Journal of Experimental Botany, Vol. 62, No. 10, pp. 3659–3669, 2011
doi:10.1093/jxb/err070 Advance Access publication 11 March, 2011
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Functional characterization of various algal carotenoid ketolases reveals that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic *Arabidopsis*

Yu-Juan Zhong[1], Jun-Chao Huang[1,*], Jin Liu[1], Yin Li[2], Yue Jiang[3], Zeng-Fu Xu[4], Gerhard Sandmann[5] and Feng Chen[1,6,*]

[1] School of Biological Sciences, University of Hong Kong, Pokfulam Road, Hong Kong, China
[2] School of Life Sciences, Sun Yat-Sen University, 510275 Guangzhou, China
[3] Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China
[4] Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, 88 Xuefu Road, Kunming 650223, China
[5] Biosynthesis Group, Molecular Biosciences, J.W. Goethe University, Frankfurt, Germany
[6] Institute for Food and Bioresource Engineering, College of Engineering, Peking University, Beijing, China

* To whom correspondence should be addressed. E-mail: huangjc@hku.hk, sfchen@hku.hk

Received 5 January 2011; Revised 16 February 2011; Accepted 18 February 2011

Abstract

Extending the carotenoid pathway to astaxanthin in plants is of scientific and industrial interest. However, expression of a microbial β-carotene ketolase (BKT) that catalyses the formation of ketocarotenoids in transgenic plants typically results in low levels of astaxanthin. The low efficiency of BKTs in ketolating zeaxanthin to astaxanthin is proposed to be the major limitation for astaxanthin accumulation in engineered plants. To verify this hypothesis, several algal BKTs were functionally characterized using an *Escherichia coli* system and three BKTs were identified, with high (up to 85%), moderate (~38%), and low (~1%) conversion rate from zeaxanthin to astaxanthin from *Chlamydomonas reinhardtii* (*CrBKT*), *Chlorella zofingiensis* (*CzBKT*), and *Haematococcus pluvialis* (*HpBKT3*), respectively. Transgenic *Arabidopsis thaliana* expressing the *CrBKT* developed orange leaves which accumulated astaxanthin up to 2 mg g^{-1} dry weight with a 1.8-fold increase in total carotenoids. In contrast, the expression of *CzBKT* resulted in much lower astaxanthin content (0.24 mg g^{-1} dry weight), whereas *HpBKT3* was unable to mediate synthesis of astaxanthin in *A. thaliana*. The none-native astaxanthin was found mostly in a free form integrated into the light-harvesting complexes of photosystem II in young leaves but in esterified forms in senescent leaves. The alteration of carotenoids did not affect chlorophyll content, plant growth, or development significantly. The astaxanthin-producing plants were more tolerant to high light as shown by reduced lipid peroxidation. This study advances a decisive step towards the utilization of plants for the production of high-value astaxanthin.

Key words: *Arabidopsis thaliana*, astaxanthin, β-carotene ketolase, carotenoid, *Haematococcus pluvialis*.

Introduction

In plants and algae, carotenoids play important roles in the assembly of photosystems, in light harvesting, and in photoprotection. As precursors of vitamin A and as antioxidants in protecting cells from the damaging effects of free radicals and singlet oxygen, carotenoids are also essential components of human nutrition (Krinsky, 1989).

Abbreviations: BKT, β-carotene ketolase; MDA, malondialdehyde; LHC, light harvesting centre; PG, plastoglobulin; PS, photosystem; TEM, transmission electron microscope.

Increasing or changing carotenoid composition of crop plants to improve consumer health represents an important goal of plant breeding and genetic engineering efforts (Hirschberg, 1999; Giuliano *et al.*, 2000; Römer *et al.*, 2000; Paine *et al.*, 2005).

Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione) is a high-value ketocarotenoid that has powerful antioxidative activity and protection against a broad range of human diseases (Kurihara *et al.*, 2002; Hussein *et al.*, 2006; Ikeda *et al.*, 2008;). In nature astaxanthin is biosynthesized only in a few bacteria, certain green algae, and the basidiomycetous yeast *Xanthophyllomyces dendrorhous*. Rather small amounts of astaxanthin are also found in the flower of *Adonis* species. To date, the astaxanthin yields from these organisms are still too low to compete economically with that produced by chemical synthesis (Cunningham and Gantt, 2005; Misawa 2009; Zhu *et al.*, 2009). Nevertheless, the health benefits and high cost of natural astaxanthin (~US$7000 per kg) have attracted considerable interest in engineering astaxanthin pathways in suitable hosts, especially in plants.

Exhibiting high activity of carotenoid hydroxylase but lacking β-carotene ketolase (BKT/crtW) activity, plants are capable of forming 3-hydroxy carotenoids (e.g. lutein and zeaxanthin) but are unable to synthesize 4-ketocarotenoids (e.g. canthaxanthin and astaxanthin) (Fig. 1). In general, nuclear transgenic plants overexpressing a *BKT*/*crtW* gene from various sources are poor in astaxanthin synthesis (Mann *et al.*, 2000; Stalberg *et al.*, 2003; Ralley *et al.*, 2004; Gerjets and Sandmann, 2006; Morris *et al.*, 2006; Gerjets *et al.*, 2007; Suzuki *et al.*, 2007; Jayaraj *et al.*, 2008). Typically, adonixanthin (4-keto zeaxanthin) is the major keto product, which cannot be further metabolized into astaxanthin. This is due to the nature of the ketolases and the hydroxylases which compete for β-carotene as the same substrate. Since the ketolases known to date prefer an unsubstituted β-ionone ring, astaxanthin synthesis is possible only when ketolase concentration in transgenic plants is significantly higher than the endogenous hydroxylase level (see Zhu *et al.*, 2008 for detailed discussion). This explanation is supported by the fact that higher amounts of astaxanthin were obtained only in the leaves of tobacco plastid transformants with very high expression of a ketolase (Hasunuma *et al.*, 2008) and in carrot roots with very low endogenous hydroxylase activity transformed with *Haematococcus pluvialis BKT1* (Jayaraj *et al.*, 2008).

A strategy alternative to high expression of ketolase over hydroxylase is searching for a ketolase with increased substrate specificity for 3-hydroxy β-carotene derivatives. Therefore, in this study, an *Escherichia coli* expression

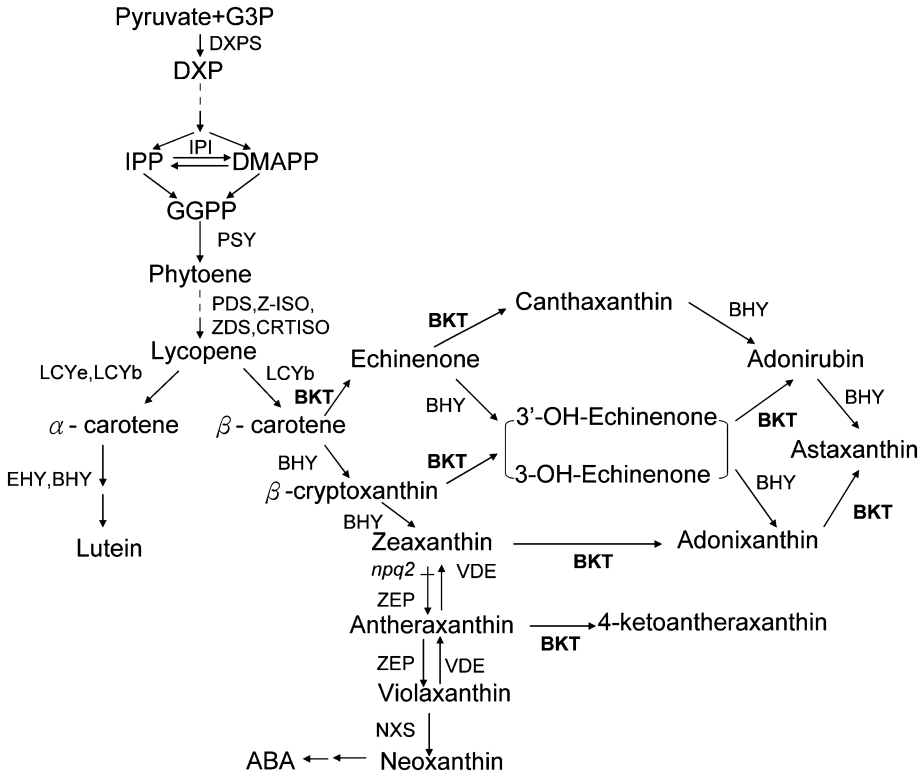

Fig. 1. Possible biosynthetic pathways to astaxanthin in transgenic *Arabidopsis* expressing an algal *BKT*. The abbreviations for intermediates and genes are as follows: G3P, D-glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; IPI, isopentenyl diphosphate isomerase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, 15-*cis*-ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYe, lycopene ε-cyclase; LCYb, lycopene β-cyclase; EHY, ε-carotene hydroxylase; BHY, β-carotene hydroxylase; BKT, β-carotene ketolase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase.

