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ORIGINAL ARTICLE

Cloning and selection of carotenoid ketolase genes for the engineering of high-yield astaxanthin in plants

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Abstract β-Carotene ketolase (BKT) catalyzes the rate-limiting steps for the biosynthesis of astaxanthin. Several *bkt* genes have been isolated and explored to modify plant carotenoids to astaxanthin with limited success. In this study, five algal BKT cDNAs were isolated and characterized for the engineering of high-yield astaxanthin in plants. The products of the cDNAs showed high similarity in sequence and enzymatic activity of converting β-carotene into canthaxanthin. However, the enzymes exhibited extremely different activities in converting zeaxanthin into astaxanthin. *Chlamydomonas reinhardtii* BKT showed the highest conversion rate (ca 85 %), whereas, *Neochloris wimmeri* BKT exhibited very poor activity of ketolating zeaxanthin. Expression of *C. reinhardtii* BKT in tobacco led to a twofold increase of total carotenoids in the leaves with astaxanthin being the predominant. The *bkt* genes described here provide a valuable resource for metabolic engineering of plants as cell factories for astaxanthin production.

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Keywords Astaxanthin · β-Carotene ketolase · Green algae · Plant

Abbreviations

BKT	β-Carotene ketolase
CHYb	β-Carotene hydroxylase
HPLC	High-performance liquid chromatography
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcriptase-PCR
WT	Wild type

Introduction

Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) is a high-value ketocarotenoid that is biosynthesized only by a few organisms typically at low levels. This red pigment (produced through chemical synthesis) has been used in large amounts in aquaculture. Currently, natural astaxanthin is employed as a health food and is investigated for the treatment of a number of human diseases including cancers (Guerin et al. 2003; Hussein et al. 2006). The limited renewable sources and growing demand for natural astaxanthin have attracted tremendous interest in engineering heterologous hosts, especially plants with the ability of sequestering 10- to 50-fold higher carotenoids than microorgansims, to produce the high-value pigment during the past decade (Mann et al. 2000; Misawa 2009; Zhu et al. 2009). However, none of these attempts has led to satisfactory yields of astaxanthin. The most promising approach reaching high astaxanthin yields was by chloroplast transformation of a bacterial ketolase gene (Hasunuma et al. 2008).

The biosynthesis of astaxanthin from β-carotene requires a β-carotene hydroxylase (CHYb) and a β-carotene ketolase (BKT, also known as *CrtW* or *CrtO*), which compete for β-carotene leading to the formation of various intermediates (Fig. 1). BKT genes are rare and only a very few *bkt* genes have been isolated and characterized (Misawa 2009). Typically, BKTs can efficiently add two keto-groups to β-carotene but not to its hydroxylated derivatives, e.g., zeaxanthin (3,3′-dihydroxy-β-carotene) to form astaxanthin (Lotan and Hirschberg 1995; Breitenbach et al. 1996; Fraser et al. 1997). Transgenic plants expressing such a BKT mostly synthesized low levels of an array of ketocarotenoids including astaxanthin (Zhu et al. 2009). Thus, BKT enzymes with broader substrate specificity may overcome the problem. One strategy towards this goal is to improve the known BKTs using directed evolution approaches as described by Tao et al. (2006) and Ye et al. (2006). Alternatively, new *bkt* genes can be isolated from the green microalgae with differential ketocarotenoid profiles that could result directly from their BKT functions (Orosa et al. 2000; Ma and Chen 2001; Zhang and Lee 2001; Qin et al. 2008).

The aim of this study was to screen and select functionally enhanced *bkt genes* from microalgae for the engineering of high-yield astaxanthin in plants. The products of the identified *bkts* were found to exhibit various catalytic activities, especially in converting zeaxanthin to astaxanthin. The functionality of the most efficient BKT redirected the carbon flux to carotenoids in tobacco leaves, resulting in a twofold increase of total carotenoids with astaxanthin being the major pigment.

Materials and methods

Strains and culture conditions

The green microalgae, *Neochloris wimmeri*, *Protosiphon botryoides,* and *Scotiellopsis oocystiformis* were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). *Chlorococcum* sp. was isolated from a rocky wall of Victoria Peak, Hong Kong (Ma and Chen 2001). *Chlamydomonas reinhardtii* cc-124 was obtained from Chamydomonas Center (Duke University, Durham, NC, USA). The algae were maintained at 4 °C on an agar slant containing the modified Bristol's medium CZ-M1 (Ip et al. 2004). Briefly, 10 ml of CZ-M1 broth was inoculated with cells from slants and the alga was grown aerobically in flasks at 27 ± 1 °C for 4 days with orbital shaking at 150 rpm and illuminated with continual florescence light at 60 μmol m^{-2} s^{-1} measured at the surface of the flask. The cells were then inoculated at 5 % (v/v) into a 200 ml Erlenmeyer flask containing 36 ml of the medium and were grown for 4 days and used as seed for batch culture. All media were adjusted to pH 6.5 prior to autoclaving at 121 °C for 20 min. High light irradiation (200 μmol m^{-2} s^{-1}) with nitrogen deficiency was used to induce astaxanthin biosynthesis. Samples (15 ml) were collected after 12 h of the treatment.

