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Engineering ketocarotenoid biosynthesis in potato tubers

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Abstract

Consumption of astaxanthin is increasingly associated with a range of health benefits. Attempts to engineer ketocarotenoid biosynthesis in plants have been successful although there are no reports of nutritionally significant levels of astaxanthin in plant storage organs. Thus, in this study, ketocarotenoid biosynthesis was engineered in potato tubers. Both *Solanum tuberosum* and *Solanum phureja* transgenic lines were produced that expressed an algal *bkt1* gene, encoding a β-ketolase, and accumulated ketocarotenoids. Two major ketocarotenoids were detected, ketolutein and astaxanthin. The level of unesterified astaxanthin reached ca. 14 $\mu g\, g^{-1}$ DW in some *bkt1* expressing lines of *S. phureja* but was much lower in the *S. tuberosum* background. Co-transformation of *S. tuberosum* with *crtB*, encoding phytoene synthase, and the *bkt1* gene was achieved in order to determine whether this would enhance the levels of *S. tuberosum* ketocarotenoid.

Keywords: Ketocarotenoid; *bkt1*; Astaxanthin; Co-transformation; Ketocarotenoid *Solanum phureja*; *Solanum tuberosum*

1. Introduction

Carotenoids form a large class of lipophilic molecules, synthesised by plants, algae and many bacteria. In plants they are components of the photosynthetic machinery, intermediates in the biosynthesis of abscisic acid and other apocarotenoids and act as coloured pigments particularly in floral and fruit tissues (reviewed in Demmig-Adams et al., 1996; Fraser and Bramley, 2004; Taylor and Ramsay, 2005). Humans benefit in a number of ways from dietary carotenoids present in green leaf tissue and many fruits, seeds, roots and tubers (reviewed in van den Berg et al., 2000; Fraser and Bramley, 2004). Carotenoids with a β-ring end group are required for the synthesis of vitamin A, and deficiency for this vitamin remains a major health problem in some parts of the world (Mayne, 1996). Evidence is rapidly accruing that different carotenoids have other different beneficial effects, although the mechanisms of action remain unclear. For example, lycopene appears to have a protective effect against prostate cancer (Gann and Khachik, 2003). Lutein and zeaxanthin intake appear to provide protection against age-related macular degeneration (Krinsky et al., 2003; Beatty et al., 2004).

Consumption of ketocarotenoids, most notably astaxanthin, is also increasingly associated with a range of health benefits. Some evidence suggests astaxanthin boosts immune function in humans (Jyonouchi et al., 1995), reduces oral carcinogenesis in rats (Tanaka et al., 1995) and inhibits the growth of mammary tumours in mice (Chew et al., 1999). Other studies in mice indicated that astaxanthin treatment could inhibit gastric infections of *Helicobacter pylori* (Bennedsen et al., 1999; Wang et al., 2000). Consequently astaxanthin is now marketed as a dietary supplement or so-called neutraceutical and also finds applications in the cosmetics, food and feed industries

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(Guerin et al., 2003). In nature, the main sources of astaxanthin are marine bacteria and microalgae. Fish and crustaceans, accumulate astaxanthin from their diet, resulting in characteristic colourations of flesh and/or carapace. For farmed aquatic organisms, separated from their natural diet, astaxanthin must be included as a dietary supplement to develop the appropriate colouration acceptable to consumers. Additionally, astaxanthin is associated with benefits to fish health as in salmon a lack of astaxanthin is associated with an increase in yolk sac mortality (Pettersson and Lignell, 1999).

Currently, for economic reasons, most astaxanthin produced commercially is chemically synthesised. However, the synthetic pigment only contains 25% of the naturally occurring stereoisomer (*3S, 3′S*) although the biological significance of the chirality of astaxanthin is not known. Additionally, chemically synthesised astaxanthin may be contaminated with reaction intermediates. Production of astaxanthin from natural sources has been investigated and several microorganisms are known to produce high levels of ketocarotenoids. Examples include the unicellular green alga *Haematococcus pluvialis*, an organism that can produce 4–5% dry weight ketocarotenoid (Yuan and Chen, 2000). Although used commercially, this alga requires high light intensities and grows slowly. Another astaxanthin-producing microorganism is *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*), which can produce up to 0.5% dry weight astaxanthin, however this is in the *3R, 3′R* form (Johnson and An, 1991). The biotechnological and health benefits of ketocarotenoids such as echinenone and ketolutein remain to be characterised in detail.

Although uncommon, some plants do produce astaxanthin and other ketocarotenoids (Czeczuga, 1987; Goodwin, 1980). Most notably, the flowers of *Adonis aestivalis* accumulate ketocarotenoids at levels of up to 1% of dry weight (Renstrom et al., 1981). Recently, the enzymes involved in the conversion of β-carotene to astaxanthin have been characterised in *Adonis* (Cunningham and Gantt, 2005). The cDNAs of the genes encoding the biosynthetic enzymes may be useful for the transgenic introduction of the astaxanthin pathway into other plants.

