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ORIGINAL PAPER

Metabolic engineering of novel ketocarotenoid production in carrot plants

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Abstract Carotenoids constitute a vast group of pigments that are ubiquitous throughout nature. Carrot (*Daucus carota* L.) roots provide an important source of dietary β-carotene (provitamin A), α-carotene and lutein. Ketocarotenoids, such as canthaxanthin and astaxanthin, are produced by some algae and cyanobacteria but are rare in plants. Ketocarotenoids are strong antioxidants that are chemically synthesized and used as dietary supplements and pigments in the aquaculture and neutraceutical industries. We engineered the ketocarotenoid biosynthetic pathway in carrot tissues by introducing a β-carotene ketolase gene isolated from the alga *Haematococcus pluvialis*. Gene constructs were made with three promoters (double CaMV 35S, Arabidopsis-ubiquitin, and *RolD* from *Agrobacterium rhizogenes*). The pea Rubisco small sub-unit transit peptide was used to target the enzyme to plastids in leaf and root tissues. The phosphinothricin acetyl transferase (*bar*) gene was used as a selectable marker. Following *Agrobacterium*-mediated transformation, 150 plants were regenerated and grown in a glasshouse. All three promoters provided strong root expression, while the double CaMV 35S and Ubiquitin promoters also had strong leaf expression. The recombinant ketolase protein was successfully targeted to the chloroplasts and chromoplasts. Endogenous expression of carrot β-carotene hydroxylases was up-regulated in transgenic leaves and roots, and up to 70% of total carotenoids was converted to novel ketocarotenoids, with accumulation up to 2,400 μg/g root dry weight. Astaxanthin, adonirubin, and canthaxanthin were most prevalent, followed by echinenone, adonixanthin and β-cryptoxanthin. Our results show that carrots are suitable for biopharming ketocarotenoid production for applications to the functional food, neutraceutical and aquaculture industries.

Keywords β-carotene ketolase · Carotenoid pathway engineering · Ketocarotenoids · Astaxanthin · Root pigmentation · Biopharming

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Introduction

An increased emphasis on dietary sources of health-promoting foods (functional foods) that contain carotenoids is leading to improvements in plant cultivars using traditional breeding methods and biotechnology (Fraser and Bramley et al. 2004; Botella-Pavia et al. 2006). The benefits of dietary-derived carotenoids to human health are well

recognized, and efforts to further enhance the levels of these compounds in crop plants for human health applications are ongoing (Botella-Pavia et al. 2006). Metabolic engineering of agriculturally important plant species has already yielded significant scientific breakthroughs related to the potential applications of plant-based foods in improving human health. These include introduction of novel pathways leading to accumulation of β-carotene in rice (golden rice) (Ye et al. 2000) and enhanced levels of lycopene and β-carotene in tomato fruits (Fraser et al. 2002; D'Ambrosio et al. 2004). The pursuit and applications of further developments in this area of agricultural biotechnology rely on the availability of cloned genes from appropriate metabolic pathways and commercially important crop plants as targets.

The carotenoid biosynthetic pathway in higher plants has been extensively studied and most of the genes have been identified (Hirschberg 2001; Cunningham 2002; Fraser and Bramley et al. 2004). Furthermore, these studies have been extended to other organisms, which naturally accumulate significant levels of carotenoids in their cells eg: the alga *Haematococcus pluvialis* (Lotan and Hirschberg 1995), the yeast *Xanthophyllomyces dendrorhous* (Visser et al. 2003) and several types of bacteria (Goodwin 1980). Ketocarotenoids represent a group of oxygenated carotenoids which impart a distinct pink to red colour to tissues that accumulate them, such as the flesh of salmon, shells of shrimp and other crustaceans, and feathers of birds such as flamingoes and quails (Guerin et al. 2003; Higuera-Ciapara et al. 2006).

Ketocarotenoids such as astaxanthin and canthaxanthin are of particular interest in the functional food, neutraceutical and aquaculture industries where they have broad utility. Recent research has demonstrated that astaxanthin has high antioxidant potential (surpassing that of lycopene, β-carotene and α-tocopherol), prevents cardiovascular disease, boosts the immune system, has antibacterial and anticancer properties, and can prevent cataracts and tissue damage from ultraviolet radiation (Guerin et al. 2003; Higuera-Ciapara et al. 2006; Hussain et al. 2006). The addition of astaxanthin to the feed of farmed salmonids and crustacea is also essential for flesh pigmentation and to achieve adequate growth and reproduction (Guerin et al. 2003; Higuera-Ciapara et al. 2006; Hussain et al. 2006). Currently, astaxanthin used as colourants or feed supplements is derived from chemical synthesis (Hussain et al. 2006), although alternative sources from algae and microbes are being explored to meet the increasing demand for this compound (Higuera-Ciapara et al. 2006; Hussain et al. 2006). One of the drawbacks to chemically synthesized astaxanthin is contamination with reaction intermediates. While *H. pluvialis* can accumulate ketocarotenoids up to 4–5% of dry weight, this alga requires high light intensities and special growing conditions, and grows slowly during commercial cultivation (Yuan and Chen 2000). Astaxanthin and other ketocarotenoids occur rarely in plants (Cunningham and Gantt 2005). The ornamental flowering plant *Adonis aestivalis* accumulates ketocarotenoids (Renstorm et al. 1981), while ketocarotenoids such as capsanthin and capsorubin are found in *Capsicum annuum* (Camara 1980).

There is considerable interest in developing a plant-based biological production system for these valuable ketocarotenoids. However, demonstration of biologically and industrially relevant levels of ketocarotenoid accumulation in transgenic plants for commercial utility (biopharming) has not been achieved despite previous reports of ketocarotenoid production in transgenic tobacco and tomato (Mann et al. 2000; Ralley et al. 2004), arabidopsis (Stalberg et al. 2003) and potato (Gerjets and Sandmann 2006; Morris et al. 2006). In these studies, the β-carotene levels as an initial substrate was limiting and the total amount of ketocarotenoids produced on a whole plant basis was low. In the present study, we demonstrate that carrot storage roots, which naturally accumulate high levels of α-carotene and β-carotene in chromoplasts (Simon 1989), have broad potential utility for ketocarotenoid production for commercial use. We show significant ketocarotenoid accumulation in various tissues (callus, leaf and roots) of carrot. To our knowledge, this is the first report of a high rate of conversion (up to 70%) of β-carotene to ketocarotenoids in a widely-grown and economically important root crop, with accumulation to levels up to 2400 μg/g dry weight.