RNA isolation and reverse transcription

The RNA technique was followed according to the standard methods described in Sambrook et al. (1989). RNA

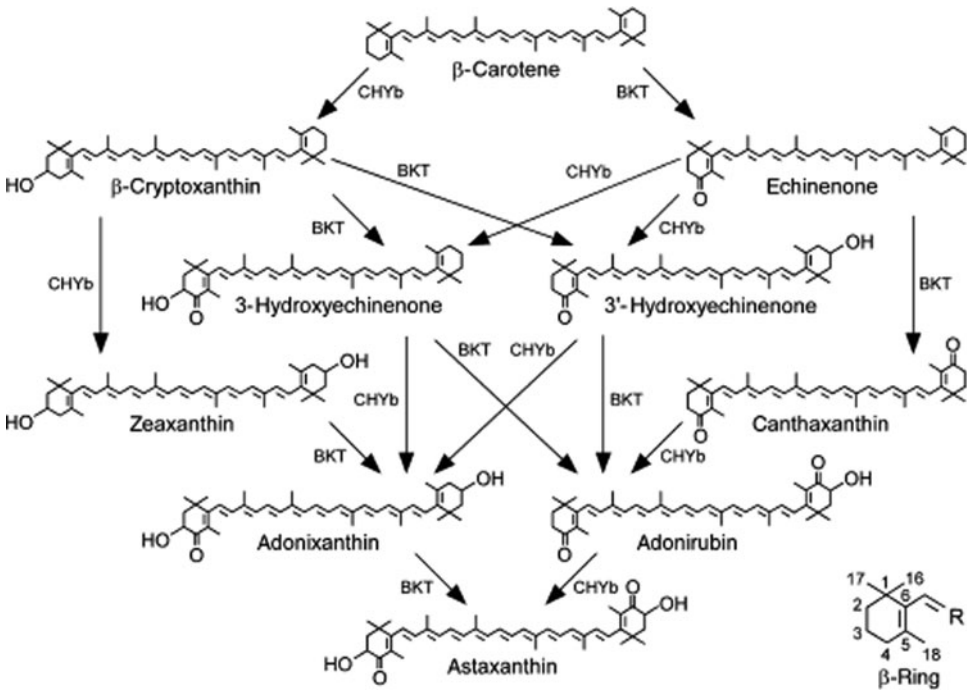

Fig. 1 Possible combionations of hydroxylase and ketolase reactions in the biosynthetic pathways to astaxanthin in green algae

was isolated from aliquots of about 10^8 cells or tobacco leaves using the TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm. Reverse transcription was performed according to Huang and Chen (2006).

Cloning of *BKT* cDNA

The *BKT* cDNAs were isolated by using a pair of degenerate primers designed for the amplification of a partial *BKT* cDNA, from which specific primers were derived for 3′ and 5′ RACE by the approach we previously developed (Huang and Chen 2006). The primers used for the cloning of the cDNAs were listed in Table 1. One-step RT-PCR with primers dbktf and dbktr was performed to amplify a portion of a *bkt* cDNA. Total RNA (100 ng) was from induced cells for 12 h. RT-PCR conditions were 50 °C for 15 min for cDNA synthesis, followed with PCR amplification (32 cycles of 94 °C for 20 s, 52 °C for 20 s, 72 °C for 30 s) using the SuperScript™ III One-Step RT-PCR system (Invitrogen). The PCR products were cloned into pTZ57R/T vector (Fermentas) according to the manufacturer's instructions. Positive clones were selected and sequenced in both directions using an automated ABI3100 sequencer. The sequence of putative insert was used for designing specific primers for 5′ and 3′ RACE (Table 1). The RACE products were gel purified and sequenced. Specific primers were designed from the sequences of the 5′ and 3′ RACE fragments to amplify the full length and coding sequences of the *bkt* cDNAs.

Functional analysis of *bkt* cDNA

The *bkt* open reading frames were PCR amplified and cloned into the vector pUC19 (Stratagene) as an in-frame fusion to the *lacZ* gene resulting in plasmids pChlbkt, pCrbkt, pNbkt, pPbkt, pSbkt, respectively. *Escherichia coli* strain JM109 was used as a host for complementation experiments by co-transformation of pChlbkt, pCrbkt, pNbkt, pPbkt, or pSbkt with plasmids either pACCAR16ΔcrtX or pACCAR25ΔcrtX that harbor the carotenoid biosynthetic genes for producing β-carotene or zeaxanthin, respectively (Misawa et al. 1995). Transformants were cultivated in LB medium containing chloramphenicol (30 μg/ml) and ampicillin (100 μg/ml) in the dark at 28 °C.

