RESEARCH PAPER

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

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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.
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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 ketoproduct, 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.](https://academic.oup.com/jxb/article-abstract/62/10/3659/484859)
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., 2006a) and C. zofingiensis BKT (Huang et al., 2006b) were inserted as a HindIII-SacI 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 (HindIII) 5'-GAGAAGCTTCTAGGCGCTTGAGATACA-3' and reverse primer (XhoI) 5'-GCGCTAGATAGCGGCTTGCGCCG-3'. The PCR product was inserted as a HindIII-XhoI fragment into pBluescript II KS (pCRBKT) and sequenced, revealing an open reading frame of 984 bp, encoding the following individual BKT coding sequences were transformed into BKT-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 peptide of RBCS1 was amplified from A. thaliana genomic DNA with the following primers: forward primer (SalI-SmaI) 5'-GGAGTCGACGGCCGGCCGAGATACA-3' and reverse primer (XhoI) 5'-GAGAAGCTTCTAGGCGCTTGAGATACA-3'. The PCR product was inserted as a HindIII-SalI fragment into pBluescript II KS (pCRBKT) and sequenced, revealing an open reading frame of 984 bp, encoding a protein of 324 amino acid residues and a stop codon. Plasmids containing individual BKT coding sequences were transformed into BKT-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.

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The cloning and sequencing of the corresponding sites of binary vector of pBI121 (Chen et al., 2003). Binary plasmids were transformed 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 T0 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⁻²s⁻¹ with 16-h light, 22 °C and 8-h dark, 20 °C. T2 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⁻²s⁻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., 2006a; 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 000g 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 ml min⁻¹ with a linear gradient from 100% solvent A [acetonitrile/methanol/0.1 M TRIS-HCl (pH 8.0), 84:2:4, 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-Ketoastaxanthin 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 Yam and Chen (1999a). 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 ultra centrifuged 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, sections 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’- CGTTTCCCCGCTTACGTTTA-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−ΔΔCt 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 adduct was separated and quantified by HPLC as described by Hayaux et al. (2005). The average retention time of the MDA-TBA adduct was 8 min. The levels of MDA were calculated using tetraethoxypropane (Sigma) as a standard.

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., 2006a), C. zofingiensis (CzBKT) (Huang et al., 2006b), 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 1. Primers used for RT-PCR analysis of transgene expression and endogenous carotenogenic genes in Arabidopsis

| Primer | Sequence | GenBank accession No. |
|--------|----------|-----------------------|
| Hp-BKT F | TCGCCGCACTGCATCTTTTGA | AY605347 |
| Hp-BKT R | AGGCTGACGATGATGGGAGAT | |
| Cz-BKT F | GGGCCTCCCTGGGAAGAATACACTAC | AY772713 |
| Cz-BKT R | GGACGGTATGGGTCATGAT | |
| Cr-BKT F | CGGCGTAGACAGAGGGAGTTC | |
| Cr-BKT R | GGGCGGATGGGGTAACAG | |
| Actin F | CGGCAAAAGGAATCGCGC | |
| Actin R | TGGCGATGAAGCTCAATCC | NM_179953 |
| DXSP3 F | GGACGGCCTGGTTCGCTTGTA | NM_001085101 |
| DXSP3 R | TGGCGCGATGGAGTCATG | |
| PSY F | CCCGCGTGATATCAGCCATT | NM_001036818 |
| PSY R | CTGGCAACCGAGGAGTGGAC | NM_202816 |
| CRTISO F | TGGCGGCACTGGCATGAT | NM_100559 |
| CRTISO R | GCGCGGGAATCAGGGACGAT | |
| LCY F | CGGCGGTAAGACAGGAGGATG | NM_11858 |
| LCY R | GGGAGAAGACCGCTTGAGGAG | |
| CHY1 F | CCGGCTCCCGAAATCTTATC | NM_118702 |
| CHY1 R | GCCAAACAGCAAGCGCATAC | |

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 (Miswaawa et al., 1990), which accumulate, respectively, β-carotene or zeaxanthin. Car, β-carotene; Ech, echinenone; Can, canthaxanthin; Zea, zeaxanthin; Ado, adonixanthin; Ast, astaxanthin; pHpBKT3, pC2BKT, 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  |
| pHpBKT3 | 1  | 3  | 96 | 87 | 12 |
| pHpBKT3 | 6  | 11 | 83 | 34 | 28 |
| pHpBKT3 | 0  | 2  | 98 | 11 | 4  | 86 |
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-bisphosphate carboxylase small subunit 1 (RBCS1) and driven by the cauliflower 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 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 BKT3 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; npq2, zeaxanthin-accumulating mutant; NPQ2-9, C. reinhardtii BKT transgenic npq2 line. (D) Relative expression levels of isoprenoid biosynthetic genes (dxps3, psy, pds, criso, 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 (1999b). Surprisingly, all the transformants with \textit{C. reinhardtii} \textit{BKT} accumulated much more total carotenoids than wild type, e.g. \textit{npq2-9} accumulated total carotenoids up to 4.82 mg g\textsuperscript{-1} DW, which is \textgreater\textsuperscript{2}-fold higher than in wild type. Much lower amounts of ketocarotenoids and astaxanthin were synthesized in the \textit{C. zofingiensis} \textit{BKT} transformants. CR-4 was detected as a heterozygous line harbouring a T-DNA insertion at the +3530 bp site of the \textit{Tocl59} gene, which is required for the quantitative import of photosynthetic proteins (Bauer \textit{et al}., 2000). Its homozygous plant had an albino phenotype and did not survive.

