time quantitative RT-PCR of endogenous carotenogenic genes was performed according to Zhong et al. (2011). Primers used in this study are listed in Table 1. PCR was run in a BIO-RAD iCycler IQ Multi-Color RealTime PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative levels of the amplified mRNA were evaluated according to the $2^{-\Delta\Delta Ct}$ method (Blois, 1958) using *actin* gene for normalization.

2.6. Photosynthesis analysis

The maximum quantum yield of PSII photochemistry was measured in dark-adapted samples by $(F_m-F_0)/F_m=F_v/F_m$ ratio, where F_0 is the dark-adapted initial fluorescence level and F_m is the maximum fluorescence level. CO_2 uptake (gas exchange) was measured using an LI-6400 portable photosynthesis system (LI-COR, http://www.licor.com). The leaf cuvette enclosed 6 cm^2 of leaf and was equipped with a thermostat to control leaf temperature. Light was provided by a Li-Cor light-emitting diode (LED). Leaves were measured the rate of CO_2 uptake (μmol CO_2 $m^{-2}s^{-1}$) in light photons of 0–1600 μmol photons $m^{-2}s^{-1}$ until the CO_2 rate is not increasing.

2.7. Antioxidant activity

Homogenized tomato samples (1 g of fresh full ripe fruit) were each extracted twice with acetone. DPPH (1.1-diphenyl-2-picrylhydrazyl) radical scavenging activity was determined according to the method of Livak and Schmittgen (2001). Equal volume (1 ml) of tomato extract and DPPH solution (0.2 mM in ethanol) was kept at room temperature for 30 min before the decrease of the solution