Plasmid construction for transformation

The truncated *C. reinhardtii BKT* was fused with the *Nicotiana tabacum* transit peptide sequence of ribulose-1,5-bisphosphate carboxylase small subunit (RBCS, PCR amplified with specific primers RBCS-Tpf and RBCS-Tpr as listed in Table 1). This fused fragment was then inserted between the *Sma*I and *Sac*I sites of pBI121, resulting in the transformation construct pNTPCRBKT. The construct were transformed into *Agrobacterium tumefaciens* strains harboring LBA4404 by electroporation.

Table 1 Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′–3′)
dbkt-F	CTGCAYMRSAARCAYTGGGAGCA
dbkt-Rq	GGCCASCKRTGRTGYTCCCA
Chlbkt-3F1	GCCACCAGATGCCAAGGAAGT
Chlbkt-3F2	GTGGCAGAAGAGTCATAGTAGCG
Chlbkt-5R1	GCTTGCAGGACTTGGCTCA
Chlbkt-5R2	TTGCCCCTGTGGAAGTCTG
Chlbkt-F	GAGAAGCTTC**ATG**GGACGTGGCGGGCAAA
Chlbkt-R	GAGTCTAGACCCAAG**TTA**CTCTGCGCCTGC
Nbkt-3F1	GCCTGAGCCCGGCCCCTC
Nbkt-3F2	CTGGTTCCGGTCCCGGACTA
Nbkt-5R1	ACCTGCAGCACCACTGTCC
Nbkt-5R2	TTGCCCCGGTGGAAGTCAG
Nbkt-F	GAGAAGCTTC**ATG**CAGCTGGCCGCGACA
Nbkt-R	GAGTCTAGAG**CTA**AGCAGGGACCAGGCCAC
Pbkt-3F1	CCCTGAGCCCGGCCCAGCT
Pbkt-3F2	TTGGACAATGTCACGTACGTCC
Pbkt-5R1	AGTTGCATGCCTGTGGCCC
Pbkt-5R2	TTGCCTCTGTGGAAGTCTG
Pbkt-F	GAGAAGCTTC**ATG**GTTGTGTCACAGGGTATG
Pbkt-R	GAGTCTAGAATGCATTCT**TTA**ACCCCCGTG
Sbkt-3F1	GCCACCAGATGCCAAGGAAAAT
Sbkt-3F2	GTGGCAGAAGAGTCACAGCAGTG
Sbkt-5R1	GCCTGTAGGACCTGACTGG
Sbkt-5R2	TTGCCTCTGTGAAAGTCTG
Sbkt-F	GAGAAGCTTC**ATG**GGACGTGGCGGGCAAA
Sbkt-R	GAGTCTAGAACAGCTGCTGGCAGTCATT**CTA**C
Crbkt-F	GAGAAGCTTC**ATG**GGCCCTGGGGATACA
Crbkt-R	GCGTCTAGA**TCA**GGCCAGGGCTGCGCCGCG
Crbkt-df	CCGCCTTCCGCCTGTTCTACTA
Crbkt-dr	GGCGGCACTTGGGCAGCT
RBCS-Tpf	GCGTCGACCCGGGAATCTTTCTGTCTTAAG
RBCS-Tpr	CCCAAGCTTGGCATGCATTGCACTCTTC

F Forward, *R* reverse, *K* G or T, *M* A or C, *Y* C or T

Generation and growth of transgenic tobacco

Wild-type tobacco plants (*Nicotiana tabaccum* cv. Xanthi) were grown on MS medium in pots (Murashige and Skoog 1962) and aseptic leaves were transformed with *Agrobacterium* according to the leaf disc method developed by Horsch et al. (1985). After shoot regeneration and root

regeneration, the resultant T_0 plants were grown on soil in a growth chamber with 16-h light and 8-h dark, 24 °C at a photon flux density of 300 μmol m^{-2} s^{-1}. Homozygous T_1 tobacco plants were used in all of the experiments. Putative transformants were confirmed by detecting the expression of the transgene using RT-PCR with one pair of specific primers Crbkt-df and Crbkt-dr (Table 1).

Carotenoid extraction and analysis

Carotenoids were extracted and analyzed according to McCarthy et al. (2004). *Escherichia coli* cells (1.5 ml) were collected by centrifugation and extracted with 100 μl acetone for 5 min. For quantifying carotenoids of plant transformants, 6 mg of freeze-dried leaves were grounded with acetone and liquid nitrogen until the cell debris was almost colorless. 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. Samples were separated on a 5-μm ODS2 4.6 × 250 mm analytical column (Waters Spherisorb®) with a Waters high-performance liquid chromatography. Individual carotenoids were identified by absorption spectra and their typical retention times compared with standard samples of pure carotenoids. Carotenoids were finally quantified by integrating peak areas and converting them to concentrations by comparison with authentic standards purchased from Sigma and Wako (Osaka, Japan). 4-Ketoantheraxanthin and antheraxanthin were quantified using violaxanthin as standard. Astaxanthin was measured at 480 nm and other carotenoids were measured at 450 nm.