system was used to screen several green algal *BKT* cDNAs for their catalytic function with respect to astaxanthin synthesis. The *BKT* cDNA from *Chlamydomonas reinhardtii* (Lohr *et al.*, 2005), a green alga with no astaxanthin accumulation in vegetative cells, was found to encode a bifunctional ketolase that can convert β-carotene to canthaxanthin and zeaxanthin to astaxanthin efficiently. The *C. reinhardtii BKT* and the corresponding cDNAs from *H. pluvialis* and *Chlorella zofingiensis*, which encode ketolases with different efficiencies in ketolating zeaxanthin in *E. coli*, were then expressed in *Arabidopsis thaliana* in order to reveal the limiting step for astaxanthin biosynthesis in plants. It is shown here that only ketolases with high activity of converting zeaxanthin to astaxanthin trigger transgenic *Arabidopsis* to accumulate large amounts of astaxanthin in leaves and seeds. The locality of non-native ketocarotenoids and their effect on plant growth were also investigated.

Materials and methods

Plasmid constructs for expressing BKT genes in E. coli and Arabidopsis

The coding regions of *H. pluvialis BKT* (Huang *et al.*, 2006*a*) and *C. zofingiensis BKT* (Huang *et al.*, 2006*b*) were inserted as a *Hin*dIII–*Sac*I fragment into pBluescript II KS (Stratagene, USA) as in-frame fusion to the *lacZ* gene, generating plasmids pHPBKT3 and pCZBKT. *C. reinhardtii* BKT was found to contain a 120 amino acid extension at the carbonyl end compared with other BKTs (Lohr *et al.*, 2005). A truncated *C. reinhardtii BKT* that encodes protein of length compatible with the BKTs from the other two algae was amplified using RT-PCR with the following primers (restriction site extensions underlined, initiating and stop codons in bold): forward primer (*Hin*dIII) 5'-GAGAAGCTTC**ATG**GGCCCTGGGGATACA-3' and reverse primer (*Xba*I) 5'-GCGTCTAGA**TCA**GGCCAGGGCTGCGCCGCG-3'. The PCR product was inserted as a *Hin*dIII–*Xba*I fragment into pBluescript II KS (pCRBKT) and sequenced, revealing an open reading frame of 984 bp, encoding a protein of 328 amino acid residues and a stop codon. Plasmids containing individual *BKT* coding sequences were transformed into β-carotene/zeaxanthin-accumulating *E. coli* JM109 cells (Misawa *et al.*, 1995). Transformed *E. coli* cells were cultured in 4 ml of LB medium containing 30 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin with shaking in darkness at 28 °C for 48 h.

The coding sequence for the transit peptede of RBCS1 was amplified from *A. thaliana* genomic DNA with the following primers: forward primer (*Sal*I–*Sma*I) 5'-GAGGTCGACCCGGGCTCAGTCACACAAAGAGT-3' and reverse primer (*Hin*dIII) 5'-GAGAAGCTTGGCATGCAGTTAACTCTT-3'. The amplified fragment was inserted between *Sal*I and *Hin*dIII cutting sites of pHPBKT3, pCZBKT, and pCRBKT. The fused *BKT*s were released with *Sma*I and *Sac*I, which were then inserted into the corresponding sites of binary vector of pBI121 (Chen *et al.*, 2003). Binary plasmids were transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Production and growth of transgenic A. thaliana

Wild-type *A. thaliana* Col-0 and mutant *npq2*, provided by TAIR (http://www.arabidopsis.org/), was transformed using the *Agrobacterium* floral dip method (Clough and Bent, 1998). The *npq2* mutant accumulates zeaxanthin due to a mutation in the zeaxanthin epoxidase gene (*zep*). It was used to investigate whether zeaxanthin is a preferred precursor for astaxanthin in this study. Kanamycin-resistant *A. thaliana* T_0 plants were selected on MS medium plates (Murashige and Skoog, 1962) and then grown in soil in a chamber at a photon flux density of 250 μmol m^{-2} s^{-1} with 16-h light, 22 °C and 8-h dark, 20 °C. T_2 *A. thaliana* transformants were used for all the experimental analyses. For high-light treatment, 4-week-old wild-type and transgenic *A. thaliana* were transferred to a growth chamber at 22 °C and a photon flux density of 1000 μmol m^{-2} s^{-1} (photoperiod, 12 h) for a week.

Pigment analysis

Carotenoid-producing *E. coli* cells were collected by centrifugation, and pigments were extracted and analysed by HPLC as described previously (Huang *et al.*, 2006*a*; Li *et al.*, 2008). For quantifying carotenoids of plant transformants, 6 mg of freeze-dried leaves or 60 mg of freeze-dried mature seeds were ground with acetone and liquid nitrogen until the cell debris was almost colourless. The combined acetone extracts were pooled and centrifuged at 15 000×*g* for 20 min to remove particulate matter and then brought to dryness under a stream of nitrogen. Twenty microlitres of each extract was separated by HPLC on a Waters Spherisorb® 5 μm ODS2 4.6 250 mm analytical column with a Waters HPLC system (Waters, Milford, MA, USA) using a modified method previously described by Baroli *et al.* (2003). Pigments were eluted at a flow rate of 1.2 ml min^{-1} with a linear gradient from 100% solvent A [acetonitrile/methanol/0.1 M TRIS–HCl (pH 8.0), 84:2:14, v/v/v] to 100% solvent B (methanol/ethyl acetate, 68:32, v/v) over a 15-min period, followed by 10 min of 100% solvent B. Pigments were identified on the basis of their absorption spectra and retention times relative to standard compounds. Pigments were finally quantified by integrating peak areas and converting them to concentrations by comparison with authentic standards purchased from Sigma (St Louis, MO, USA) and Wako (Osaka, Japan). 4-Ketoantheraxanthin and antheraxanthin were quantified using violaxanthin as standard considering their similarity in light absorbance. Astaxanthin was measured at 480 nm and other carotenoids were measured at 450 nm. Saponification of astaxanthin esters was performed according to Yuan and Chen (1999*a*). A freshly prepared 1-ml aliquot of 0.107 M NaOH dissolved in methanol was added to 5 ml of pigment extract solution under a nitrogen atmosphere. The mixture was evaporated and concentrated to 5 ml under nitrogen and then placed at ambient temperature (22 °C) in darkness under nitrogen atmosphere for complete saponification of astaxanthin esters. After saponification, the extract solutions were directly analysed by HPLC.

Analysis of pigments in thylakoid membranes

Chloroplast thylakoid membranes were isolated from 4-week-old leaves of *A. thaliana* according to the method described by Huber *et al.* (2004). Membrane pigment–protein complexes were extracted and ultracentrifuged as described by Dall'Osto *et al.* (2005). Four visible bands were harvested, from which pigments were extracted and analysed by HPLC.