Materials and methods

Construction of vectors for plant transformation

A cDNA clone of β-carotene ketolase (*bkt1*) (Keto-2a), isolated from a *Haematococcus pluvialis* expression

library, was used. The cDNA had an ORF of 987 nt with 23 nt 5′ UTR and 377 nt 3′ UTR and had no predicted transit peptide sequence. When compared to previously published β-carotene ketolase sequences of *H. pluvialis*, the clone had 94% homology to GenBank CAA60478 and 79% homology to GenBank BAA08300. Plant transformation constructs were made in the binary vector *pCAMBIA 1300* (CAMBIA, Canberra, Australia) utilizing different constitutive promoters, including Double CaMV 35S (provided by Dr. Shawn Mansfield, Canada), and Arabidopsis Ubiquitin-3 (this study) and a root specific promoter RolD from *Agrobacterium rhizogenes* (provided by Dr. Stephane Garberk, France). The transit peptide (TP) of the small sub-unit of Rubisco (SSU) was synthesized as three overlapping oligonucleotide fragments and subsequently PCR–amplified and fused with the *bkt* gene. The selectable marker gene encoding phophinothricin acetyl transferase (*bar*) driven by the CaMV 35S promoter (provided by Dr. Subbaratnam Muthukrishnan, USA) was used. The resulting plasmids *pCAMBIA*- D-*CaMV 35S/Ubi/RolD-bkt-Bar* (Fig. 1.1) were used for plant transformation. A cDNA of β-carotene hydroxylase gene (*chyB*) (GenBank NM_118702) (930 nt) was PCR - amplified from *Arabidopsis thaliana* mRNA and a plant transformation construct was made in *pCAMBIA1300* as described above utilizing the Arabidopsis Ubiquitin-3 promoter. All plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 through electroporation and

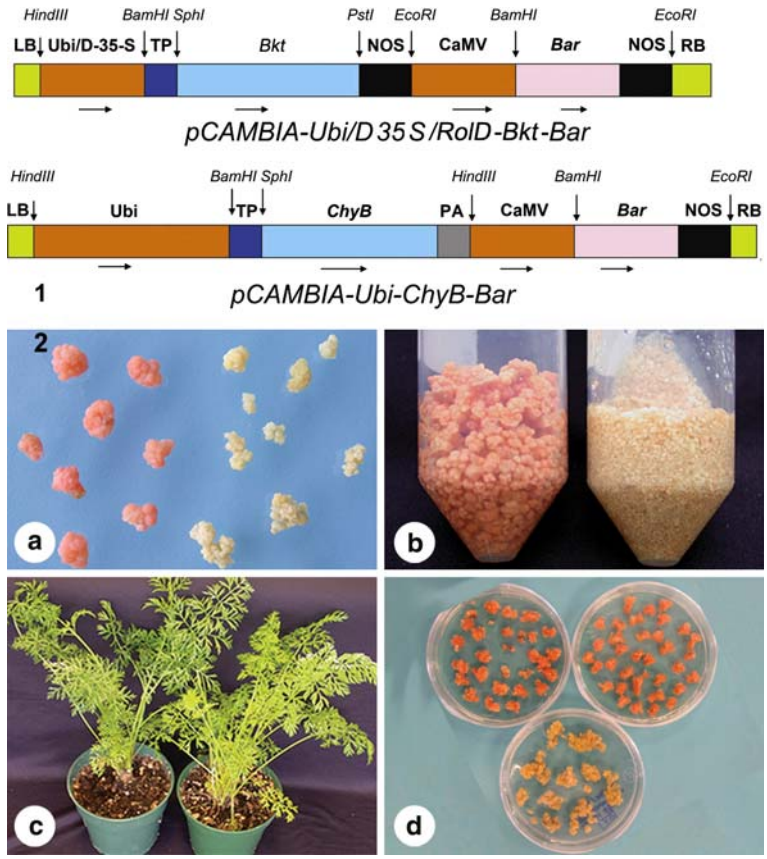

Fig. 1 (**1**) Schematic map of plasmid constructs used for carrot transformation TP, transit peptide; LB, left border; RB, right border; PA, polyA tail; NOS, nos terminator; *Bkt*, β-carotene ketolase; Ubi, Arabidopsis ubiquitin promoter; D-35-S, Double CaMV 35S promoter, *ChyB*, β-carotene hydroxylase; *Bar*, phosphinothricin acetyl transferase gene. (**1.2**) Phenotype of transgenic calli and carrot roots expressing *bkt* gene compared to wild-type. (**a**, **b**) Comparison of transgenic (*left*) and wild-type (*right*) calli from (**a**) solid medium and (**b**) suspension cultures. (**c**) Transgenic (*left*) and wild-type (*right*) plants growing in the glasshouse. (**d**) Callus development on tissues derived from transgenic plant (top row) compared to wild-type (*bottom dish*)

used for plant transformation individually or in combination.

Bacterial complementation experiments

Bacterial expression constructs utilizing *bkt* and *chyB* were made in plasmids *pET28A* and *pQE60*, respectively. The resulting plasmids (*pET28A-bkt* and *pQE60-chyB*) were mobilized into *Escherichia coli* strain JM101 harboring *pACCAR25Δ-crtX* or *pACCAR16Δ-crtX* (provided by Dr. Norihiko Misawa, Japan). The *E. coli* transformants were cultivated in LB medium supplemented with 35 μg/ml chloramphenicol, 100 μg/ml ampicillin/50 μg/ml kanamycin as required. The bacterial cultures were grown at 37°C to $OD_{600} = 0.4$ and induced with varying concentrations of IPTG (0.5 and 1 mM) and grown at 28°C for 6–24 h. The cells were harvested by centrifugation (3000 g for 15 min at 4°C), washed in sterile distilled water, lyophilized and used for carotenoid extraction.