Results and discussion

Isolation of *bkt* cDNAs from astaxanthin-producing green algae

In addition to *Haematococcus pluvialis*, which is currently served as the primary source for natural astaxanthin, several green algae including *Chlorococcum* sp., *N. wimmeri*, *P. botryoides,* and *S. oocystiformis* were reported to synthesize various arrays of astaxanthin and its intermediates, e.g., canthaxanthin, adonirubin, and adonixanthin (Orosa et al. 2000; Ma and Chen 2001; Zhang and Lee 2001). Differential carotenoid profiles of the algae indicate distinct pathways to astaxanthin, possibly resulting from individually interacting activities of β-carotene ketolases and hydroxylases. To address this issue, we isolated the *bkt* cDNAs from the astaxanthin-producing algae using the simultaneous 5′ and 3′RACE approach (Huang and Chen 2006). An approximately 400-bp fragment was amplified from each of the algal cDNAs using a pair of degenerate primers (Table 1, dbktf and dbktr). The DNA fragments were cloned and sequenced to confirm them as putative *bkt* cDNA fragments based on sequence comparison. According to their sequences, specific primers were designed (Table 1) for RACE of full-length cDNAs. Four corresponding fragments of putative *bkt* cDNAs were finally cloned, sequenced, and deposited in GenBank (Accessions JF451848-JF451851). The deduced amino acid sequences from the cDNAs show high similarity with each other and other known algal BKT (ca 70 % identities) as shown in Fig. 2. The conserved domains of di-iron binding motifs (Masamoto et al. 1998) are found in all the algal putative ketolases (Fig. 2). The amino acid sequences of the motifs are identical among the green algal BKTs, suggesting their important role in the enzymatic function.

Functional characterization of green algal BKTs

To investigate the functions of the algal BKT enzymes, we cloned their coding sequences into the expression vector pUC19 as an in-frame to the *lac*Z gene. In addition, we also cloned the corresponding sequence of the putative *bkt* from *C. reinhardtii* to characterize the undocumented ketolase (Lohr et al. 2005). Plasmids containing the *bkt* coding sequences were transferred into β-carotene or zeaxanthin-producing *E. coli* respectively (Misawa et al. 1995) for the characterization of the gene products. The HPLC profiles of the carotenoids from the complemented cells were shown in Figs. 3, 4. All the *bkt* gene products except for that from *S. oocystiformis* are able to convert β-carotene (Fig. 3 peak 1) to canthaxanthin (peak 3) via echinenone (peak 2) efficiently. In contrast, the BKT enzymes exhibit differential efficacies in converting zeaxanthin to astaxanthin (Fig. 4). The *C. reinhardtii* BKT has the highest conversion rate of ketolating zeaxanthin to astaxanthin (ca 85 %), followed by the *Chlorococcum* BKT. The *P. botryoides* and *S. oocystiformis* BKT have lower activities in converting zeaxanthin to astaxanthin (Fig. 4e, f). Interestingly, the *N. wimmeri* BKT, which shares the closest homology to *H. pluvialis* BKT1 (Fig. 2), showed very poor activity in ketolating zeaxanthin to astaxanthin (Fig. 4e). *Haematococcus pluvialis* BKT1-3 and bacterial type CrtW were previously found to be efficient in the conversion of β-carotene to canthaxanthin but to be very poor in converting zeaxanthin to astaxanthin (Lotan and Hirschberg 1995; Breitenbach et al. 1996; Fraser et al. 1997; Huang et al. 2006). As a result, only the reaction sequence with ketolation of β-carotene first and subsequent hydroxylation (Fig. 1) is considered to be the favorite pathway for astaxanthin biosynthesis in these organisms and possibly also in *N. wimmeri* as shown in this study. More than one pathway to astaxanthin was proposed to occur in