Other transgenic experiments have led to the construction of the astaxanthin biosynthetic pathway in plant organs. β-Carotene ketolases (or oxygenases) catalyse the formation of a carbonyl group at the carbon at position 4 of each ring of β-carotene. Genes encoding this activity have been identified in bacteria (for example Harker and Hirschberg, 1999; Misawa et al., 1995a,b) and green algae (Lotan and Hirschberg, 1995). The bacterial genes are generally designated *crtW* whereas a green algal gene was designated *bkt1* (Lotan and Hirschberg, 1995; Huang et al., 2005), although sharing sequence similarity with the bacterial genes. Expression of the *bkt1* gene from *H. pluvialis* in transgenic tobacco, led to the accumulation of ketocarotenoids in the nectary tissue (Mann et al., 2000). The tissue specificity of accumulation was attributed to the activity of the gene promoter chosen to drive transgene expression (the tomato phytoene desaturase). As the conversion of β-carotene to astaxanthin requires the action of a hydroxylase as well as a ketolase (Fig. 1), Ralley et al. (2004) introduced transgenically, bacterial genes encoding both these activities simultaneously to tobacco and tomato. In transgenic tobacco leaves ketocarotenoids accumulated to high levels (up to $800\,\mu g\,g^{-1}$ DW) whereas in tomato, levels 50-fold lower were measured in transgenic leaves and the maximal level in tomato fruit was $10\,\mu g\,g^{-1}$ DW. In both the tobacco and tomato transgenics the major ketocarotenoid that accumulated was echinenone. Transformation of *Arabidopsis* with an algal β-carotene oxygenase also leads to the accumulation of ketocarotenoids (Stalberg et al., 2003). In this case the major ketocarotenoids were 4-keto-lutein, adonirubin and canthaxanthin. In order to enhance the levels of ketocarotenoids, crosses were made with transgenic *Arabidopsis* over-expressing an endogenous phytoene synthase gene (Lindgren et al., 2003), resulting in an increase in ketocarotenoid content of up to 13-fold over that measured in the single β-carotene oxygenase transgenics.

Although several studies have demonstrated the possibility to produce transgenic plants containing ketocarotenoids, the accumulation of high levels of astaxanthin in plant storage organs has not yet been achieved. The focus in this study was to investigate the levels and types of ketocarotenoid that can accumulate in a staple part of our diet, potato tubers. Previously, we have characterised carotenogenesis in *Solanum tuberosum* and *Solanum phureja* cultivars that produce yellow-fleshed tubers (Morris et al., 2004). We have also shown that potato tubers can accumulate β-carotene when transformed with a bacterial phytoene synthase gene (Ducreux et al., 2005a). In this study we describe the levels and types of carotenoid that accumulate in both *S. tuberosum* cultivar Desiree and *S. phureja* cultivar Mayan Gold on transformation with an algal β-carotene oxygenase gene. We also describe the effects of introducing the bacterial phytoene synthase gene and the algal β-carotene oxygenase gene simultaneously into *S. tuberosum* cultivar Desiree by co-transformation.

2. Materials and methods

2.1. Plant material

S. tuberosum L. cultivar Desiree and *S. phureja* cultivar Mayan Gold were grown from seed tubers or in vitro propagated tissue culture plants in 30 cm diameter pots containing a compost mix of: Irish moss peat (bedding grade), 1200 L; Pavoir sand, 100 L; limestone (magnesium), 2.5 kg; limestone (calcium), 2.5 kg; Sincrostart base fertiliser, 1.5 kg (William Sinclair, Lincoln, UK); Celcote wetting agent, 1.5 kg (LBS Horticulture, Lancashire, UK); Perlite, 100 L; Osmocote mini controlled release fertiliser, 2.0 kg (Scotts, Humberside, UK) and Intercept insecticide,