Histological analysis and electron microscopy

For cryostat microtome section preparation, *A. thaliana* rosette leaf samples were embedded with tissue freezing medium and then under liquid nitrogen sectioned at 15 μm in a cryostat microtome at –20 °C. The sections were melted onto glass slides precoated with gelatin. For conventional microscopy, senescent *A. thaliana* rosette leaves were fixed in 2.5% (v/v) glutaraldehyde and 0.1 M sodium phosphate (pH 7.2) with post-fixation in 1% (v/v) osmium tetroxide (OsO_4). The fixed specimens were dehydrated and embedded in Epoxy resin. Samples were stained with uranyl acetate and lead citrate, and finally viewed under a transmission electron microscope (TEM) (EM208s; Philips, Netherlands).

PCR and RT-PCR analysis

Standard DNA/RNA techniques were carried out according to the methods described in Sambrook *et al.* (1989). DNA fragment flanking the T-DNA was cloned by a PCR-walking method using BD GenomeWalking Kit (BD Biosciences) (Cottage *et al.*, 2001). Specific primers were 5'-CCGCTCATGATCAGATTGTCG-3' and 5'- CGTTTCCCGCCTTCAGTTTA-3'. Total RNA was extracted from *A. thaliana* leaves using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The concentration of total RNA was determined spectrophotometrically at 260 nm coupled with agarose gel electrophoresis. RNA samples were treated with DNase I (Invitrogen). Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA by using a SuperScript III First-Strand Synthesis System primed with oligo(dT) according to the manufacturer's instructions (Invitrogen). RT-PCR analysis of transgene expression and endogenous carotenogenic genes in *Arabidopsis* was performed using the primers listed in Table 1. The expression of endogenous actin gene was used to demonstrate equal amounts of templates and loading. Real-time quantitative PCR of endogenous carotenogenic genes was performed from 1 μl of the RT reaction mixture in a total volume of 20 μl with 0.5 μM each pair of specific primers, 10 μl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). 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 (Livak and Schmittgen, 2001) using the *actin* gene for normalization.

Biochemical determination of lipid peroxidation

Lipid peroxidation was assessed by measurement of malondialdehyde (MDA) using HPLC. MDA is a three-carbon, low molecular weight aldehyde that is produced from radical attack on polyunsaturated fatty acids. Samples (several leaf discs of 1 cm in diameter) were ground in 750 μl of chilled 80% ethanol. After centrifugation, 250 μl of supernatant was combined with 250 μl of the following reaction mixture: 20% trichloroacetic acid, 0.01% butylated hydroxytoluene, and 0.65% thiobarbituric acid (TBA). After heating at 95 °C for 20 min and centrifugation, the MDA-$(TBA)_2$ adduct was separated and quantified by HPLC as described by Havaux *et al.* (2005). The average retention time of the MDA-$(TBA)_2$ adduct was 8 min. The levels of MDA were calculated using tetraethoxypropane (Sigma) as a standard.

Table 1. Primers used for RT-PCR analysis of transgene expression and endogenous carotenogenic genes in *Arabidopsis*

Primers were designed using the GeneTool program (Biotool, Edmonton, AB, Canada) to generate 100- to 250-bp PCR products.

Primer	Sequence	GenBank accession No.
Hp-BKT3 F	TCGCCGCAGTCTTCATTGTA	AY603347
Hp-BKT3 R	AGGCGTACAGTGATATGCAGATG	
Cz-BKT F	GGCTGCTGGCAAATCAAACTCAC	AY772713
Cz-BKT R	CGACGGTTCCTGATGGCAATAG	
Cr-BKT F	CCGCCTTCCGCCTGTTCTACTA	AY860820
Cr-BKT R	GGCGGCACTTGGGCAGCT	
Actin F	TGGCGATGAAGCTCAATCC	NM_179953
Actin R	CACTGGCATAAAGAGAAAG	
DXSP3 F	GGGACCGGGTTGTTGCTGTGA	NM_001085101
DXSP3 R	TGCGCTGATGGATGCCTTGG	
PSY F	CCCGGTCGATATTCAGCCATTT	NM_001036818
PSY R	CTCGCATCTTCGCCTACGTCTCT	
PDS F	TGCGGAACAACGAGATGCTGAC	NM_202816
PDS R	GTCGGTCACGCGCTCAGGTATT	
CRTISO F	TCGGTTCTGGGATTGGTGGATTA	NM_100559
CRTISO R	TTGGGGAGATGGAAATGGACAGT	
LCY F	CGGCGTAGACAGAGGGAGTTC	NM_111858
LCY R	GGGAGAAACAGCCTGGAAGAC	
CHY1 F	CCGCCTCCCGAAATCCTTATC	NM_118702
CHY1 R	GCCAAACGCAACGCCAGATAC	

Fluorometry

Chlorophyll fluorescence emission of leaves attached to the plant was measured with an OS5p Portable Fluorometer (Opti-Sciences, USA). The maximum quantum yield of photosystem (PS) II 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.

Results

Functional comparison of green microalgal BKTs in an *E. coli* system

β-Carotene ketolase is the rate-limiting enzyme for astaxanthin biosynthesis in transgenic plants (Zhu *et al.*, 2008). To engineer an efficient biosynthetic pathway in plants, a search was conducted for novel *BKT* cDNAs from green microalgae encoding enzymes with high efficiency for converting zeaxanthin to astaxanthin. The functionality of *BKT* cDNAs from the green microalgae *H. pluvialis* (*HpBKT3*) (Huang *et al.*, 2006*a*), *C. zofingiensis* (*CzBKT*) (Huang *et al.*, 2006*b*), and *C. reinhardtii* (*CrBKT*) (Lohr *et al.*, 2005) were compared using β-carotene or zeaxanthin as substrate in *E. coli*. The results are summarized in Table 2. All the three BKT enzymes are able to convert β-carotene to canthaxanthin via echinenone efficiently with the *C. zofingiensis* BKT having lower efficiency. However, the BKT function varied greatly in ketolating zeaxanthin to astaxanthin. *H. pluvialis* BKT3 catalysed this reaction poorly and the *C. zofingiensis* BKT moderately. In contrast, high activity for zeaxanthin conversion (~85% conversion rate) was found for *C. reinhardtii* BKT. Therefore, HpBKT3 is a β-carotene ketolase while CzBKT and CrBKT are β-carotene/zeaxanthin ketolases.

Table 2. Activity of β-carotene ketolase with β-carotene or zeaxanthin as substrate in *E. coli*

Data given as mol percentage (%), which was the average of five to eight repeats. Carotenoids accumulated in a strain of *E. coli* harbouring the plasmid pACCAR16ΔcrtX or pACCAR25ΔcrtX (Misawa *et al.*, 1990), which accumulate, respectively, β-carotene or zeaxanthin. Car, β-carotene; Ech, echeninone; Can, canthaxanthin; Zea, zeaxanthin; Ado, adonixanthin; Ast, astaxanthin; pHPBKT3, pCZBKT, and pCRBKT represent pBluescript II KS vectors harbouring *H. pluvialis BKT3*, *C. zofingiensis BKT*, and modified *C. reinhardtii BKT*, respectively.

Vector	pACCAR16ΔcrtX			pACCAR25ΔcrtX		
	Car	Ech	Can	Zea	Ado	Ast
None	100	0	0	100	0	0
pHPBKT3	1	3	96	87	12	1
pCZBKT	6	11	83	34	28	38
pCRBKT	0	2	98	11	4	85

Construction of transgenic A. thaliana overexpressing different BKT cDNAs

To address whether the functionality of BKT enzymes is the limiting step for plants to accumulate astaxanthin, three expression cassettes were constructed containing each of the BKT coding sequences fused to the transit peptide sequence of *A. thaliana* ribulose-1,5-biosphosphate carboxylase small subunit 1 (RBCS1) and driven by the cauliflour mosaic virus (CaMV) 35S promoter (Fig. 2A). The constructs, namely pHpBKT3, pCzBKT, and pCrBKT, were delivered to wild-type *A. thaliana* by the *Agrobacterium*-mediated method. Transformations per construct generated 48, 42, and 47 kanamycin-resistant lines, respectively. Marked differences in performance of the BKTs were observed in terms of the transgenic plant phenotypes (Fig. 2B). pHpBKT3 transformants had the same green leaves as wild type. pCzBKT transformants exhibited a light-green leaf pigmentation, whereas pCrBKT transformants displayed reddish brown leaves, inflorescence, and flowers (Fig. 2B, CR-5). Encouraged by these results, pCrBKT was also introduced into zeaxanthin-accumulating *A. thaliana* (*npq2* mutant) due to a mutation in the zeaxanthin epoxidase gene (*ZEP*) to investigate whether zeaxanthin is a preferred precursor for astaxanthin (Fig.1). Thirty-three transgenic lines of *npq2* plants were obtained which also showed reddish brown leaves. Selected transformants HP-13 directed from *HpBKT3*, CZ-3 from *CzBKT*, two lines, named CR-4 and CR-5, from *CrBKT*, and a transformant of the *npq2* line named NPQ2-9 were further analysed. Transcripts of *BKT* genes were detected by RT-PCR in all transgenic lines but were absent as expected in non-transformed controls (Fig. 2C).