Since astaxanthin was not sequestered at an 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 transsected tobacco leaves (Kumagai \textit{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 \textit{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 \textit{et al}., 2008). Real-time quantitative RT-PCR revealed that the transcript levels of \textit{PSY}, \textit{PDS}, and \textit{CRTISO} in \textit{CrBKT} transformants are \textless\textsuperscript{2}- to 3-fold that in wild type (Fig. 2D). An increase in other carotenogenic genes including 1-deoxy-a-xylulose-5-phosphate synthase (\textit{DYPs3}), and \textgreek{beta}-carotene hydroxylase (\textit{CHY1}) was not statistically significant.

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

### Table 3. Carotenoid content (mg g\textsuperscript{-1} DW of tissues) in leaves of wild-type and transgenic \textit{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 \textit{a posteriori} 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 \textit{Arabidopsis} plants, \pm SE. Values followed by the same letter are not significantly different (\textit{p}<0.05). Values in parentheses are the percentage of esterified astaxanthin/adonixanthin in total astaxathin/adonixanthin. nd, not detected.

| Pigment-protein complex | Plants | Carotenoids | \textgreek{beta}-Carotene | Lutein | Violaxanthin | Neoxanthin | Antheraxanthin | Chlorophyll a | Chlorophyll b |
|-------------------------|--------|-------------|--------------------------|-------|-------------|------------|---------------|--------------|--------------|
| LHCII monomers, minor antennae | WT | nd | 1.15\textsuperscript{a} | nd | nd | nd | nd | 0.13\textsuperscript{a} | 0.40\textsuperscript{a} |
| LHCII trimers | WT | nd | 0.17\textsuperscript{a} | nd | nd | nd | nd | 0.17\textsuperscript{a} | 0.40\textsuperscript{a} |
| PSII core proteins | WT | nd | 0.22\textsuperscript{a} | nd | nd | nd | nd | 0.22\textsuperscript{a} | 0.40\textsuperscript{a} |
| LHCI, PSI core proteins | WT | nd | 1.16\textsuperscript{a} | nd | nd | nd | nd | 1.16\textsuperscript{a} | 0.40\textsuperscript{a} |

### Table 4. Carotenoid and chlorophyll composition in pigment–protein complexes of wild-type and CR-5 transgenic \textit{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 \pm SE of three experiments. nd, not detected.
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 Haematococcus 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 (Loht 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 senescent (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.
Ketolating zeaxanthin is essential for high production of astaxanthin

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 astaxanthin/adonixanthin. nd, not detected.

| Carotenoid content (μg g⁻¹ DW) | Wild-type | CR-5 | CZ-3 | npq2 | NPQ2-9 |
|--------------------------------|-----------|------|------|------|-------|
| Total carotenoid              | 32.63±1.30 | 69.03±1.02 | 57.36±1.40 | 66.62±3.88 | 113.97±0.43 |
| Total ketocarotenoid          | nd         | 36.80±1.16 | 29.37±0.42 | nd   | 59.00±2.97 |
| 4-Ketoantheraxanthin          | nd         | 0.14±0.00 | 0.06±0.00 | nd   | nd   |
| Astaxanthin                   | nd         | 17.23±1.55 | 11.97±0.75 | 32.12±2.21 | 0 (89) |
| Adoxanthin                    | nd         | 16.64±0.49 | 13.06±0.74 | 23.59±0.26 | 42 (69) |
| Adonirubin                    | nd         | nd   | 1.48±0.01 | nd   | 0.13±0.03 |
| 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 | 0.98±0.07 | nd   | 1.46±0.17 |
| Echinenone                    | nd         | 2.27±0.07 | 1.48±0.21 | nd   | 1.71±0.36 |
| Lutein                        | 30.62±1.31 | 25.85±0.10 | 26.20±0.83 | 22.86±1.45 | 16.39±0.73 |
| Zeaxanthin                    | nd         | nd   | nd   | 39.27±6.58 | 54.39±3.54 |
| Neoxanthin                    | 0.09±0.01  | 0.08±0.01 | 0.07±0.01 | nd   | nd   |
| Violaxanthin                  | 0.30±0.00  | 0.26±0.02 | 0.26±0.05 | nd   | nd   |
| Antheraxanthin                | 0.13±0.00  | 0.05±0.01 | 0.09±0.02 | nd   | nd   |
| β-Carotene                    | 1.49±0.11  | 1.20±0.02 | 1.37±0.11 | 4.49±1.23 | 2.31±0.02 |

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

| Parameter                   | Wild type | CR-4 | CR-5 |
|-----------------------------|-----------|------|------|
| Rosette diameter (cm)       | 9.80±0.57 | 9.10±0.33 | 8.20±0.79 |
| Chlorophyll a (mg g⁻¹ DW)   | 8.72±0.3  | 8.54±0.37 | 7.98±0.19 |
| Chlorophyll b (mg g⁻¹ DW)   | 4.16±0.05 | 4.11±0.11 | 3.75±0.34 |
| Chlorophyll a/b             | 2.22±0.09 | 2.14±0.09 | 2.14±0.15 |
| Fv/Fm                        | 0.80±0.01 | 0.75±0.04 | 0.76±0.01 |
| MDA (mg cm⁻²)               | 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 LHCCI 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 scenscent leaves (Fig. 3) supports the involvement of plastoglobules in carotenoid storage (Bréhélin 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.

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