Plant tissue culture and transformation

Carrot (*Daucus carota* L.) transformation was conducted as described previously (Wally et al. 2006), utilizing petiole explants (1 cm length) of a high carotene producing line (HCM) (provided by Dr. Phil Simon, USA). High Carotene Mass (HCM) was derived from the F1 hybrid [(Kokubu × B2158) F4] × [(B3079× Imperial Long Scarlet) F3] (Simon et al. 1989). For cotransfomation of carrot petioles, equal amounts of *Agrobacterium* containing *bkt* and *chyB* were mixed and cocultivated with the explants. Selection was imposed with phosphinothricin (1, 10 mg/l) and the resulting plantlets were placed inside a humid chamber for 2 weeks and transplanted into sterilized potting medium and grown in a glasshouse (22–28°C; 70–85% relative humidity; light intensity 600–800 μ mol/m^2/s; 12 h photoperiod). Putatively transgenic carrot plants at the 5-leaf-stage were treated with Liberty herbicide (0.2% v/v, 100 μl) (Monsanto Canada Inc, Winnipeg, Canada) and evaluated for phytotoxicity symptoms after 10 days. Surviving plants (150 lines in total) were maintained in the glasshouse for periods of up to one year while the molecular and biochemical analyses were conducted.

Molecular analyses of transgenic plants

DNA was extracted from 300 mg of leaf material and 500 mg callus/root tissue using a CTAB procedure (Sambrook et al. 1989). PCR analysis for *bar* was carried out using forward primer: 5′- AAGCACGGT CAACTTCCGTA 3′; and reverse primer: 5′- GAAG TCCAGCTGCCAGAAAC -3′). The *bkt* gene specific primers were: forward: 5′- TGGATTCCCAGTGAA GAAGG -3′; and reverse: 5′-GTAGCAGGTCAGGA AGCTG -3′.

For Southern analysis, genomic DNA (10 μg) was digested with *Eco*RI or *Hind* III, separated on 1% (wt/vol) agarose gels and blotted onto nylon membranes (Hybond N+, Amersham) and probed with a [α-^{32}P] dCTP- labelled PCR-derived *bkt* (980 nt)/*chyB* (930 nt) fragment using random primers (Prime-a-gene, Promega) (Sambrook et al. 1989). For Northern analysis, total RNA was extracted using Trizol reagent (Invitrogen) as per the manufacturer's protocol. RNA (10 μg) was run on a 1.4% (wt/vol) agarose formaldehyde gel and transferred onto membrane (Hybond N+, Amersham) and probed as before. Total RNAs were also probed with the coding fragments of two carrot β-carotene hydroxylase cDNAs, *DC-Chy1* (GenBank: DQ192193) and *DC-Chy2* (GenBank: DQ192194) cloned from carrot. cDNA synthesis and RT-PCR was carried out using 500 ng total RNA utilizing appropriate gene primers.

For Western analysis, 500 mg of leaf or root tissue was ground in buffer (50 mM Tris, pH 8.0, 2% SDS, 20% glycerol and 1 mM DTT), vortexed and boiled for 10 min, and centrifuged at 5,000 × *g* for 15 min. Protein content was quantified using BCA reagent (Pierce, Rockford, IL) and 50–100 μg was loaded per lane in a 12% SDS-PAGE gel. Chromoplasts were isolated from carrot roots (20 g) by blending the tissues followed by centrifugation at 2,000 × *g* for 30 min. The pellet was resuspended in 0.05 M Tris buffer (pH 8.0) and processed in an Aminco French pressure cell at 12000 psi. The mixture was centrifuged at 20,000 × *g* for 30 min and the supernatant was used for immunoblotting (Brynt et al. 1992). Chloroplasts were extracted from 20 g leaf tissue and purified according to Robinson (1983), and transferred to SDS-PAGE sample buffer. About 25–50 μg of chromoplast/chloroplast protein was used. After electrophoresis, proteins were transferred to a polyvinylene difluoride (PVDF) membrane by electroblotting.

Western blotting was carried out as described earlier (Wally et al. 2006) using a polyclonal antibody raised against a synthetic 15 AA (HTGEVGKDPDFHRGC) *BKT* peptide (GenScript Corporation, Piscataway, NJ).

Carotenoid analysis

Carotenoids from bacterial cells (100 mg) and carrot tissues (1 g) were extracted with hexane-acetone (1:1 vol/vol). The extracts were evaporated and concentrated under a nitrogen hood and the residue was dissolved in 1 ml acetone. The carotenoids were separated by high-performance liquid chromatography (HPLC) on a reverse phase Analytical YMC Carotenoid Column C30 (Waters, Milford, MA) using the mobile phase consisting of methanol-MTBE (0–100%). The mobile phase was pumped by a HP 1100 high-pressure pump at a flow rate of 0.4 ml/min. The pigments were separated by a linear gradient between the two solvents for 60 min. Peaks were detected at UV/VIS 350 to 600 nm by a HP 1100 series diode-array detector. Individual carotenoids were identified by their absorption spectra and typical retention times and compared to chemically pure standards of astaxanthin, adonoxanthin, adonirubin, β-cryptoxanthin, echinenone (Carotenature, Switzerland), lutein, zeaxanthin, β-carotene (Prodemex, Mexico), α-carotene and canthaxanthin (Hoffmann-La Roche, Switzerland). Peak fractions were collected, evaporated under nitrogen flow and checked for purity by analytical HPLC. Molecular masses of carotenoids were determined by mass spectra obtained from a Bruker Esquire ion trap mass spectrometer using an APCI (atmospheric pressure chemical ionization) ion source (Department of Chemistry, University of British Columbia, BC, Canada). The samples were dissolved in chloroform and a 10 µl volume was injected using Agilent 1100 and delivered into the APCI ion source with methanol at a flow rate of 200 µl/min.