Carotenoid composition of transgenic A. thaliana

To reveal the functionality of the various BKTs in *A. thaliana*, the carotenoid composition in the leaves (4 weeks old) of representative transgenic lines was analysed. The results are listed in Table 3. No ketocarotenoids were found in leaves of wild type or *npq2* controls or in transgenic lines overexpressing the *H. pluvialis BKT3* (Table 3). In contrast, astaxanthin and other ketocarotenoids mainly adonixanthin, adonirubin, and canthaxanthin were detected in the leaves of transformants carrying either the *C. zofingiensis* or the *C. reinhardtii BKT* (Table 3). The reddish leaves of CR-4 and CR-5 lines accumulated larger amounts of the red pigment astaxanthin [1.37 and 0.85 mg g^{-1} (DW)] together with other ketocarotenoids. Astaxanthin and other ketocarotenoids are the major carotenoids in the leaves of CR-4 and CR-5 accounting for 75% and 73% of total carotenoid, respectively. The transformants of the *npq2* line (NPQ2-9) accumulated even higher amounts of astaxanthin (2.07 mg g^{-1} DW) and total ketocarotenoids (4.51 mg g^{-1} DW). Astaxanthin existed predominantly in a free form (84–96%) with the rest being in monoester or diester form in young leaves. Astaxanthin from the transformants was identified mainly as the *trans*-isomer (~95%) with minor amounts of 9-*cis*- and 13-*cis*-isomers

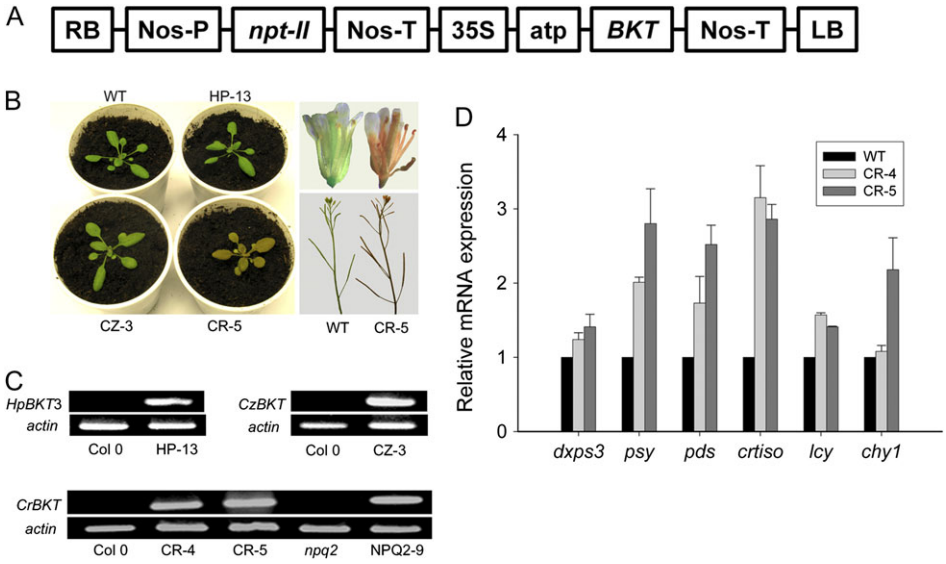

Fig. 2. Molecular characterization of transgenic *Arabidopsis*. (A) Map of T-DNA region of the binary vector used for transformation. LB and RB, left and right borders of the T-DNA; Nos-P, nopaline synthase gene (*nos*) promoter; *nptII*, neomycin methyltransferase II gene; Nos-T, the *nos* terminator; 35S, CaMV 35S promoter; atp, the transit peptide sequence from *Arabidopsis* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU); *BKT*, β-carotene ketolase from *H. pluvialis*, *C. zofingiensis*, or *C. reinhardtii*. (B) Phenotypes of wild-type and transgenic *Arabidopsis* lines. HP-13, *H. pluvialis BKT*3 transformant; CZ-3, *C. zofingiensis BKT* transformant; CR-5, *C. reinhardtii BKT* transformant. (C) RT-PCR analysis of transgene expression in leaves, *actin* serves as internal control. Col-0, wild-type *Arabidopsis*; *npq*2, zeaxanthin-accumulating mutant; NPQ2-9, *C. reinhardtii BKT* transgenic *npq*2 line. (D) Relative expression levels of isoprenoid biosynthetic genes (*dxps3*, *psy*, *pds*, *crtiso*, *lcy*, and *chy1*) in wild type and *C. reinhardtii BKT* transformants (CR-4 and CR-5). The levels of expression were normalized to *actin* relative to the wild-type plants, which was set to 1. The data represent average values from the analysis of three different *Arabidopsis* plants. Error bars indicate ±SEM.

according to the method of Yuan and Chen (1999*b*). Surprisingly, all the transformants with *C. reinhardtii BKT* accumulated much more total carotenoids than wild type, e.g. NPQ2-9 accumulated total carotenoids up to 4.82 mg g^{-1} DW, which is >2-fold higher than in wild type. Much lower amounts of ketocarotenoids and astaxanthin were synthesized in the *C. zofingiensis BKT* transformants. CR-4 was detected as a heterozygous line harbouring a T-DNA insertion at the +3530 bp site of the *Toc159* gene, which is required for the quantitative import of photosynthetic proteins (Bauer *et al.*, 2000). Its homozygous plant had an albino phenotype and did not survive.

Since astaxanthin was not sequestrated by esterification, this non-native pigment may be specifically recruited into photosynthetic pigment–protein complexes as is the case for non-native capsanthin and capsorubin in transfected tobacco leaves (Kumagai *et al.*, 1998). To prove this expectation, the location of ketocarotenoids was determined by analysing the pigment composition of different photosynthetic complexes and the result is shown in Table 4. As expected, ketocarotenoids were only found in monomers and trimers of light harvesting centre II (LHCII), the complexes of PSII antenna.

The increase in total carotenoid content in *CrBKT* transformants may be due to a transcriptional deregulation of the pathway resulting from the engineered formation of ketocarotenoids as observed before for tobacco plastid transformants (Hasunuma *et al.*, 2008). Real-time quantitative RT-PCR revealed that the transcript levels of *PSY*, *PDS*, and *CRTISO* in *CrBKT* transformants are ~2- to 3-fold that in wild type (Fig. 2D). An increase in other carotenogenic genes including 1-deoxy-D-xylulose-5-phosphate synthase (*DXPS3*), and β-carotene hydroxylase1 (*CHY1*) was not statistically significant.

During ageing, the green leaves of wild-type *A. thaliana* gradually turned yellow. In contrast to wild type, CR-4

Table 3. Carotenoid content (mg g^{-1} DW of tissues) in leaves of wild-type and transgenic *Arabidopsis*

Total carotenoid content was calculated as the sum of the content of each carotenoid. Carotenoid content was analysed by one-way *ANOVA*, carried out by a *post-hoc* Tukey's honestly significant difference (HSD) test for more than three group means or a paired-samples *t* test for two group means. Values are the averages from measurements of three individual *Arabidopsis* plants, ±SE. Values followed by the same letter are not significantly different (*P*>0.05). Values in parentheses are the percentage of esterified astaxanthin/adonixanthin in total astaxathin/adonixanthin. nd, not detected.