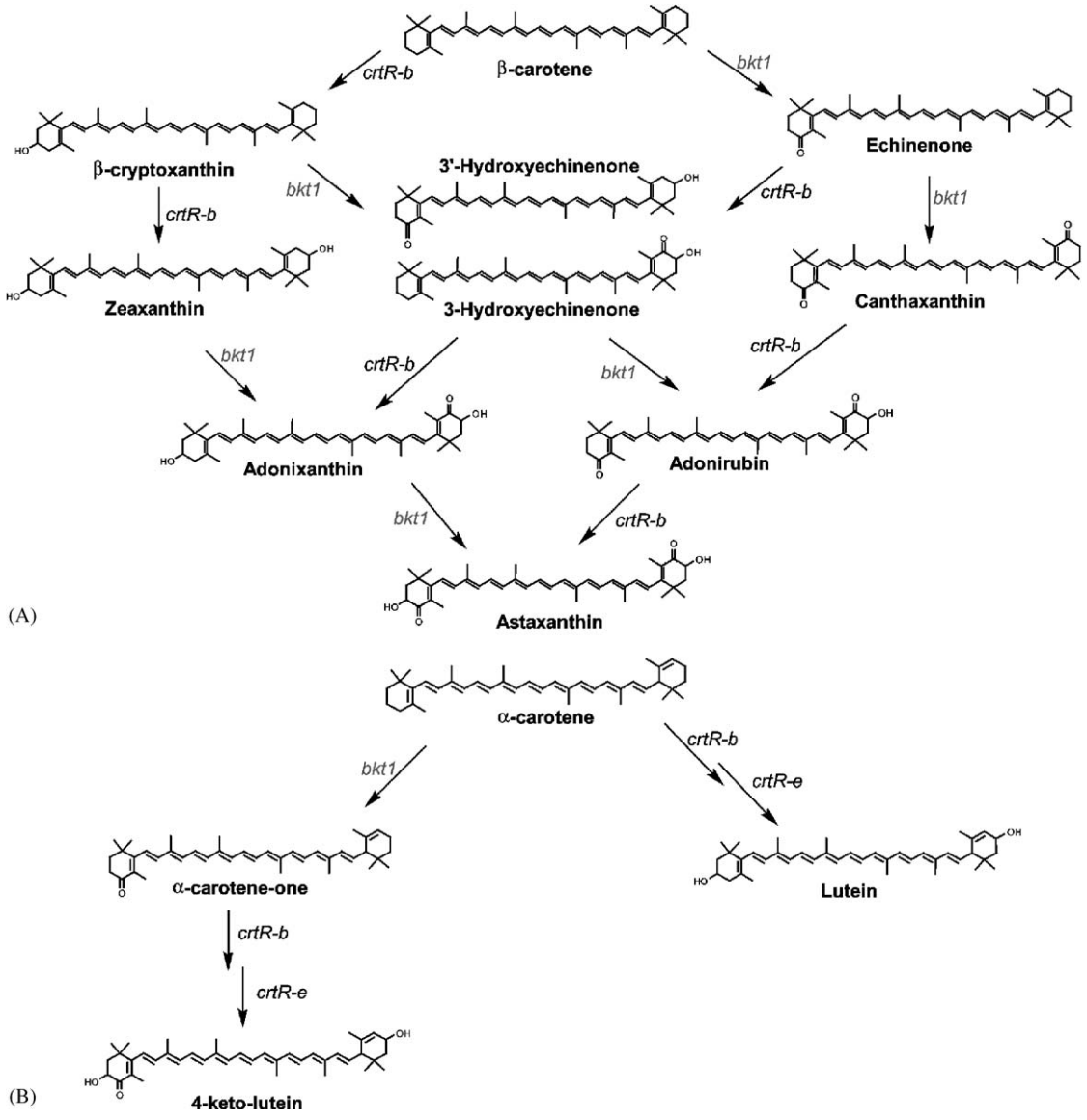

Fig. 1. Biochemical routes to ketocarotenoid formation from β-carotene (A) and α-carotene (B). *bkt1*, β-carotene ketolase; *crtR-b*, β-carotene 3,3′-hydroxylase; *crtR-e*, α-carotene 3,3′ hydroxylase (adapted from Mann et al., 2000).

0.39 kg (Bayer CropScience, UK). Plants were raised in a glasshouse maintained at a daytime temperature of 20 °C and a nocturnal temperature of 15 °C. The maximum irradiance was approximately 1000 μmol m^{-2} s^{-1} and the mean day length was 16 h.

2.2. Binary vector construction and transformation of potato

The β-carotene ketolase gene (*bkt1*) from the unicellular green alga *H. pluvialis* was kindly provided by Prof. J. Hirschberg, The Hebrew University of Jerusalem, Israel in the form of plasmid pPTCRTOBIB (Mann et al., 2000). Primers were designed to amplify region 1009–1448 bp of the plastid targeting PDS transit peptide (accession number X71023), along with region 211–1155 bp of the β-carotene ketolase coding region (accession number X86782), while engineering *Bam*HI sites (underlined) at both termini to facilitate sub-cloning. Primer sequences were: forward primer (5pdslead): 5′-TGGATCCAAAATGCCTCAAATTGGACTT-3′ and reverse primer (3bkt1): 5′-AGTGGATCCAGCTAGGCAGGAACCAG-3′. The resultant fragment was sub-cloned into pGEM-T Easy (Promega, UK) and sequenced on a 377 automated DNA sequencer (Applied Biosystems), using a cycle sequencing protocol and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), in order to determine the authenticity of the cloned fragment. For tuber-specific expression, the recombinant plasmid was digested with *Bam*HI and cloned behind a patatin promoter contained in binary vector pBI140.5 (essentially pBIN19 containing the 3.5 kb class I patatin cassette from pBI240.7; Bevan et al., 1986). Constructs were screened for orientation by restriction digestion and sense derivatives selected for plant transformation.

The phytoene synthase gene (*crtB*) from *Erwinia uredovora* was a gift from Dr N. Misawa, Marine Biotechnology Institute, Iwate, Japan. A construct enabling tuber specific expression was created as detailed in Ducreux et al. (2005a). Plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformed *Agrobacterium* cells were selected by their resistance to kanamycin (100 μg ml^{-1}) and rifampicin (100 μg ml^{-1}). Potato transformation (*S. tuberosum* cultivar Desiree) was carried out as described previously (Ducreux et al., 2005a). The procedure for co-transformation of *S. tuberosum* (cv. Desiree) was essentially the same as described above except that approximately equal amounts of binary vector harbouring *Agrobacteria* (two constructs), as determined by cell density measurements, were co-cultivated with explants. Transformation of *S. phureja* (cv. Mayan Gold) was performed using a modified potato transformation protocol (Ducreux et al., 2005b).

2.3. Screening of selected transformants

Screening for putative co-transformants was not possible by antibiotic selection alone because both binary constructs contained the same selectable marker for kanamycin resistance. Screening was therefore achieved by polymerase chain reaction analysis of genomic DNA extracted from tissue culture plantlets. Primers were designed to the coding regions of the *crtB* and *bkt1* transgenes and had the following sequences: Forward (HP*bkt1*for): 5′-ATCCAAGACCAGAGCTGGAC-3′; Reverse (HP*bkt1*rev): 5′-AAACCAGGGCACA-ATGCCAG-3′; and Forward (EU*crtB*for): 5′-TACTCAATCA-TGCGGTCGAA-3′; Reverse (EU*crtB*rev): 5′-AATAGCGCAGCGTATCATCC-3′. Genomic sequences were amplified in 20 μl PCR reactions containing 25–50 ng of template DNA, in the presence of 20 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 50 mM KCl, 200 nM of each primer, 100 μM of each dNTP and 0.5 units of *Taq* DNA polymerase. Thermal cycling conditions were: 3 min denaturation at 95 °C followed by 30 cycles (30 sec at 95 °C, 30 sec at 54°C, 1 min at 72 °C) followed by 5 min at 72 °C.

2.4. Analysis of tuber carotenoids

Peeled whole tuber samples (pooled samples of three tubers) were freeze-dried and stored at −80 °C prior to analysis. Triplicate samples from the different transgenic and control lines were analysed. Total potato tuber carotenoids were extracted and analysed by reverse phase HPLC. Ethyl acetate was used to dissolve samples for HPLC. Chromatography was carried using a Waters system (Watford, Hertfordshire, UK) consisting of a Waters Alliance 2600S system with no. 996 diode array. Data were collected and analysed using the Waters Empower software. Throughout chromatography, the eluate was monitored continuously from 200 to 650 nm. Column temperature was maintained at 25°C by a no. 7955 column oven (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). A reverse-phase $C_{30,}$ 5 μm column (250 × 4.6 mm) coupled to a 20 × 4.6 mm C_{30} guard (YMC Inc., Wilmington, NC, USA) with mobile phases consisting of methanol (A), water/methanol (20/80 by volume) containing 0.2% ammonium acetate (B) and *tert*-methyl butyl ether (C) was used. The gradient elution used with this column was 95%A, 5% B isocratically for 12 min, a step to 80% A, 5% B, 15% C at 12 min, followed by a linear gradient to 30% A, 5% B, 65% C by 30 min. A conditioning phase (30–60 min) was then used to return the column to the initial concentrations of A and B.