Light and electron microscopy

Transgenic and wild-type carrot root pieces were sectioned to 75 µm thickness on a sliding microtome and fresh sections were mounted in glycerol and viewed under a light microscope equipped with a Zeiss Illuminator 100 lamp and HBO 50 W high pressure mercury source. A violet band pass filter was used (405 and 436 nm transmission) together with a 530 nm barrier filter (Zeiss Canada). For electron microscopy, root pieces were fixed in 3% gluteraldehyde in 0.05 M phosphate buffer, rinsed, and post-fixed in 2% osmium tetroxide. Samples were dehydrated through an acetone series (10–100%) and transferred to propylene oxide. Samples were infiltrated and embedded in Epon 812/Araldite 6005 and sectioned to 90 nm thickness using a diamond knife on a Reichert-Jung Ultracut E microtome (Vasquez-Caicedo et al. 2006). Sections were placed on formvar/carbon-coated nickel grids, stained with 2% uranyl acetate, washed and post-stained with 0.04% lead citrate. The grids were viewed at 1,000× magnification in a Phillips EM301 transmission electron microscope at 60 kV.

Results and discussion

Bacterial expression of *bkt* and *chyB* genes

Bacterial complementation experiments using *Escherichia coli* strain JM101 with pACCAR25Δ-*crtX* expressing zeaxanthin and pACCAR16Δ-*crtX* expressing β-carotene confirmed the conversion of these two carotenoid substrates by the *bkt* and *chyB* gene products. Different IPTG concentrations and incubation times were tested; IPTG concentration of 1 mM and 24 h of induction at 28°C maximized the substrate utilization and the highest conversion rates were seen under these conditions. Expression of *bkt* in zeaxanthin-producing *E. coli* cells led to the production of astaxanthin with a maximum substrate conversion rate of around 95–100%. When expressed in β-carotene-producing *E. coli*, canthaxanthin and echinenone were produced by *bkt* with a substrate conversion rate of around 90–100%. Similarly, the functionality of the cloned *chyB* gene was verified in β-carotene-producing *E. coli*, leading to the production of zeaxanthin and β-cryptoxanthin at about 90–100% substrate conversion rate.

Lotan and Hirschberg (1995) studied the substrate utilization by a β-C-4 oxygenase gene from *H. pluvialis* through bacterial complementation experiments. They observed a high rate of conversion of β-carotene to canthaxanthin and echinenone in bacterial cells containing pBCAR (with genes for β-carotene synthesis) and pHPK (containing β-C-4 oxygenase

cDNA). Our results with a different *bkt* gene corroborate these findings. However, when pHPK was expressed in *E. coli* cells complemented with pZEAX (containing genes coding for zeaxanthin synthesis), no synthesis of astaxanthin was observed (Lotan and Hirschberg 1995), implying that zeaxanthin was not a substrate. However, we observed close to 100% utilization of zeaxanthin and its conversion to astaxanthin with our clone. Kajiwara et al. (1995) similarly demonstrated astaxanthin synthesis in *E. coli*, expressing a beta-carotene ketolase gene from *H. pluvialis* and the *E. uredovora* genes required for zeaxanthin biosynthesis.

Carrot transformation

Following *Agrobacterium* transformation of petiole explants, they were transferred to Murashige-Skoog (MS) medium (Murashige and Skoog 1962) containing 1 mg 2,4-dichlorophenoxy acetic acid (2,4-D), 1 mg phospinothricin (PPT) and 300 mg/l Timentin for 10 days and subsequently transferred to 10 mg/l PPT. Callus development was observed within 5 weeks and they were subcultured onto a similar concentration of PPT (10 mg/l). In contrast, non-transformed calli died on PPT-containing medium. Some callus lines displayed a distinct pink pigmentation (Fig. 1.2a) when compared to the normal yellow color of wild-type calli. These lines represented about 10% of the putatively transgenic lines recovered after phosphinothricin selection. When propagated in suspension culture, calli retained this distinct pigmentation (Fig. 1.2b). Embryos developed after 25 weeks and plantlets were recovered on MS medium without growth regulators and transferred to potting mix. Transformed plants (lines) were considered to have been derived from different transformation events if they originated from different calli. Around 150 putatively transgenic independent plant lines derived from three different promoter constructs were grown in the glasshouse (Fig. 1.2c). This included 80 lines with the double CaMV 35S promoter, 35 lines with the Ubiquitin promoter and 35 lines with *RolD*. Nearly 80% of the plants were resistant to Liberty herbicide (0.2%). When surface-sterilized leaf petioles from these plants were cultured in MS medium containing 1 mg/l and 10 mg PPT, the callus colour was a distinct

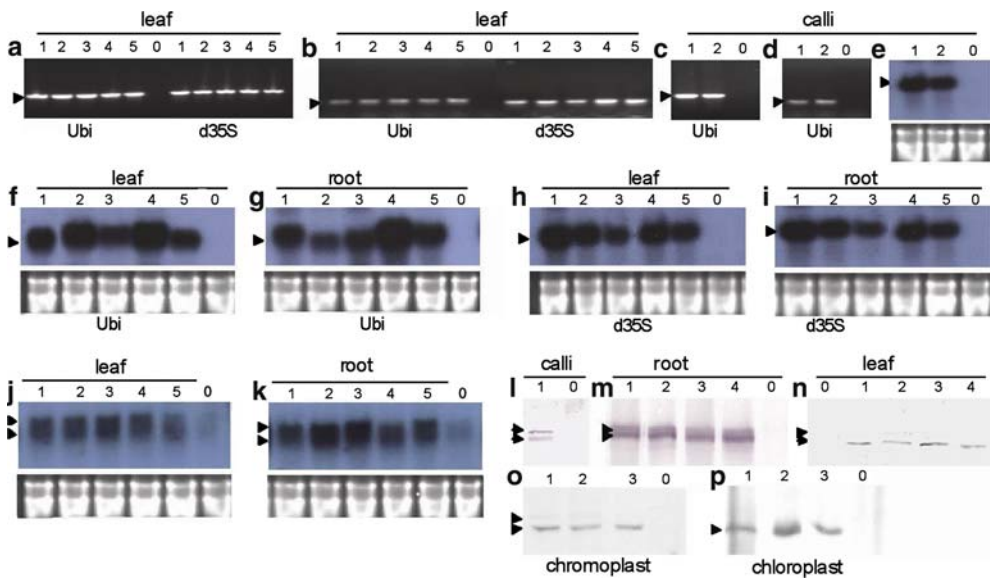

Fig. 2 Analysis of transgene expression in carrot tissues. (**a–d**) PCR for ketolase (**a, c**) or *bar* (**b, d**). (**e**) Ketolase mRNA levels in calli. (**f–i**) Ketolase mRNA levels in leaves and roots with two promoter constructs. (**j–k**) Hydroxylase mRNA levels in leaves and roots. (**l–n**) Western blots against ketolase protein of calli, roots and leaves. (**o–p**) Western blots of partially purified chromoplast (**o**) or chloroplast (**p**) fractions. Lower panels (**e–k**) show RNA loading control. d35S, double CaMV 35S promoter; ubi, Arabidopsis *ubiquitin* promoter. Numbers above lanes indicate transgenic lines. 0, wild-type carrot-control

pink-orange compared to the yellow callus obtained from wild-type plants (Fig. 1.2d).