	Wild-type	CR-4	CR-5	HP-13	CZ-3	*npq2*	NPQ2-9
Total carotenoid	2.11±0.01^{a}	3.69±0.15^{b}	3.42±0.25^{b}	2.14±0.02^{a}	2.30±0.32^{a}	2.26 ±0.34^{a}	4.82 ±0.38^{c}
Total ketocarotenoid	nd	2.76±0.10^{a}	2.49±0.28^{a}	nd	0.97±0.10^{b}	nd	4.51 ±0.33^{c}
4-Ketoantheraxanthin	nd	0.19±0.03^{a}	0.05±0.01^{b}	nd	0.24±0.14^{a,c}	nd	nd
Astaxanthin	nd	1.37±0.05^{a} (4)	0.85±0.04^{b} (7)	nd	0.27±0.04^{c} (5)	nd	2.07±0.19^{d} (14)
Adonixanthin	nd	0.39±0.01^{a} (6)	0.37±0.05^{a,b} (8)	nd	0.26±0.00^{b} (4)	nd	1.24±0.10^{c} (7)
Adonirubin	nd	0.37±0.02^{a}	0.46±0.04^{b}	nd	0.16±0.00^{c}	nd	0.42±0.02^{a,b}
3'-OH-echinenone	nd	0.10±0.01^{a}	0.16±0.03^{a}	nd	nd	nd	0.44±0.03^{b}
Canthaxanthin	nd	0.24±0.01^{a}	0.55±0.12^{b}	nd	nd	nd	0.33±0.02^{a}
Echinenone	nd	0.08±0.01^{a,b}	0.10±0.02^{a}	nd	0.06±0.01^{b}	nd	0.01±0.00^{c}
Lutein	1.30±0.01^{b}	0.64±0.03^{a}	0.67±0.02^{a}	1.19±0.03^{b}	0.74±0.09^{a}	0.98±0.15^{c}	0.29±0.05^{d}
Zeaxanthin	nd	nd	nd	nd	nd	0.99±0.12	nd
Neoxanthin	0.21±0.01^{a}	0.02±0.00^{b}	0.01±0.00^{b}	0.18±0.01^{a}	0.07±0.03^{c}	nd	nd
Violaxanthin	0.25±0.01^{a}	0.08±0.00^{b,c}	0.02±0.00^{b}	0.35±0.02^{d}	0.16±0.05^{c}	nd	nd
Antheraxanthin	0.03±0.01^{a}	nd	nd	0.06±0.00^{a}	nd	nd	nd
β-Carotene	0.33±0.00^{c}	0.22±0.01^{a}	0.24±0.04^{a}	0.37±0.01^{c}	0.35±0.01^{c}	0.30±0.07^{c}	0.02±0.00^{b}

Table 4. Carotenoid and chlorophyll composition in pigment–protein complexes of wild-type and CR-5 transgenic *Arabidopsis*

Carotenoid and chlorophyll composition are expressed as mol percentage of total pigments (carotenoids and chlorophylls) in each pigment–protein band. The value given is the mean±SE of three experiments. nd, not detected.

Pigment–protein complex	Plants	Carotenoids						Chlorophylls	
		Ketocarotenoids	β-Carotene	Lutein	Violaxanthin	Neoxanthin	Antheraxanthin	Chlorophyll a	Chlorophyll b
LHCII monomers, minor antennae	WT	nd	2.32±0.02	18.57±1.31	5.37±0.06	0.30±0.01	0.24±0.01	46.06±2.43	27.14±1.08
	CR-5	24.17±1.86	0.20±0.01	5.23±0.11	0.40±0.02	0.45±0.02	nd	46.96±0.53	22.60±1.49
LHCII trimers	WT	nd	0.21±0.02	11.72±0.89	2.57±0.10	1.36±0.09	nd	51.20±2.66	32.95±1.94
	CR-5	8.08±0.01	0.10±0.01	7.23±0.13	0.24±0.01	0.10±0.00	nd	44.03±1.21	40.21±1.10
PSII core proteins	WT	nd	8.12±0.50	6.70±0.64	1.65±0.17	0.77±0.18	nd	71.10±2.33	11.66±0.83
	CR-5	nd	6.72±0.23	3.14±0.03	nd	nd	nd	66.16±0.81	23.98±1.02
LHCI, PSI core proteins	WT	nd	21.50±1.46	5.10±0.12	1.13±0.01	0.30±0.05	0.11±0.01	58.88±2.85	12.98±1.45
	CR-5	nd	13.58±1.63	3.63±0.09	nd	nd	nd	62.45±1.93	20.44±0.21

and CR-5 developed orange senescent leaves. Carotenoid composition and content in senescent leaves of wild type and CR-5 were analysed and shown in Fig. 3A. Carotenoid content is lower in both wild-type and transgenic senescent leaves than in young leaves. However, senescent leaves of CR-5 transformant kept 70% of total carotenoids of young leaves with 85% being ketocarotenoids, whereas wild-type senescent leaves maintained only 30% of total carotenoids of young leaves (Fig. 3A). Furthermore, 88% of astaxanthin was found in ester forms in CR-5 senescent leaves; in contrast, astaxanthin was found predominantly in free form in young leaves (Fig. 3A, Table 3). To locate the storage site of astaxanthin and adonixanthin, cryostat microtome section was performed for senescent leaves (7 weeks old) of wild type and CR-5 at a similar site on their rosettes. The wild-type leaves contained only some yellow grains, whereas CR-5 contained many more grains in red (Fig. 3B). In senescent leaves, chloroplasts degenerate to gerontoplasts with the disappearance of thylakoids and an increase in plastoglobulins (PGs) that contain a variety of compounds including carotenoids (Biswal *et al.*, 2003). TEM study showed that many more PGs occurred in CR-5 gerontoplasts than in wild-type ones (Fig. 3B). This result indicated that esterified astaxanthin might be stored in PGs together with other carotenoid esters.

It is interesting to investigate the ketocarotenogenesis in seeds that contain different content and profiles of carotenoids from leaves. The carotenoids from mature seeds of wild type, *npq2*, CZ-3, CR-5, and NPQ2-9 were measured by HPLC (Table 5). *A. thaliana* contains much lower amounts of total carotenoids in seeds (e.g. 32.63 μg g^{-1} DW in wild type and 66.62 μg g^{-1} DW in *npq2*) than in leaves (2110 μg g^{-1} DW in wild type and 2260 μg g^{-1} DW in *npq2*). Total carotenoids in the transformants were 2-fold higher than controls with about half being ketocarotenoids and a quarter being astaxanthin. The efficiency of astaxanthin biosynthesis in seeds of *C. reinhardtii BKT* transformants was much lower than in leaves, possibly resulting from the different status of BKT cofactors between the two organs.

Growth, photosynthesis, and high-light tolerance of transgenic Arabidopsis plants

To study the impact of carotenoid alteration in leaves on plant growth and photosynthesis, the maximum diameter, chlorophyll content, and the photosynthetic parameter F_v/F_m were measured for maximum quantum yield of wild type and transgenic CR-4 and CR-5 plants grown at 22 °C under a light intensity of 250 μmol m^{-2} s^{-1} (Table 6). Compared with the wild type, the values for all these parameters were slightly but not significantly lower for the transformants. This result indicates that the drastic changes in carotenoid composition do not affect photosynthesis and growth of the transgenic plants.

Astaxanthin was proposed to protect algal cells from high-light stress (Wang *et al.*, 2003). To investigate whether the accumulation of astaxanthin and other ketocarotenoids confers any improvement in stress tolerance, the amount of MDA, a product of the oxidation of polyunsaturated fatty acids, as stress indicator (Davison *et al.*, 2002) was determined in wild-type, CR-4, and CR-5 plants exposed to high light (1000 μmol m^{-2} s^{-1}). As shown in Table 6, the mean values for wild type, CR-4, and CR-5 were 16.43, 9.28, and 9.71 ng cm^{-2} leaf area, respectively. This resembles at least a 40% decrease in lipid peroxidation in the transgenic lines, indicating higher tolerance of CR-4 and CR-5 to high-light stress than wild type.