2.5. DNA extraction and Southern analysis

Plant genomic DNA was extracted from leaves as described previously (Draper and Scott, 1988). Ten micrograms of DNA was digested with *Bam*HI or *Kpn*I, as these enzymes only cut once within the T-DNA region, and resolved by electrophoresis on 0.8% agarose gels. DNA was transferred to nylon membranes (Hybond-N$^+$, Amersham) as previously described (Sambrook et al., 1989). Filters were hybridised with *crtB* cDNA labelled to high specific activity (ca. 1×10^9 cpm μg^{-1}) with [α-^{32}P] dCTP using random primers (HiPrime, Boehringer). Following hybridisation, filters were washed at low stringency (0.5 × SSC, 0.1% SDS at 45°C) and exposed to X-ray film for 24 hours at −80°C with intensifying screens.

2.6. Northern blot analysis

Northern blots were performed using total RNA extracted from tuber samples using an RNA isolation kit (Qiagen GmbH, Hilden, Germany). Ten micrograms per track RNA was analysed by denaturing agarose gel electrophoresis. Nucleic acids were transferred to positively charged nylon membrane (Hybond-N+, Amersham biosciences) as previously described (Sambrook et al., 1989) Filters were probed with random-primed (HiPrime, Boehringer) [α-^{32}P] dCTP labelled DNA for 16 h at 42 °C in formamide hybridisation buffer (Sambrook et al., 1989). A potato 18S ribosomal RNA probe was used as a control. Filters were washed in low and high stringency saline sodium citrate/sodium dodecyl sulphate solution at 42 °C until an acceptable signal to noise ratio was achieved. Relative gene expression level was determined by autoradiography.

2.7. Western blot analysis

Protein was extracted from freeze-dried tuber powder. Approximately 0.25 g of powder was extracted with 2 ml of extraction buffer (2% SDS, 40 mM Tris pH 8.0, 20% glycerol, 1 mM DTT). Samples were vortexed and then boiled for 10 min. Samples were then centrifuged at 4000*g* for 15 min. Supernatants were collected and the protein

quantified by DC Protein Assay (Bio-Rad). Sixty micrograms of protein per track was separated by electrophoresis on a pre-cast 4–12% acrylamide gel (Novex) prior to electroblotting on to nitrocellulose membrane. Membranes were blocked in phosphate-buffered saline (PBS) containing 3% (w/v) BSA, 2% (w/v) non-fat dry milk for 30 min at room temperature. The membrane was then incubated overnight at room temperature with rabbit polyclonal antibodies raised against CRTB (Fraser et al., 2002) or BKT1 (Grunewald et al., 2001) at a dilution of 1:1000 in PBS solution and 1:250, respectively. Secondary antibody alkaline phosphatase conjugate was used at a dilution of 1:7000. Western blots were developed using an alkaline phosphatase system according to the manufacturer's instructions (Bio-Rad).

2.8. Quantitative RT-PCR

Total RNA (10 μg) was treated with DNase I (Ambion Inc., Austin, Texas) before undergoing reverse transcription, using random hexamers as primer and SuperScript™ II reverse transcriptase (Invitrogen life technologies, Carlsbad, CA, USA), to generate first strand cDNA template. Potato ubiquitin primers were used as a control. Samples (1 μl of a 10-fold dilution) were amplified in 25 μl using a Perkin Elmer ABI Prism 7700 sequence detector in conjunction with the Quantitect SYBR green PCR kit (Qiagen GmbH, Hilden, Germany). Thermal cycling conditions were: 15 min denaturation at 95 °C followed by 40 cycles (15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C). Relative expression levels were calculated and the primers validated using the $\Delta\Delta$Ct method (Livak, 1997) using data obtained with the ubiquitin-specific primers as an internal control. Primer sequences were: BCHF, 5′-GTCTAAATTGGTCATCCCCACAA-3′; BCHR, 5′-TCCTGAAGTAGAAGAGGCGGAA-3′; PSYF, 5′-TGGCTTCAATCTCATCGAGAATC-3′; PSYR, 5′-AGGAGTGACAGAATTAAGCGCAG-3′; UBIF, 5′-ACACCATTGATAATGTCAAGGCTAAG-3′; UBIR, 5′-GCCATCCTCCAATTGCTTTC-3′.

3. Results

3.1. Generation of transgenic potato plants expressing an algal β-carotene ketolase gene (bkt1) in the tuber

The *bkt1* gene from *H. pluvialis*, encoding β-carotene ketolase was expressed specifically in the potato tuber with the aim of producing ketocarotenoid-accumulating tubers. The patatin promoter was chosen as a well-characterised tuber-specific promoter (Verhees et al., 2002) and the *bkt1* open reading frame was fused to the tobacco phytoene desaturase transit peptide (Mann et al., 2000) to ensure the β-carotene ketolase product was directed to the tuber plastid.