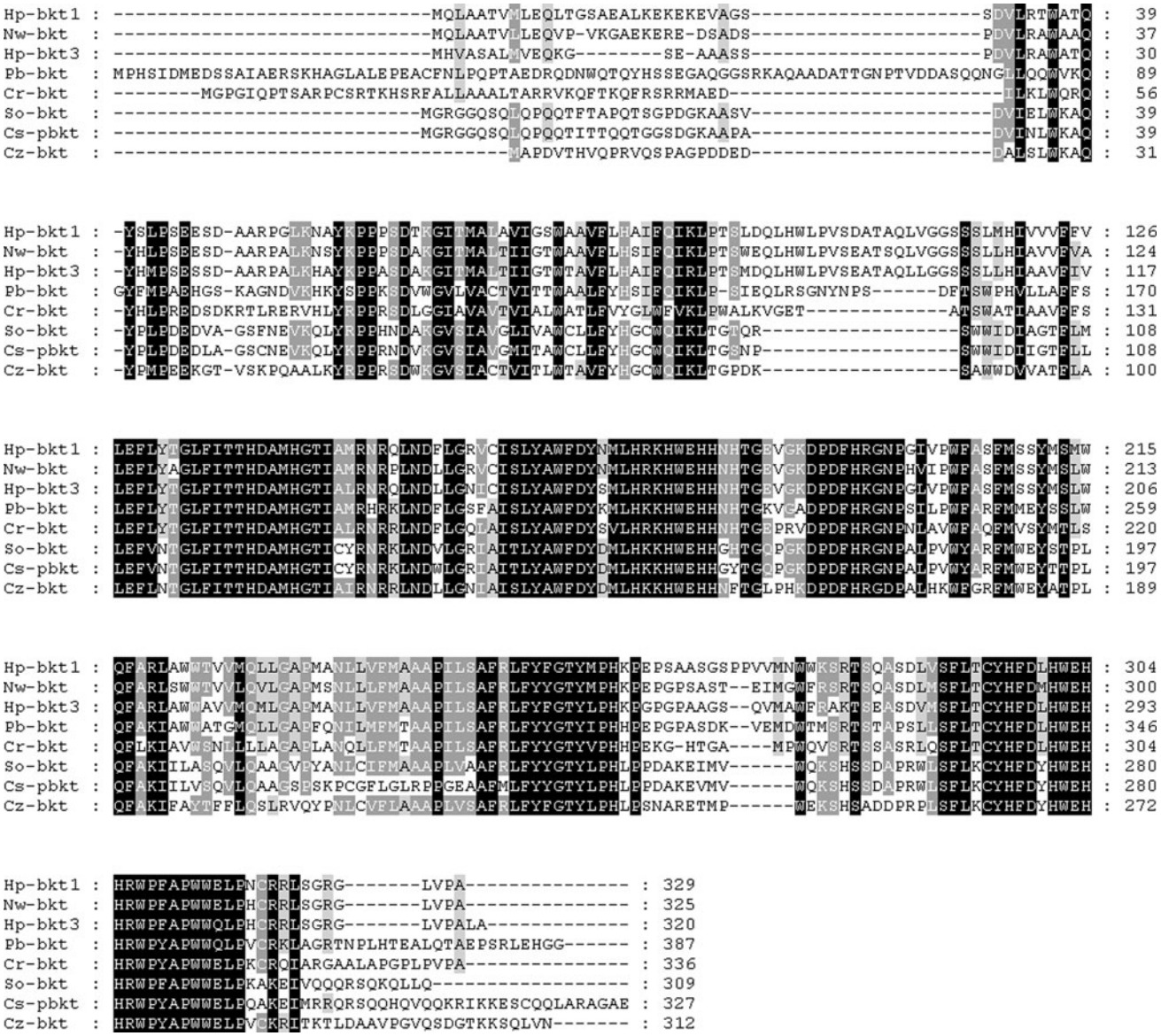

Fig. 2 Amino acid sequence alignment of *β*-carotene ketolases from *Chlorococcum* sp. (Csp-bkt), *Neochloris wimmeri* (Nw-bkt), *P. botryoides* (Pb-bkt), *S. oocystiformis* (So-bkt), and three known BKT from *Chlamydomonas reinhardtii* (Cr-bkt; GenBank accession AAX54908.1), *Chlorella zofingiensis* (Cz-bkt; GenBank accession AAV41372.1), and *Haematococcus pluvialis* (Hp-bkt1, GenBank accession X86782.1; Hp-bkt3, GenBank accession AAT35555.1)

Chlorococcum sp. based on its ketocarotenoid profile (Zhang and Lee 2001; Yuan et al. 2002). The *Chlorococcum* BKT function revealed in this study supports this suggestion. Multiple pathways for astaxanthin may also occur in *P. botryoides* and *S. oocystiformis* since their BKTs can also accept both *β*-carotene and zeaxanthin as substrates for astaxanthin formation. Surprisingly, the most efficient BKT in ketolating both *β*-carotene and zeaxanthin is derived from *C. reinhardtii* that has not been reported to produce astaxanthin. Why *C. reinhardtii* does not synthesize astaxanthin remains unknown. Possibly, the expressed ketolase and their substrates are not present in the same compartment. Nevertheless, this unique BKT gene may serve as a very useful tool for metabolic engineering of higher plants for astaxanthin production.