Molecular analyses of transformants

The presence of the *bar* and *bkt* genes in transgenic tissues was confirmed by PCR (Fig. 2a–d). Pink-pigmented calli (Fig. 2c, d) and leaf tissues (Fig. 2a, b) showed the presence of *bar* and ketolase genes. Out of the 150 lines, 137 were positive for both *bkt* and *bar* genes; the remaining 13 were negative for both genes, indicating they were probably escapes. All the *bkt* positive lines were tested for *bkt*–mRNA expression by Northern blotting. A total of 70 plants expressed *bkt* transcripts at moderate to high levels in leaf and root tissues. The remaining 67 plants had very low or non-detectable levels of mRNA despite the fact that they were PCR positive for *bkt* gene (data not shown). The double CaMV 35S and ubiquitin promoters provided high ketolase mRNA expression in callus, leaf and root tissues (Fig 2e–i) and constituted the majority (75%) of the lines recovered. Ketolase expression in leaves of *RolD*-derived plants was low or non-detectable (data not shown). The majority of the transgenic lines (71%) out of 120 plants had a single copy of the ketolase gene and some lines (22.5%) had two and very few (6.5%) had multiple copies (data not shown).

Western analysis

Since accumulation of β-carotene occurs in chromoplasts, we targeted ketolase expression to the plastids by utilizing the Rubisco small sub-unit transit peptide. In transgenic calli and roots with ketolase mRNA expression, two bands approx. 37 and 43 kDa in size were detected in Western blots (Fig. 2l, m), which likely represent mature processed (transit peptide deleted) and unprocessed protein, respectively. In transgenic leaves (2n) and partially purified chromoplast (2o) and chloroplast (2p) extracts, the 37 kDa band was predominant, reflecting the correctly processed and targeted plastid form. We assume that the additional presence of unprocessed protein in calli and roots reflects lower peptidase levels in these non-photosynthetic tissues. In a previous study of expression of an *Erwinia uredovora crtl* gene targeted to plastids of tobacco, some of the plants showed a protein band of higher molecular weight than expected, suggesting an incomplete processing of the transgenic protein (Misawa et al. 1993). However, potato tubers expressing an algal β-carotene ketolase gene with the phytoene desaturase

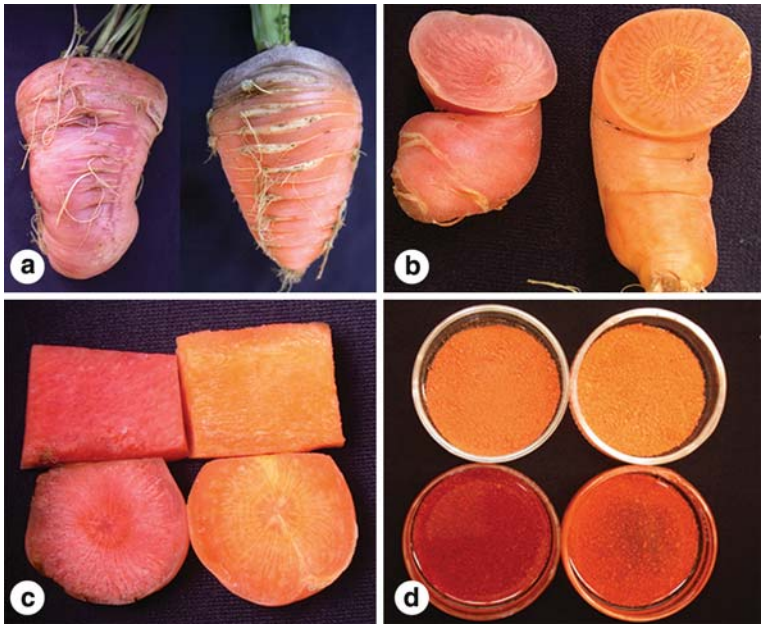

Fig. 3 Phenotype of transgenic carrot roots expressing an algal ketolase gene compared to wild-type. (**a**, **b**) Transgenic (left) and wild-type (right) roots. (**c**) Sections through transgenic (*left*) and wild-type (*right*) roots. (**d**) Dried root powder from transgenic (*left*) wild-type (*right*) roots. Top row is of dried root powder alone; bottom row with canola oil as a suspension

(PDS) transit peptide showed a single protein band of 37 kDa, reflecting a complete processing of the recombinant protein (Morris et al. 2006).

Phenotypic changes in roots

Roots of high mRNA expressing lines developed a distinct pink to deep red colour compared to the typical orange colour in wild-type roots (Fig. 3). The unique pink pigmentation was visible both externally and internally (Fig. 3a–c). In contrast, no significant change in root colour (internal or external) was observed in lines that were shown to be weakly expressing at the mRNA level or in wild-type control roots. After wild-type and transgenic carrot roots were dried at 40°C for 48 h and ground to a fine powder, the distinct tissue colour differences were retained (Fig. 3d). Suspending the powder in canola oil enhanced the deep red color of the transgenic root extract compared to the orange colour of the wild-type extract (Fig. 3d).

Microscopic observations

Microscopically, an accumulation of carotenes as orange aggregates (representing chromoplasts) as seen in the light microscope in wild-type roots (Fig. 4a) was markedly reduced in transgenic roots, which instead displayed a reduced orange pigmentation (Fig. 4b). Transmission electron micrographs of chromoplasts in wild-type roots (Fig. 4c) showed the presence of amorphous and crystalline deposits of carotenes as well as small plastoglobuli. In previous studies on carrot, β-carotene was shown to accumulate in crystalline form and spherical lipoidal globules were also present in chromoplasts (Israel et al. 1969). In transgenic roots, there were much larger plastoglobuli presumed to contain ketocarotenoids within the chromoplasts (Fig. 4d). In mango fruits, large plastoglobuli similar to those observed in carrot were also associated with lipid globules, which contained partially solubilized carotenes (Vasquez-Caicedo 2006). Plastoglobules are commonly found in several fruits and flowers where they serve as the storage vesicles for different types of carotenoids (Howitt and Pogson 2006).