A. tumefaciens-mediated transformation was used to introduce the *bkt1* transgene to potato germplasm. We chose to transform both low tuber carotenoid *S. tuberosum* cultivar Desiree and also *S. phureja* cultivar Mayan Gold, which produces tubers containing ca. 5–8 fold higher levels of carotenoids than the Desiree tubers (Morris et al., 2004). A total of 30 transgenic lines were generated. Southern analysis confirmed the presence of the *bkt1* gene with copy number ranging from 1 to 3 (data not shown). Northern blot analysis demonstrated the presence of the *bkt1*-specific transcript in tubers from several transgenic lines from Desiree (bkt1-1, bkt1-3 and bkt1-5, Fig. 2A) and Mayan Gold (bkt1-1 and bkt1-2, Fig. 2B). No *bkt1*-specific transcript was detected in wild-type controls (Figs. 2A and B). Using polyclonal antibodies raised against the *H. pluvialis* β-carotene ketolase (Grunewald et al., 2001), BKT1 protein could be detected in several transgenic lines from both Desiree and Mayan Gold transformants, but was absent from wild-type control samples (Figs. 3A

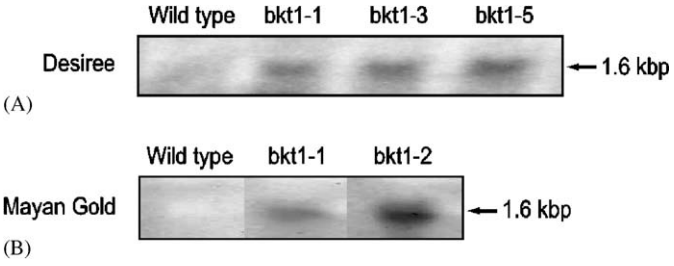

Fig. 2. (A) Northern analysis of total RNA from developing Desiree tubers; *bkt1* lines 1, 3, and 5 compared with wild-type control. (B) Northern analysis of total RNA from developing Mayan Gold tubers; *bkt1* lines 1 and 2 compared with wild-type controls. *Haematococcus pluvialis bkt1* cDNA was used as probe.

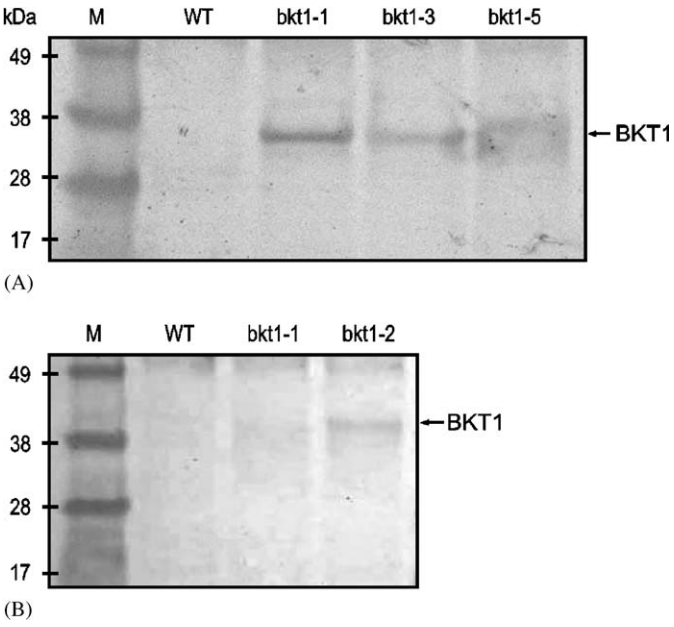

Fig. 3. (A) Western analysis of 60 μg total protein, using an anti-BKT1 polyclonal antibody, from developing Desiree tubers; *bkt1* lines 1, 3, and 5 compared with wild-type (WT) control (B) Western analysis of 60 μg total protein from developing Mayan Gold tubers; *bkt1* lines 1 and 2 compared with wild-type control.

and B). The apparent molecular mass of the BKT1 protein is ca. 37 kD, similar to the predicted size of the mature BKT1 protein (Fig. 3). The PDS transit peptide has a molecular mass of 12 kD (Mann et al., 2000) and so the absence of the precursor protein of 49 kD, implies that BKT1 is processed and targeted to the plastid in the potato extracts analysed.

3.2. Carotenoid analysis of bkt1 expressing tubers

Visual inspection of harvested tubers, clearly demonstrated marked changes in tuber pigmentation, with similar changes occurring in the different tissue types within the tuber flesh (Fig. 4). The carotenoid contents of tubers from several lines with the most obviously altered pigmentation were analysed in detail by HPLC-DAD. The carotenoid composition of the samples was determined on the basis of co-chromatography and spectral characteristics using a range of authentic standards (Table 1). In *bkt1* expressing lines of both Desiree and Mayan Gold, tuber ketocarotenoid accumulation could be detected. The only ketocarotenoids that were detected were ketolutein and astaxanthin. No intermediate ketocarotenoids such as echinenone, canthaxanthin, adonixanthin or adonirubin were present at detectable levels. Lutein, although a major constituent carotenoid in wild-type lines of both Desiree and Mayan Gold, was completely absent in *bkt1* expressing lines. Zeaxanthin, a major carotenoid in wild type Mayan Gold tubers was also greatly reduced in the *bkt1* expressing lines. Although clearly detectable, the total ketocarotenoid levels in Desiree *bkt1* lines were approximately 5% of the total ketocarotenoid levels detected in the *S. phureja* lines. Even these levels however were sufficient to cause a distinct colouration of the Desiree *bkt1* tubers compared with the