Overexpression of Chlamydomonas *BKT* gene in tobacco

Previous studies found that plants overexpressing the *H. pluvialis bkt* or bacterial *crtW* failed to synthesize astaxanthin efficiently in plant tissues (Mann et al. 2000; Stälberg et al. 2003; Ralley et al. 2004; Morris et al. 2006; Gerjets et al. 2007; Suzuki et al. 2007). To investigate

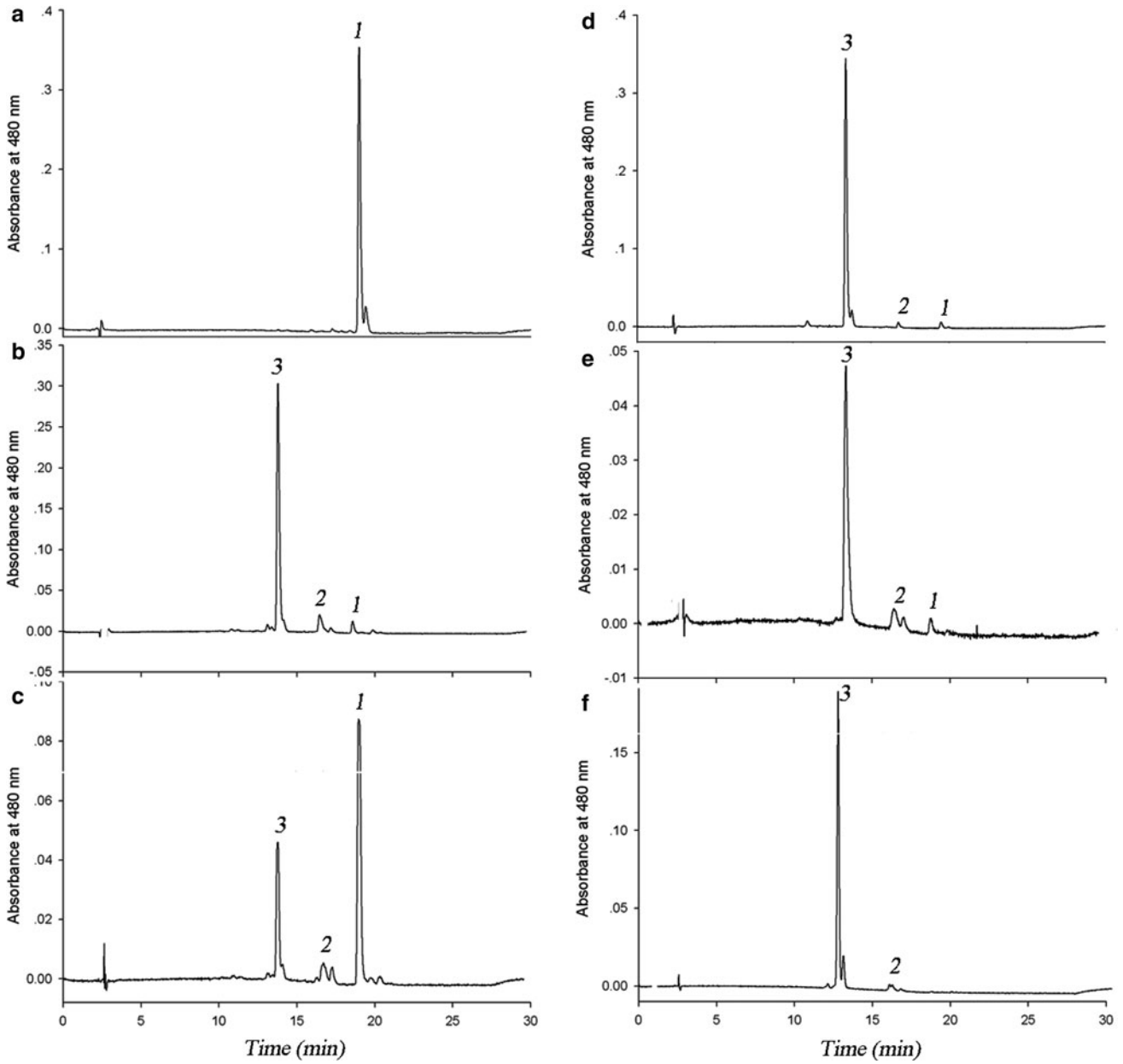

Fig. 3 HPLC analysis of carotenoids extracted from *β*-carotene-producing *E. coli* (**a**), *β*-carotene-producing *E. coli* expressing *bkt* from *Chlorococcum* sp. (**b**), *C. reinhardtii* (**c**), *N. wimmeri* (**d**), *P. botryoides* (**e**) and *S. oocystiformis* (**f**). Retention times and identified carotenoids of peaks *1* 19.8 min, *β*-carotene; *2* 17 min, echeninon; *3* 13 min, canthaxanthin

whether the most efficient BKT in ketolating zeaxanthin can trigger plants to efficiently synthesize astaxanthin, we generated transgenic tobacco expressing the *C. reinhardtii bkt* coding sequence fused with the RBSC2 transit peptide sequence of tobacco to direct the enzyme to the chloroplast where carotenoids are synthesized. The transformation construct, phenotypes, and RT-PCR detection in transformants are shown in Fig. 5. In contrast to WT with green leaves, transformants overexpressing *C. reinhardtii bkt* display brown leaves (Fig. 5b). In addition, there are distinct differences in flower color between WT and transgenic plants (Fig. 5b). The carotenoid compositions in the leaves of representative transgenic lines were analyzed and the results are listed in Table 2. No ketocarotenoids were found in leaves of WT. In contrast, astaxanthin and other ketocarotenoids mainly adonirubin, canthaxanthin, and adonixanthin were detected in the leaves of transformants (Table 2). The reddish leaves of transformants accumulated astaxanthin up to 1.60 mg g^{-1} DW. This content of astaxanthin is much higher than those ever