Discussion

The algal BKT genes

The functions of three algal BKTs were elucidated first in *E. coli* and then in *A. thaliana*. They all ketolate β-carotene but differ in their substrate specificity for zeaxanthin (Table 2). In this respect, the ketolase from *C. reinhardtii* was superior to the enzyme from *C. zofingiensis*. Transgenic *A. thaliana* overexpressing the *BKT* cDNAs accumulated large amounts of astaxanthin in leaves at concentrations of up to 2 mg g^{-1} DW for the *C. reinhardtii* BKT in combination with a zeaxanthin-accumulating background (Table 3). This is the highest level ever reached by nuclear transformation. Other previous plant transformants with different β-carotene ketolase genes, e.g. *BKT1* and *BKT2* from *H. pluvialis*, *crtW* from bacteria, and *crtO* from cyanobacteria (Mann *et al.*, 2000; Stalberg *et al.*, 2003; Ralley *et al.*, 2004; Gerjets and Sandmann 2006; Morris *et al.*, 2006; Suzuki *et al.*, 2007; Zhu *et al.*, 2007; Jayaraj *et al.*, 2008) resulted in much lower yields or even failed to synthesize astaxanthin. Even the amounts of astaxanthin in non-photosynthetic tissues, e.g. in carrot roots transformed with *H. pluvialis BKT1*, did not exceed ~1 mg g^{-1} DW (calculated from Jayaraj *et al.*, 2008). It is difficult to decide whether the high astaxanthin content (1.88 mg g^{-1} DW in the *crtW*-expressing line) in the transplastomic tobacco plants can be attributed to a better efficiency of astaxanthin synthesis by the *Brevundimonas* sp. SD212 *crtW* and/or to the high levels of protein accumulation in this plastid transformant (Hasunuma *et al.*, 2008).

This study showed that screening for a catalytically improved *BKT* gene can be successful. The *C. reinhardtii* BKT proved more efficient in ketolating zeaxanthin than the *C. zofingiensis* and *H. pluvialis* BKTs, both of which are involved in high astaxanthin accumulation in the algae. Interestingly, BKT3 from the richest astaxanthin-producing organism, *H. pluvialis*, although active in converting β-carotene to canthaxanthin in *E. coli* (Table 2), failed to trigger astaxanthin biosynthesis in *A. thaliana* (Table 3), whereas BKT1 and BKT2 from *Haemotococcus* are active in plants (Mann *et al.*, 2000; Stalberg *et al.*, 2003). Differences in transgene expression are unlikely to be a primary factor because all the *BKT* transgenes were driven by the same expression cassette (Fig. 2B). It should also be noted that the green alga *C. reinhardtii* has not been reported to synthesize astaxanthin (Lohr *et al.*, 2005) even though it

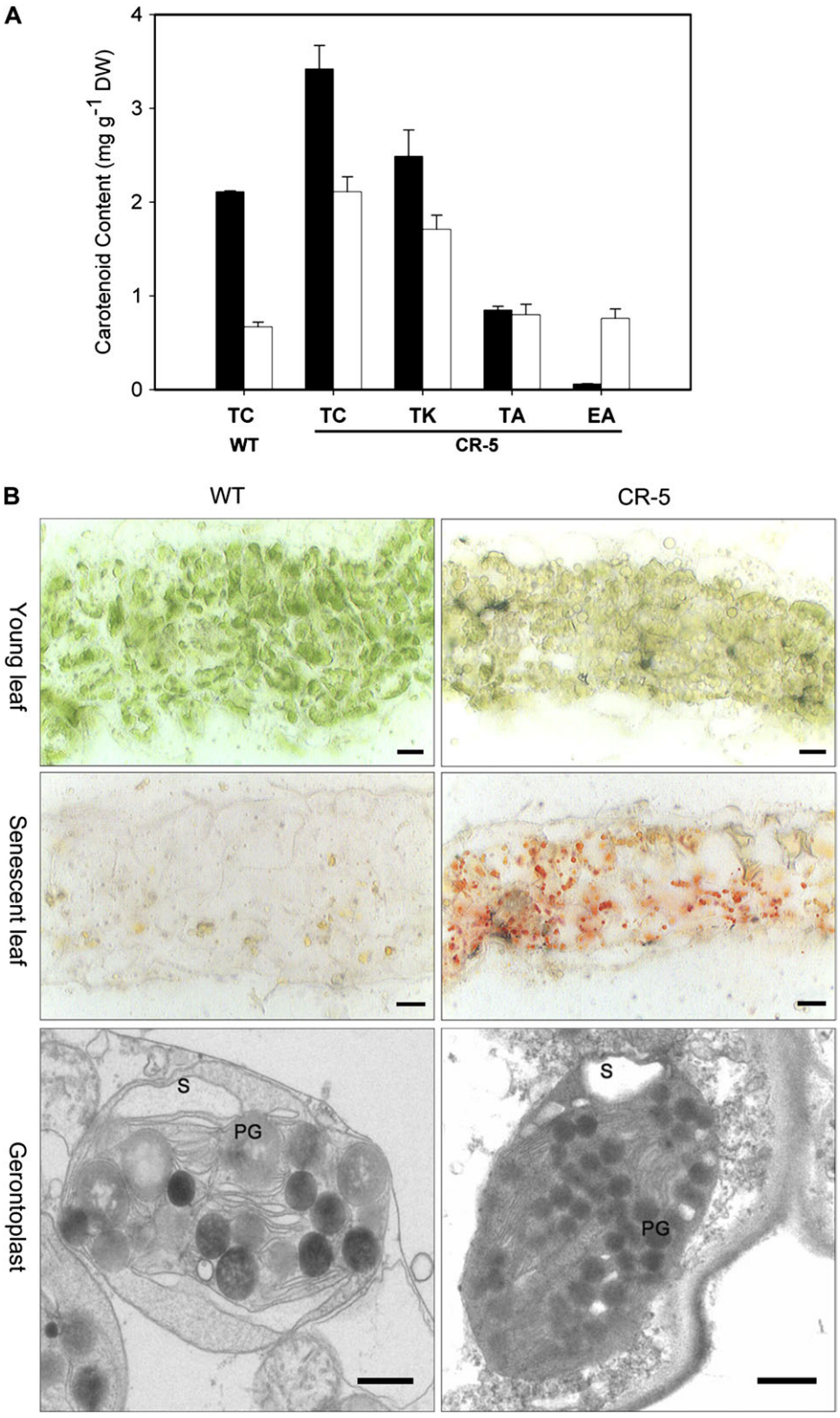

Fig. 3. Differential carotenoid content and cell structure between young leaves and senescent leaves. (A) Carotenoid content of young (black column) and scenescent (white column) leaves from wild type (WT) and CR-5 (a *C. reinhardtii BKT* transformant). TC, total carotenoids; TK, total ketocarotenoids; TA, total astaxanthin; EA, esterified astaxanthin. (B) Microscope images of WT (left) and CR-5 (right) leaf samples. Transverse sections of young (upper row) and senescent (middle row) leaves in WT and CR-5 (bar 5μm). TEM analysis of senescent leaves (lower row) showed the gerontoplast of WT and CR-5 plants (bar 0.5μm). S, starch; PG, plastoglobulin.

Table 5. Carotenoid content of wild-type and transgenic *Arabidopsis* mature seeds

Total carotenoid content was calculated as the sum of each carotenoid content. Carotenoid content was analysed by one-way ANOVA, carried out by a *post-hoc* Tukey's HSD test for more than three group means or a paired-samples *t* test for two group means. Values are the averages from measurements of seed collections of three individual plants, ±SE. Values followed by the same letter are not significantly different ($P>0.05$). Values in parentheses are the percentage of esterified astaxanthin/adonixanthinin total astaxathin/adonixanthin. nd, not detected.