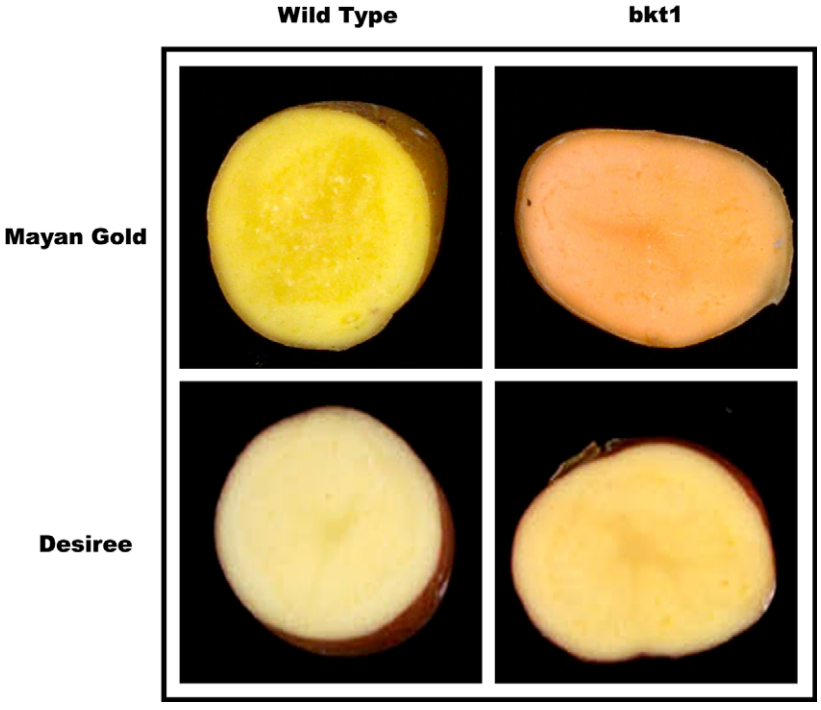

Fig. 4. Variation in flesh colour of *bkt1* transgenic lines compared to wild-type controls for Mayan Gold and Desiree freshly harvested mature tubers.

Table 1
Carotenoid content of mature tubers from Mayan Gold (MG) and Desiree (Des) transformed with *bkt1* compared with controls; values given are in $\mu g\,g^{-1}$ DW ± standard error of the means

	Vio	Lut	Zea	Xan est	Ketolut	Asta	Cant	β-car	Neo	Ant	Total	Total KC
Deswt	3.5±0.3	2.0±1.5	0.0±0.0	1.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.2	0.5±0.1	7.5±0.5	0.0±0.0
Desbkt1-1	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.2±0.0	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.1	0.4±0.1
Desbkt1-3	0.4±0.2	0.0±0.0	0.0±0.0	1.7±0.9	0.5±0.3	0.6±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	3.2±0.6	1.1±0.3
MGwt	0.4±0.2	11.2±0.7	11.7±2.0	14.0±2.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	37.3±2.5	0.0±0.0
MGbkt1-1	0.4±0.1	0.0±0.0	1.9±0.1	8.3±0.3	5.9±0.3	13.9±0.5	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	30.4±0.9	19.8±0.5
MGbkt1-2	1.2±0.0	0.0±0.0	0.7±0.0	5.7±0.1	9.8±0.4	9.5±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	26.9±0.3	19.3±0.2

Vio, violaxanthin; Lut, lutein; Zea, zeaxanthin; Xan est, xanthophyll esters; Ketolut, 4-ketolutein; Asta, astaxanthin; Cant, canthaxanthin; β-car, β-carotene; Neo, neoxanthin; Ant, antheraxanthin; KC, ketocarotenoids; wt, wild-type; bkt1, β-carotene ketolase 1.

wild type. Approximately 65–70% of the total carotenoid in the *S. phureja* transgenic lines accumulated as ketocarotenoid. As it was not possible to fully resolve the proportion of xanthophyll esters that were derived from ketocarotenoids, this percentage is probably an underestimate.

3.3. Co-transformation of S. tuberosum Desiree with a bacterial phytoene synthase gene (crtB) and an algal β-carotene ketolase gene (bkt1)

Previously, we have demonstrated that over-expression of an *E. uredovora crtB* gene encoding phytoene synthase, in tubers of Desiree, leads to a 5–8-fold increase in total carotenoid content and major changes in the types of carotenoids that accumulate (Ducreux et al., 2005a). In particular, the Desiree *crtB* expressing tubers accumulated significant levels of β-carotene whereas none could be detected in controls. Additionally the *crtB* expressing tubers contained levels of lutein up to 20-fold greater than in controls. Thus co-expressing *bkt1* and *crtB* simultaneously might be expected to result in a higher level of ketocarotenoid than expression of *bkt1* in a wild type background as there are enhanced levels of potential substrates of BKT1. Previously Stalberg et al. (2003) crossed *Arabidopsis* over-expressing an endogenous phytoene synthase gene (Lindgren et al., 2003) with *Arabidopsis* expressing *bkt1* and obtained 13-fold higher levels of ketocarotenoids in seeds from the cross, demonstrating that the availability of the *bkt1* substrate impacts on the levels of ketocarotenoid achievable.

In order to co-express the *bkt1* and *crtB* transgenes simultaneously a co-transformation approach was adopted (Chang et al., 2002). In this approach, the transgene constructs are multiplied separately in *A. tumefaciens* clones, and cultures of the *Agrobacterium* are mixed and incubated with the potato internode explants. Both constructs carried the neomycin phosphotransferase gene (NPT II) as a selectable marker. Transformants were selected for resistance to kanamycin and subsequently screened by PCR to identify single and double transformants. From 300 explants, 38 independent transformants were generated. Of these, 4 contained both transgenes Southern analysis verified the PCR screening strategy and gave an indication of the number of copies of each transgene (Table 2).

The expression levels of the *bkt1* and *crtB* genes were determined in four lines demonstrated by Southern analysis to be co-transformed. In developing tubers, *bkt1* and *crtB* expression could be detected in lines Bb2 and Bb3 (Fig. 5A). Immunoblotting of tuber protein extracts using antisera raised against CRTB and BKT1 demonstrated that both transcripts were translated in several co-expressed lines (Fig. 5B). As with the *bkt1* transformants, there was a visible effect on tuber flesh pigmentation (Fig. 5C). Detailed ketocarotenoid analysis was carried out on tubers from two co-expressing lines (Table 3). As with the *bkt1* transformants, low levels of ketocarotenoids were detected in the double transformants, however there was no major enhancement of ketocarotenoid content compared with tubers expressing only the *bkt1* transgene. In both the *crtB*/*bkt1* lines analysed, the levels of violaxanthin and lutein were substantially lower than in the wild type. In tubers from DescrtBb-3, substantial levels of β-carotene were also detected and a low level of canthaxanthin, an intermediate in the conversion of β-carotene to astaxanthin, was detected.