Carotenoid analyses in carrot tissues

HPLC analysis revealed the presence of astaxanthin, echinenone, adonixanthin, adonirubin, canthaxanthin and β-cryptoxanthin, as well as α-carotene, β-carotene, and zeaxanthin in the pink-pigmented calli (Table 1, Fig. 5a). Wild-type calli contained only α-carotene, β-carotene, and zeaxanthin (Fig. 5b), which have been previously reported from carrot callus cultures (Mok et al. 1976), and which provided a substrate for conversion by the ketolase enzyme to yield a unique callus phenotype.

HPLC analyses of five independent transgenic lines (ketolase gene driven by double CaMV 35S

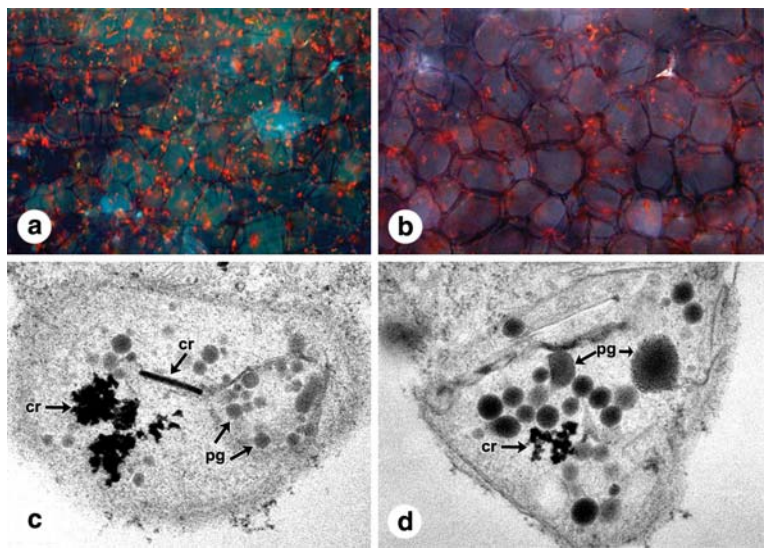

Fig. 4 Carotene accumulation in wild-type (**a**, **c**) and transgenic (**b**, **d**) carrot root tissues. (**a**, **b**) Aggregates of carotenes viewed in the light microscope. A violet band pass filter was used (405 and 436 nm transmission). (**c**, **d**) Aggregates of carotenes viewed in the transmission electron microscope. cr, crystal; pg, plastoglobuli

Table 1 Carotenoids present in non-transgenic and transgenic carrot tissues

Carotenoid compound	Range and mean level of carotenoids in different tissues (μg/g FW)					
	Callus		Leaf		Root	
	NT	T	NT	T	NT	T
α-carotene	28.6 (±3.40)	3.42 (±1.51)	19.1 (±3.29)	12.6 (±2.03)	177.3 (±22.9)	20.7 (±5.18)
β-carotene	31.1 (±4.32)	10.32 (±2.75)	20.0 (±5.8)	28.5 (±2.70)	173.4 (±31.5)	51.8 (±14.13)
Lutein	10.4 (±2.57)	–	81.5 (±16.9)	44.6 (±5.61)	13.8 (±3.94)	2.4 (±1.04)
Zeaxanthin	2.7 (±0.55)	2.0 (±0.82)	34.7 (±5.81)	39.0 (±4.74)	13.8 (±3.02)	5.5 (±1.4)
Astaxanthin	–	12.4 (±2.94)	–	34.7 (±5.86)	–	91.6 (±10.8)
Adonixanthin	–	3.2 (±1.19)	–	5.0 (±1.58)	–	15.9 (±2.8)
Adonirubin	–	3.1 (±1.3)	–	5.9 (±1.35)	–	57.0 (±9.0)
Canthaxanthin	–	5.8 (±0.69)	–	4.0 (±1.35)	–	50.1 (±10.45)
β-cryptoxanthin	–	3.1 (±0.3)	–	3.6 (±1.10)	–	6.2 (±3.11)
Echinenone	–	5.6 (±1.94)	–	2.7 (±0.90)	–	21.4 (±9.32)
Others	5.8 (±2.04)	13.6 (±3.75)	35.2 (±9.5)	45.1 (±15.33)	15.8 (±9.3)	22.8 (±7.32)
Total (μg/g FW)	78.6 (±12.9)	62.5 (±17.3)	190.5 (±41.5)	225.5 (±41.8)	394.0 (±71.0)	345.5 (±75.2)

Mean values were derived from an analysis of five transgenic lines (*bkt* gene driven by the double CaMV 35S promoter), each with two replicate samples. Values in parentheses are range values. Carotenoid compounds were identified by HPLC and mass spectrometry. NT, non-transgenic; T, transgenic; (–), not detected; FW, fresh weight

promoter) identified novel ketocarotenoids in leaves and roots (Table 1, Fig. 5c), which were similar to those in calli (Fig. 5a). Mass spectrometry analysis of purified peak fractions confirmed the identity of these compounds (data not shown). Wild-type tissues contained α-carotene, β-carotene, lutein, and zeaxanthin (Fig. 5b, d). In transgenic roots, astaxanthin, adonirubin and canthaxanthin were most abundant and accounted for 57% of the total ketocarotenoids (Table 1). We also studied the carotenoid profile of representative lines derived from *Rol-D* promoter used to drive the transgene and observed no significant differences in the root carotenoid composition and quantity from that shown in Table 1. This is in confirmation with our previous findings with expression of *gus* gene in carrot employing these promoters (Wally et al. 2005).