	Carotenoid content (μg g^{-1} DW) Wild-type	CR-5	CZ-3	*npq2*	NPQ2-9
Total carotenenoid	32.63±1.30[a]	69.03±1.02[c]	57.36±1.40[c]	66.62±3.88[c]	113.97±0.43[b]
Total ketocarotenoid	nd	36.80±1.16[b]	29.37±0.42[b]	nd	59.00±2.97[c]
4-Ketoantheraxanthin	nd	0.14±0.00[b]	0.06±0.00[c]	nd	nd
Astaxanthin	nd	17.23±1.55[b] (69)	11.97±0.75[b] (88)	nd	32.12±2.21[c] (89)
Adonixanthin	nd	16.64±0.49[b] (61)	13.06±0.74[b] (69)	nd	23.59±0.26[c] (42)
Adonirubin	nd	nd	1.48±0.01[a]	nd	0.13±0.03[b]
3'-OH-echinenone	nd	nd	0.34±0.11	nd	nd
Canthaxanthin	nd	nd	nd	nd	nd
3-OH-echinenone	nd	0.51±0.04[a]	0.98±0.07[a]	nd	1.46±0.17[b]
Echinenone	nd	2.27±0.07[b]	1.48±0.21[b]	nd	1.71±0.36[b]
Lutein	30.62±1.31[a]	25.85±0.10[c]	26.20±0.83[c]	22.86±1.45[b]	16.39±0.73[b]
Zeaxanthin	nd	nd	nd	39.27±6.58[a]	54.39±3.54[b]
Neoxanthin	0.09±0.01[a]	0.08±0.01[a]	0.07±0.01[a]	nd	nd
Violaxanthin	0.30±0.00[a]	0.26±0.02[a]	0.26±0.05[a]	nd	nd
Antheraxanthin	0.13±0.00[a]	0.05±0.01[c]	0.09±0.02[a]	nd	nd
β-Carotene	1.49±0.11[a]	1.20±0.02[a]	1.37±0.11[a]	4.49±1.23[c]	2.31±0.02[b]

Table 6. Photosynthesis parameters and high-light tolerance of wild-type and transgenic *Arabidopsis* plants

	Wild type	CR-4	CR-5
Rossette diameter (cm)[a]	9.80±0.57	9.10±0.33	8.20±0.79
Chlorophyll a (mg g^{-1} DW)[b]	8.72±0.3	8.54±0.37	7.98±0.19
Chlorophyll b (mg g^{-1} DW)[b]	4.16±0.05	4.11±0.11	3.75±0.34
Chlorophyll a/b[b]	2.22±0.09	2.14±0.09	2.14±0.15
F_v/F_m[b]	0.80±0.01	0.75±0.04	0.76±0.01
MDA (ng cm^{-2})[c]	16.43±0.76	9.28±0.16	9.71±0.80

[a] Values are the averages from measurements of 10 6-week-old plants ±SE.
[b] Values are the averages from measurements of five 4-week-old plants ±SE.
[c] Values are the averages from measurements of five 4-week-old plants exposed to a photon flux density of 1000 μmol m^{-2} s^{-1} (photoperiod, 12 h) for a week ±SE.

contains a *BKT* gene with high potential for astaxanthin biosynthesis as demonstrated in this study.

Plant leaves exhibit high activity of carotenoid hydroxylases, which can compete with BKT for β-carotene. Most ketolases are rather poor in converting the hydroxy derivative zeaxanthin to astaxanthin via adonixanthin. Therefore, the degree of adonixanthin accumulation is an indicator of restricted zeaxanthin utilization (Gerjets and Sandmann, 2006; Zhu *et al.*, 2009). Upon transformation of *A. thaliana* with the *C. reinhardtii BKT*, astaxanthin in line CR-5 accounted for almost half of total ketocarotenoids (Table 3). The corresponding transformant with the *C. zofingiensis BKT* formed less astaxanthin and exhibited an adonixanthin/astaxanthin ratio of 1. This strongly indicates much higher efficiency in converting zeaxanthin to astaxanthin for the *C. reinhardtii* BKT compared with many other ketolases. This conclusion is supported by an even higher accumulation of astaxanthin up to 2.07 mg g^{-1} DW in leaves when *C. reinhardtii BKT* is expressed in a zeaxanthin-rich mutant (*npq2*). In addition, the special *C. reinhardtii* BKT also mediated astaxanthin biosynthesis in *A. thaliana* seeds (Table 5). So far, a truncated *H. pluvialis BKT2* gene expressed in a seed-specific manner in *A. thaliana* resulted in the synthesis of small amounts of 4-ketolutein, adonirubin, and canthaxanthin (8% of total carotenoids) rather than astaxanthin in the seeds (Stalberg *et al.*, 2003).

Due to the efficient reaction of the *C. reinhardtii* BKT, significant amounts of adonirubin, the not fully hydroxylated canthaxanthin (Fig. 1), were present in transgenic *A. thaliana* leaves (Table 3). Therefore, it may be worth investigating whether simultaneous expression of an additional carotenoid hydroxylase gene can even increase the astaxanthin content in plants similar to transgenic tobacco plant that overexpressed a ketolase and a hydroxylase gene resulting in much higher amounts of astaxanthin than when transformed with the ketolase gene alone (Hasunuma *et al.*, 2008; Ralley *et al.*, 2004).

Impact of astaxanthin on pathway regulation and metabolism in A. thaliana transformants

The *C. reinhardtii* BKT not only mediated astaxanthin biosynthesis, but also resulted in a 1.8-fold increase in total carotenoids in transgenic *A. thaliana* plants (Table 3). The higher carotenoid content in leaves was shown to be associated with the up-regulation of the carotenogenic genes *PSY*, *PDS*, and *CRTSO* including *DXPS* upstream of the pathway (Fig. 2D). This resembles the situation in astaxanthin-accumulating tobacco leaves (Hasunuma *et al.*, 2008). This de-regulation may be caused either by the presence of astaxanthin or a relative depletion of lutein and

epoxy carotenoids (Table 3). A similar up-regulation effect was observed before for potato tuber in which formation of the same carotenoids was reduced at the expense of the intermediate zeaxanthin (Römer *et al.*, 2002).

For the first time it was shown here that astaxanthin and other ketocarotenoids were recruited exclusively into LHCII where they replaced native xanthophylls and performed the same functions (Table 4). Previously, the non-native ketocarotenoid capxanthin was found to be located at the same sites (Kumagai *et al.*, 1998). Leaves are specialized for photosynthesis in which carotenoids play important roles in light harvesting, photoprotection, singlet oxygen scavenging, excess energy dissipation, and structure stabilization (Frank and Cogdell, 1996; Krinsky, 1998). Nevertheless, the changes in the carotenoid profile may have an effect on plant photosynthesis and growth. This study showed that there were no significant differences in the efficiency of PSII (evaluated by F_v/F_m) and plant final size between transformants and wild-type plants (Table 6). Furthermore, the enhanced accumulation of astaxanthin and other carotenoids in the transgenic plants increased tolerance to high-light stress as indicated by the much lower lipid peroxidation levels (Table 6). The sequestration of astaxanthin in plastoglobules of scenescent leaves (Fig. 3) supports the involvement of plastoglobules in carotenoid storage (Bréhéllin and Kessler, 2008). This finding highlights the possibility of accumulating astaxanthin at high levels in plant fruits in which carotenoids become localized in plastoglobules of chromoplasts.

Conclusion

Ketolating zeaxanthin efficiently was shown to be essential for high production of astaxanthin in transgenic *Arabidopsis*. The *C. reinhardtii* BKT is a β-carotene/zeaxanthin ketolase with superior catalytic properties for astaxanthin production. Therefore, this novel *BKT* gene offers a great possibility for efficient engineering of an astaxanthin pathway into important food crops to improve their nutritional value by means of conventional nuclear transformation and for the development of a plant-based biological production system for astaxanthin. Replacement of leaf carotenoids by astaxanthin had no negative impact on photosynthesis but enhanced the stress tolerance. The present results represent an initial step for more detailed studies on astaxanthin sequestration, on its integration into photosynthesis complexes, and for the analysis of structure–function relationship of different carotenoid ketolases.