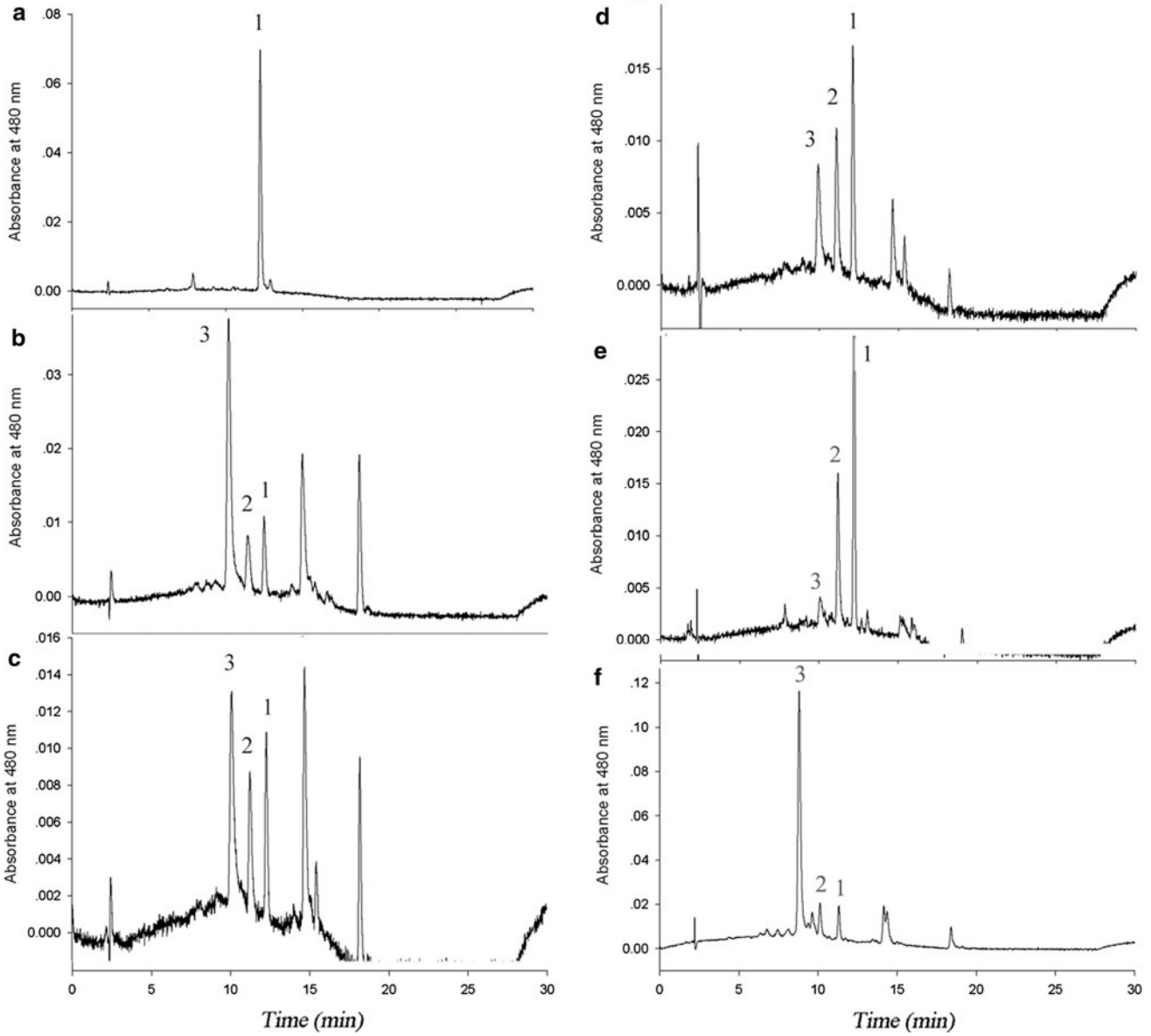

Fig. 4 HPLC analysis of carotenoids extracted from zeaxanthin-producing *E. coli* (**a**), zeaxanthin-producing *E. coli* expressing *bkt* from *Chlorococcum* sp. (**b**), *C. reinhardtii* (**c**), *N. wimmeri* bkt (**d**), *P. botryoides* (**e**) and *S. oocystiformis* (**f**). Retention times and identified carotenoids of peaks *1* 11.8 min, zeaxanthin; *2* 11 min, adonixanthin; *3* 10 min, astaxanthin