Since both ketolase and β-carotene hydroxylase activities are required in the pathway for ketocarotenoid production (Fig. 5e), we reasoned that endogenous expression of β-carotene hydroxylases must be occurring in transgenic carrot tissues engineered with the ketolase gene. This was confirmed using probes developed from two putative β-carotene hydroxylase genes cloned from carrot. Significantly higher expression levels of these endogenous hydroxylases were detected in leaves and roots of transgenic plants compared to wild-type plants (Fig. 2j, k), suggesting they were up-regulated. We had initially set out to co-transform carrots with both the algal ketolase and Arabidopsis β-carotene hydroxylase genes (Sun et al. 1996) to ensure astaxanthin production. A high degree of homology (63–71%) between the *Arabidopsis* and carrot endogenous β-carotene hydroxylase genes yielded cross-hybridization in Northern blots (data not shown). While expression of the *Arabidopsis* gene was confirmed by RT-PCR, we could not confirm mRNA levels of the heterologous gene in Northern blots in doubly transformed plants due to the upregulated endogenous β-carotene hydroxylase mRNA expression. Furthermore we did not observe any significant differences in the root carotenoid composition between ketolase and ketolase + hydroxylase transformants (data not shown). This suggests that heterologous expression of β-carotene hydroxylase was not required for ketocarotenoid synthesis due to the upregulation of endogenous hydroxylases.

Expression of hydroxylases in plant tissues leads to production of lutein from α-carotene (Kim and DellaPenna 2006). However, α-carotene and lutein levels in transgenic leaves and roots were consistently lower than wild-type (Table 1). We suggest that

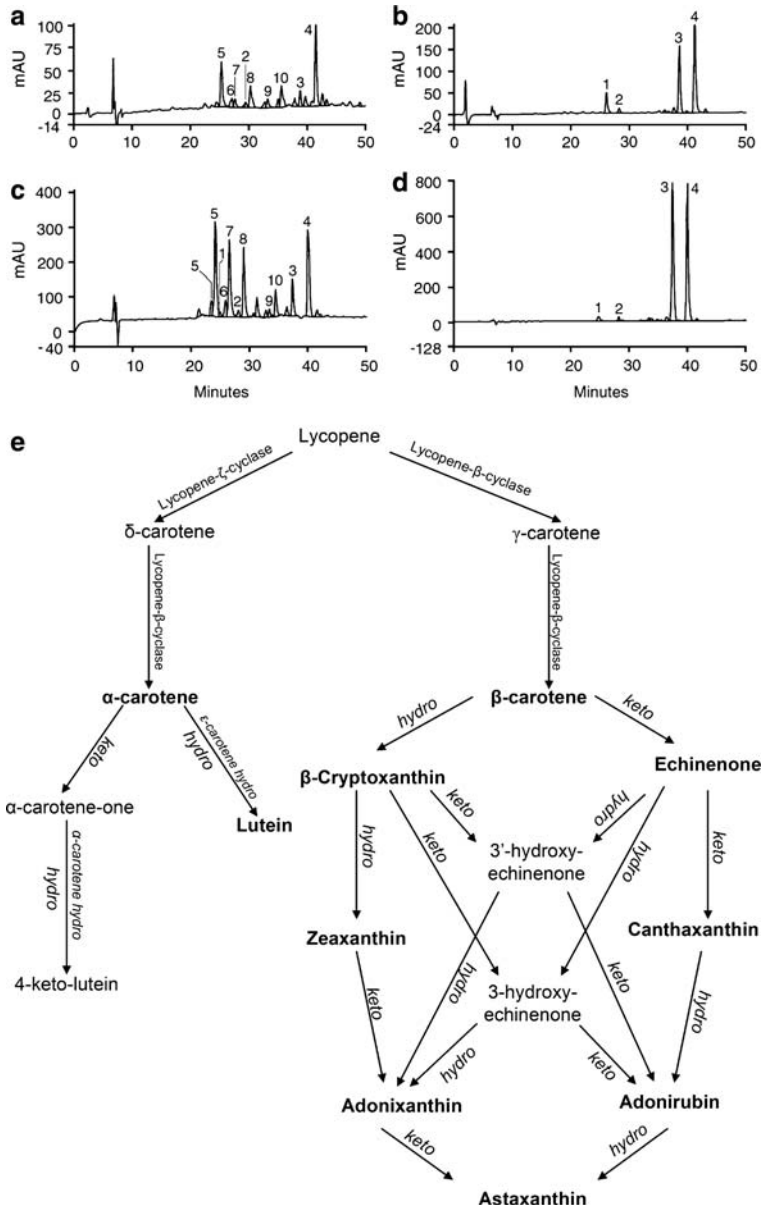

Fig. 5 HPLC scans from transgenic (**a**, **c**) and wild-type (**b**, **d**) carrot tissues. Upper panel represents calli; lower panel represents roots. Peak 1, lutein; peak 2, zeaxanthin; peak 3, α-carotene; peak 4, β-carotene; peak 5, astaxanthin; peak 6, adonixanthin; peak 7, adonirubin; peak 8, canthaxanthin; peak 9, β-cryptoxanthin; peak 10, echinenone. (**e**) Pathway for carotenoid production in plants, including xanthophylls and ketocarotenoids (adapted from Taylor and Ramsay (2005) and Morris et al. (2006)). Carotenoids shown in **bold** were detected in this study. Hydro, β-carotene-3,3′-hydroxylase; keto, β-carotene-4-ketolase. The keto gene was engineered in carrot tissues in this study while the endogenous hydro gene was up-regulated in transgenic tissues

diversion of lycopene towards β-carotene synthesis had occurred at the expense of α-carotene and lutein accumulation due to β-carotene conversion downstream to produce ketocarotenoids (Fig. 5e). Over 70% of the initial carotenoid pool in roots was converted, which significantly reduced the α-carotene and lutein pool. The β-carotene pool was not totally depleted in transgenic roots and remained at 34% of wild-type, perhaps as a consequence of feedback regulation, and was slightly enhanced in the leaves (Table 1). Previous studies on expression of β-carotene hydroxylase genes in Arabidopsis (Davidson et al. 2002; Yu et al. 2006) and tobacco (Gotz et al. 2002) did not report any undue perturbation in the carotenoid biosynthetic pathway except for a slight reduction in β-carotene content. We have also generated few plant lines overexpressing the *chyB* gene and investigation of their root carotenoid profile did not reveal any significant difference compared to wild type except for a moderate increase (14–20%) in zeaxanthin content.