Table 2
Estimation of transgene copy number by Southern analysis of Desiree genomic DNA from putative *crtB* plus *bkt1* co-transformants, compared with wild-type (WT) and empty vector (EV) controls

Sample	CrtB transgene copy number	Bkt1 transgene copy number
WT control	0	0
EV control	0	0
CrtB + Bkt1-1	2	3
CrtB + Bkt1-2	5	2
CrtB + Bkt1-3	1	4
CrtB + Bkt1-4	4	2

3.4. Expression levels of phytoene synthase and β-carotene hydroxylase genes

The expression levels of the endogenous phytoene synthase and β-carotene hydroxylase genes were compared in tubers from Desiree and Mayan Gold in both the wild type and transgenic lines. As β-carotene hydroxylase acts in concert with BKT1 to catalyse astaxanthin biosynthesis (Fig. 1), it was of interest to determine whether in Desiree there was a reduced level of β-carotene hydroxylase transcript that may account for the relatively low levels of astaxanthin accumulation. However, the level of the β-carotene hydroxylase-specific transcript was consistently higher (by up to 2-fold) than in the Mayan Gold tubers (Fig. 6A). Furthermore there was no evidence of feedback regulation of β-carotene hydroxylase transcript level in any of the Desiree transgenic lines tested, although in Mayan Gold *bkt1*, a reduced (approximately 50% of the wild type) level of β-carotene hydroxylase transcript was measured.

The expression level of phytoene synthase transcript was also measured in wild type and transgenic lines (Fig. 6B) as phytoene synthase activity is generally considered to be the rate-limiting step in carotenoid biosynthesis (Taylor and Ramsay, 2005). As we have observed previously (Morris et al., 2004), phytoene synthase transcript level was higher in the *S. phureja* tubers than in those from Desiree. In the *bkt1* transformed Desiree lines there were no consistent significant effects on phytoene synthase transcript level. In one co-transformed transgenic line (Bb3) there was however an increase of ca. 2-fold in the phytoene synthase transcript level. In the Mayan Gold *bkt1* transgenic lines there was an approximately 4-fold reduction in the phytoene synthase transcript level, indicating that there was a feedback effect on gene expression.