Acknowledgements

The authors thank M.L. Chye (HKU) for providing the plasmid pRK2301, C. S. C. Lo (HKU) for *A. tumefaciens* GV3101, and TAIR for seeds of Col-0 wild-type *A. thaliana* and *npq2* mutant. This work was supported by grants from HKU Seed Funding and the Research Grants Council of Hong Kong Special Administrative Region, China.

References

Baroli I, Do AD, Yamane T, Niyogi KK. 2003. Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects *Chlamydomonas reinhardtii* from photooxidative stress. *The Plant Cell* **15,** 992–1008.

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F. 2000. The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* **403,** 203–207.

Bréhéllin C, Kessler F. 2008. The plastoglobule: a bag full of lipid biochemistry tricks. *Photochemistry and Photobiology* **84,** 1388–1394.

Biswal C, Biswal B, Raval MK. 2003. *Chloroplast biogenesis: from proplastid to gerontoplast*. Dordrecht: Kluwer Academic Publishers.

Chen PY, Wang CK, Soong SC, To KY. 2003. Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. *Molecular Breeding* **11,** 287–293.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Cottage A, Yang AP, Maunders H, de Lacy RC, Ramsay NA. 2001. Identification of DNA sequences flanking T-DNA insertions by PCR-walking. *Plant Molecular Biology Reporter* **19,** 321–327.

Cunningham FX, Gantt E. 2005. A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flower of *Adonis aestivalis*. *The Plant Journal* **41,** 478–492.

Dall'Osto L, Caffarri S, Bassi R. 2005. A mechanism of nonphotochemical energy dissipation, independent from PsbS, revealed by a conformational change in the antenna protein CP26. *The Plant Cell* **17,** 1217–1232.

Davison PA, Hunter CN, Horton P. 2002. Overexpression of β-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. *Nature* **418,** 203–206.

Frank HA, Cogdell RJ. 1996. Carotenoids in photosynthesis. *Photochemistry and Photobiology* **63,** 257–264.

Gerjets T, Sandmann G. 2006. Ketocarotenoid formation in transgenic potato. *Journal of Experimental Botany* **57,** 3639–3645.

Gerjets T, Sandmann M, Zhu C, Sandmann G. 2007. Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species. *Biotechnology Journal* **2,** 1263–1269.

Giuliano G, Aquilani R, Dharmapuri S. 2000. Metabolic engineering of plant carotenoids. *Trends in Plant Science* **5,** 406–409.

Hasunuma T, Miyazawa SI, Yoshimura S, Shinzaki Y, Tomizawa KI, Shindo K, Choi SK, Misawa N, Miyake C. 2008. Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. *The Plant Journal* **55,** 857–868.

Havaux M, Eymery F, Porfirova S, Rey P, Dormann P. 2005. Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. *The Plant Cell* **17,** 3451–3469.

Hirschberg J. 1999. Production of high-value compounds: carotenoids and vitamin E. *Current Opinion in Biotechnology* **10,** 186–191.

Huang JC, Chen F, Sandmann G. 2006*a*. Stress-related differential expression of multiple β-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. *Journal of Biotechnology* **122,** 176–185.

Huang JC, Wang Y, Sandmann G, Chen F. 2006*b*. Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis (Chlorophyta)*. *Applied Microbiology and Biotechnology* **71,** 473–479.

Huber CG, Walcher W, Timperio AM, Troiani S, Porceddu A, Zolla L. 2004. Multidimensional proteomic analysis of photosynthetic membrane proteins by liquid extraction-ultracentrifugation-liquid chromatography-mass spectrometry. *Proteomics* **4,** 3909–3920.

Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H. 2006. Astaxanthin, a carotenoid with potential in human health and nutrition. *Journal of Nature Products* **69,** 443–449.

Ikeda Y, Tsuji S, Satoh A, Ishikura M, Shirasawa T, Shimizu T. 2008. Protective effects of astaxanthin on 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Journal of Neurochemistry* **107,** 1730–1740.

Jayaraj J, Devlin R, Punja Z. 2008. Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic Research* **17,** 489–501.

Krinsky NI. 1989. Antioxidant functions of carotenoids. *Free Radical Biology and Medicine* **7,** 617–635.

Krinsky NI. 1998. The antioxidant and biological properties of the carotenoids. *Annals of the New York Academy of Science* **854,** 443–447.

Kumagai MH, Keller Y, Bouvier F, Clary D, Camara B. 1998. Functional integration of non-native carotenoids into chloroplasts by viral-derived expression of capsanthin-capsorubin synthase in *Nicotiana benthamiana*. *The Plant Journal* **14,** 305–315.

Kurihara H, Koda H, Asami S, Kiso Y, Tanaka T. 2002. Contribution of the antioxidative property of astaxanthin to its protective effect on the promotion of cancer metastasis in mice treated with restraint stress. *Life Science* **70,** 2509–2520.

Li Y, Huang J, Sandmann G, Chen F. 2008. Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown Chlorella zofingiensis (Chlorophyceae). *Planta* **228,** 735–743.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–ΔΔC(T)) method. *Methods* **25,** 402–408.

Lohr M, Im CS, Grossman AR. 2005. Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiology* **138,** 490–515.

Mann V, Harker M, Pecker I, Hirschberg J. 2000. Metabolic engineering of astaxanthin production in tobacco flowers. *Nature Biotechnology* **18,** 888–892.

Misawa N. 2009. Pathway engineering of plants toward astaxanthin production. *Plant Biotechnology* **26,** 93–99.

Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara S, Saito T, Ohtani T, Miki W. 1995. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *Journal of Bacteriology* **177,** 6575–6584.

Morris WL, Ducreux LJM, Fraser PD, Millam S, Taylor MA. 2006. Engineering ketocarotenoid biosynthesis in potato tubers. *Metabolic Engineering* **8,** 253–263.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Paine JA, Shipton CA, Chaggar S, *et al*. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnology* **23,** 482–487.

Ralley L, Enfissi EMA, Misawa N, Schuch W, Bramley PM, Fraser PD. 2004. Metabolic engineering of ketocarotenoid formation in higher plants. *The Plant Journal* **39,** 477–486.

Römer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM. 2000. Elevation of the provitamin A content of transgenic tomato plants. *Nature Biotechnology* **18,** 666–669.

Römer S, Lübeck J, Kauder F, Steiger S, Adomat C, Sandmann G. 2002. Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation. *Metabolic Engineering* **4,** 263–272.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual.*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Stalberg K, Lindgren O, Ek B, Hoglund AS. 2003. Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. *The Plant Journal* **36,** 771–779.

Suzuki S, Nishihara M, Nakatsuka T, Misawa N, Ogiwara I, Yamamura S. 2007. Flower color alteration in *Lotus japonicus* by modification of the carotenoid biosynthetic pathway. *Plant Cell Reports* **26,** 951–959.

Wang B, Zarka A, Trebst A, Boussiba S. 2003. Astaxanthin accumulation in *Haematococcus pluvialis* (Chlorophyceae) as an active photoprotective process under high irradiance. *Journal of Phycology* **39,** 1116–1124.

Yuan JP, Chen F. 1999*a*. Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. *Journal of Agricultural and Food Chemi stry* **47,** 31–35.

Yuan JP, Chen F. 1999*b*. Isomerization of trans-astaxanthin to *cis*-isomers in organic solvents. *Journal of Agricultural and Food Chemistry* **47,** 3656–3660.

Zhu C, Gerjets T, Sandmann G. 2007. *Nicotiana glauca* engineered for the production of ketocarotenoids in flowers and leaves by expressing the cyanobacterial *crtO* ketolase gene. *Transgenic Research* **16,** 813–821.

Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T. 2008. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proceedings of the National Academy of Science, USA* **105,** 18232–18237.

Zhu C, Naqvi S, Capell T, Christou P. 2009. Metabolic engineering of ketocarotenoid biosynthesis in higher plants. *Archives of Biochemistry and Biophysics* **483,** 182–190.