Ketocarotenoid levels in transgenic roots

Levels of accumulation of total ketocarotenoids in ketolase-expressing lines reached an average of 250 μg/g root fresh weight, with a range of 230–270 μg/g (Table 1) and ketocarotenoid levels may reach up to 2.5 mg/g root on a dry weight basis (based on 90% moisture level). This reflects nearly a 70% conversion of total carotenoid content, which is much higher than that reported in previous studies (Mann et al. 2000; Stalberg et al. 2003; Ralley et al. 2004; Gerjets and Sandmann 2006). Ketocarotenoid levels (in particular astaxanthin) in specific transgenic tissues engineered to express the ketolase gene, such as tobacco nectaries (Mann et al. 2000), Arabidopsis seeds (Stálberg et al. 2003) and potato tubers (Morris et al. 2006; Gerjets and Sandmann 2006), as well as tomato and tobacco tissues (Ralley 2004), were low on a whole plant basis. In these plant species, the degree of availability of β-carotene substrate was a limiting factor in achieving high levels of ketocarotenoid accumulation (Stálberg et al. 2003; Morris et al. 2006). As well, the form in which β-carotene is sequestered inside the cell can affect conversion efficiency by the ketolase enzyme (Morris et al. 2006; Ralley et al. 2004). In tobacco nectaries, the ketocarotenoid levels reached up to 2 mg/g (dry wt. basis) (Mann et al. 2000). However, in potato transformed with an algal ketolase gene (*bkt1*), levels of accumulation of ketocarotenoids were 19 μg/g (dry wt. basis) (Morris et al. 2006). In another attempt to engineer potato to produce ketocarotenoids utilizing a β-carotene ketolase gene from *Synechocystis* sp., the ketocarotenoid conversion was up to 10–12% of total carotenoids in leaves and tubers, providing up to 7–12 μg/g dry wt. (Gerjets and Sandmann 2006). Similarly, in tomato fruit, the maximum ketocarotenoid level did not exceed 10 μg/g dry weight when a ketolase gene was expressed (Ralley et al. 2004). In transgenic carrots, the ketocarotenoid accumulation was up to 2,500 μg/g (dry wt. basis) which is the highest level reported to date. To our knowledge, this is the first demonstration of a high conversion rate of β-carotene to ketocarotenoids in an economically important root vegetable. The levels of carotene accumulation in carrots can be affected by many variables, including genotype, soil conditions, environmental factors, as well as post-harvest root storage conditions (Kopsell and Kopsell 2006). These factors may increase or decrease final ketocarotenoid levels in transgenic tissues. We selected a cultivar "HCM" with twice the level of carotenes of standard carrots for this study. An alternative approach would be to utilize carrots engineered to express higher carotene levels using the phytoene synthase gene to provide higher substrate levels for conversion to ketocarotenoids (Hauptmann et al. 1997).

Utility of transgenic carrots

An average transgenic carrot root (100 g fresh weight, with 90% moisture) can potentially accumulate up to 9 mg (fresh wt basis) of astaxanthin together with other carotenoids (Table 1). Daily ingestion of astaxanthin and ketocarotenoids were proposed to be a practical and beneficial strategy in human health management (Guerin et al. 2003) and a recommended dose was 5 mg per day (Higuera-Ciapara et al. 2006). Astaxanthin has been marketed in the United States, Canada and Europe for many years and the current human dietary sources are from consumption of seafoods and egg yolk, as well as from nutritional supplements consisting of algal or yeast extracts. Up to 6 mg of astaxanthin per day as algal supplements over 8 weeks was considered safe for human consumption (Spiller and Dewel 2003). A single transgenic carrot can surpass the daily recommended dose of astaxanthin if approved for use.

When consumed fresh or as juice, the absorption of ketocarotenoids from transgenic carrots can be affected by many factors (van den Berg et al. 2000; Yeum and Russel 2002). In general, oxygenated carotenoids are more readily taken up into lipid micelles, including those present in the human gastrointestinal tract, compared to nonoxygenated carotenoids such as β-carotene (Yeum and Russel 2002). Astaxanthin found naturally in algae or crustaceans may be sterified with fatty acids such as palmitic, oleic, stearic or linoleic in one or both hydroxyl groups (Higuera-Ciapara et al. 2006). Astaxanthin may also be found free with the hydroxyl groups not sterified (Higuera-Ciapara et al. 2006), as in the synthetically derived product. In wild-type carrot root chromoplasts, the crystalline form of β-carotene (all-*trans* isomer) (Vasquez-Caicedo et al. 2006), which occurs naturally with pectin-like fibers, has lower human bioavailability (Zhou et al. 1996). Blanching of carrot roots was

shown to result in dissolution of crystalline carotenes by cellular lipids and conversion to the *cis*-isomer, which was proposed to enhance bioavailability (Marx et al. 2003). Since transgenic carrot root chromoplasts were mostly devoid of crystalline form, this implies that ketocarotenoids may have accumulated in esterified form within lipid droplets or plastoglobuli, similar to that reported in transgenic tobacco (Mann et al. 2000) and Arabidopsis plants (Stålberg et al. 2003). In mango fruits, the partial solubilization of carotenes in lipid droplets during ripening was also associated with the formation of numerous large plastoglobuli similar to those observed in transgenic carrots (Vasquez-Caicedo 2006). Sequestration of carotenoids in fruits and flowers eg: marigold, pepper and apple is also accomplished through esterification and integration into lipid-rich plastoglobules (Howitt and Pogson 2006).

Our results are the first to demonstrate astaxanthin and other ketocarotenoid production in the storage root of a high β-carotene containing crop plant. These findings demonstrate the potential of using engineered carrots as a novel method to produce and deliver high-potency pigmented antioxidants targeted to the neutraceutical, functional food and aquaculture industries. This work supports the rationale for using plants to develop cost-effective biopharmaceuticals and edible vaccines on an agricultural scale to promote human health (Fischer et al. 2004). The advantage to using carrots is that they can be consumed directly once approved for human consumption, and are inexpensive, avoiding the need for costly extraction and formulation of the rapidly degradable ketocarotenoids.

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