obtained with nuclear transformation using other source of ketolase genes (Stälberg et al. 2003; Ralley et al. 2004; Morris et al. 2006; Gerjets et al. 2007; Suzuki et al. 2007; Zhu et al. 2007; Jayaraj et al. 2008) and is comparable with that by chloroplast transformation using the bacterial *crtW* gene (Hasunuma et al. 2008). Astaxanthin and other ketocarotenoids are the major carotenoids in the leaves accounting for 87 % of total carotenoids. Furthermore, the transgenic plants accumulated twofold higher of total carotenoids as compared with that in WT. The phenotype of transgenic tobacco is inherited (data not shown). Our study supports that ketolating zeaxanthin efficiently is essential for high production of astaxanthin in transgenic plants (Zhong et al. 2011).

Conclusions

Five carotenoid ketolase cDNAs were isolated and characterized from five green micoalgae. The BKTs demonstrate functional diversity in ketolating zeaxanthin to astaxanthin. The *C. reinhardtii* BKT can efficiently convert zeaxanthin to astaxanthin in both *E. coli* and plant systems as exemplified with tobacco. The *bkt* genes described here

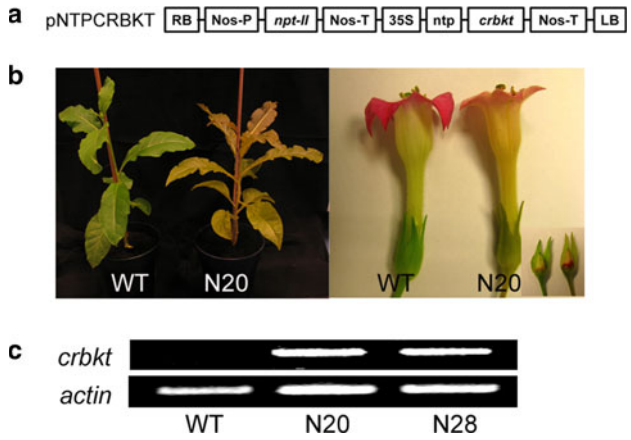

Fig. 5 Molecular characterization of transgenic tobacco. **a** Map of T-DNA region of the binary vector used for transformation. *LB* and *RB*, left and right borders of the T-DNA; *Nos-P*, the nopaline synthase gene (*nos*) promoter; *npt-II,* neomycin methyltransferase II gene; Nos-T, the *nos* terminator; 35S, cauliflower mosaic virus (CaMV) 35S promoter; ntp, the transit peptide sequence from tobacco ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU); *crbkt*, *β*-carotene ketolase from *C. reinhardtii*. **b** Phenotypes of WT and transgenic tobacco (N20) lines. **c** RT-PCR analysis of transgene expression in leaves of WT and transformants (N20 and N28)

Table 2 Carotenoid contents in leaves of wild-type and transgenic tobacco plants

	Carotenoid content (mg g^{-1}DW)		
	Wild-type	N20	N28
Total carotenoid	2.06 ± 0.10	4.11 ± 0.12	4.24 ± 0.07
Total ketocarotenoid	nd	3.58 ± 0.10	3.52 ± 0.08
4-Ketoantheraxanthin	nd	0.09 ± 0.00	0.20 ± 0.02
Astaxanthin	nd	1.49 ± 0.08	1.60 ± 0.13
Adonixanthin	nd	0.41 ± 0.02	0.44 ± 0.02
Adonirubin	nd	0.76 ± 0.03	0.67 ± 0.05
3′-OH-echinenone	nd	0.18 ± 0.01	0.16 ± 0.01
Canthaxanthin	nd	0.57 ± 0.02	0.36 ± 0.03
Echinenone	nd	0.07 ± 0.00	0.10 ± 0.00
Lutein	1.22 ± 0.26	0.45 ± 0.02	0.52 ± 0.03
Neoxanthin	0.21 ± 0.03	0.01 ± 0.00	0.01 ± 0.00
Violaxanthin	0.29 ± 0.05	0.03 ± 0.00	0.05 ± 0.00
Antheraxanthin	0.01 ± 0.00	nd	nd
β-Carotene	0.32 ± 0.06	0.07 ± 0.01	0.14 ± 0.01

Total carotenoid contents were calculated as the sum of the contents of each carotenoid. Values are the averages from measurements of three different tobacco plants, ±SE

DW dry weight of tissue

provide a valuable resource for studying the relationships of structure and catalytic function, especially substrate specificity between carotenoid ketolases, and for metabolic engineering of plants for over-production of astaxanthin and other ketocarotenoids.

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