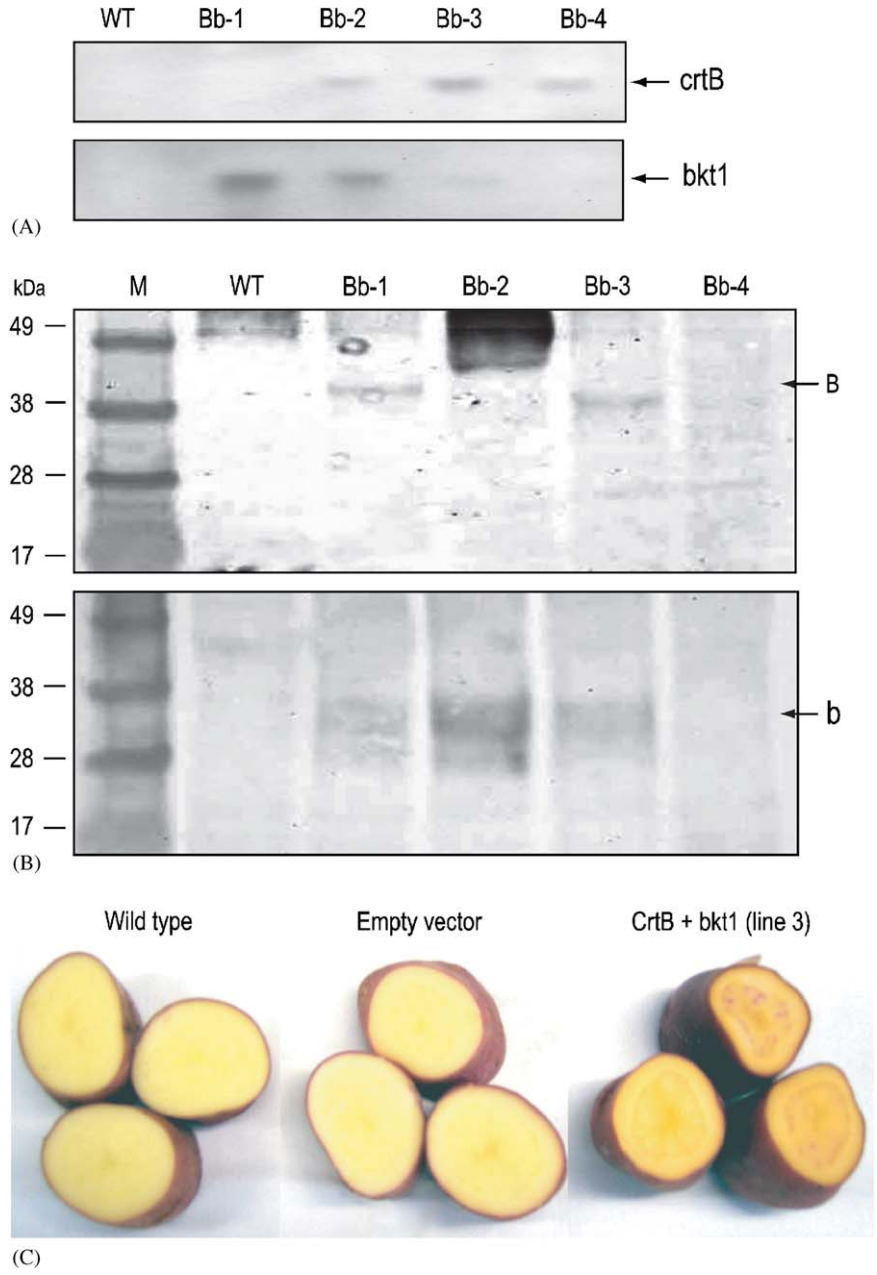

Fig. 5. (A) Northern analysis of total RNA from developing Desiree tubers; *crtB*+*bkt1* transgenic lines 2, 3 and 4 compared with wild-type (WT) controls. (B) Western analysis of 60 μg total protein from developing Desiree tubers; *crtB*+*bkt1* transgenic lines Bb-1, Bb-2, Bb-3 and Bb-4 compared to wild-type (WT) controls (arrow labelled B shows the CRTB-band, arrow labelled b shows the BKT1 band. (C) Variation in mature tuber flesh colour of *crtB*+*bkt1* transgenic line 3 compared with wild-type and empty vector controls.

Table 3
Carotenoid content of mature tubers from Desiree transformed with *crtb* and *bkt1* compared with controls; values given are in $\mu g\, g^{-1}$ DW ± standard error of the means

	Vio	Lut	Zea	Xan est	Ketolut	Asta	Cant	β-car	Neo	Ant	Total	Total KC
Deswt	3.5±0.3	2.0±1.5	0.0±0.0	1.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.2	0.5±0.1	7.5±0.5	0.0±0.0
DescrtBb-1	0.4±0.1	0.0±0.0	0.0±0.0	0.3±0.0	0.6±0.0	0.5±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	1.8±0.1	1.1±0.1
DescrtBb-3	0.0±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.0	0.2±0.0	4.7±0.4	0.0±0.0	0.0±0.0	5.2±0.2	0.4±0.0

Vio, violaxanthin; Lut, lutein; Zea, zeaxanthin; Xan est, xanthophyll esters; Ketolut, 4-ketolutein; Asta, astaxanthin; Cant, canthaxanthin; β-car, β-carotene; Neo, neoxanthin; Ant, antheraxanthin; KC, ketocarotenoids; wt, wild-type; bkt1, β-carotene ketolase 1.

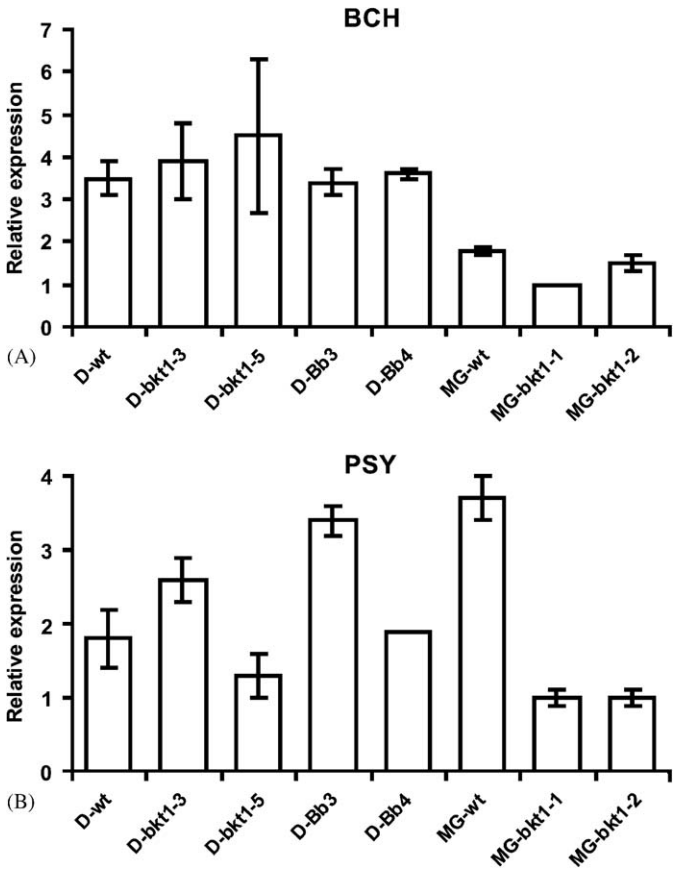

Fig. 6. Quantitative RT-PCR analysis of β-carotene hydroxylase (BCH, panel A) and phytoene synthase (PSY, panel B) transcript level in Desiree (D) and Mayan Gold (MG) tubers from wild-type (wt), bkt1 transformed (bkt1) and *crtB* and *bkt1* co-transformed tubers (Bb). Data are expressed relative to the transcript levels measured in the MG-bkt1-1 sample. Values are the means of three replicate measurements and error bars show the standard error of the mean.

4. Discussion

Previously the tomato Pds transit sequence has been shown to facilitate the efficient import of foreign peptides to both the chloroplast and chromoplast in transgenic tobacco (Mann et al., 2000). In this study, the tomato Pds transit peptide was also demonstrated to be effective in potato plastids. Western analysis of protein extracts from tubers of transgenic potato (both *S. tuberosum* and *S. phureja*) demonstrated the presence of correctly processed polypeptide and the absence of unprocessed precursor.

Ketocarotenoids were detected in both the *S. tuberosum* and *S. phureja* tubers expressing *bkt1* but were completely absent from controls. In contrast to previous attempts to engineer ketocarotenoids in storage organs, no ketocarotenoid intermediates in the pathway between β-carotene and astaxanthin were present in transgenic tubers. For example, in tomato fruit expressing bacterial 4,4′ oxygenase and 3,3′ hydroxylase activities, echinenone was the major fruit ketocarotenoid that accumulated (Ralley et al., 2004), whereas in *Arabidopsis*, adonirubin and canthaxanthin accumulated in addition to ketolutein, with very low levels of astaxanthin (Stalberg et al., 2003). As astaxanthin biosynthesis requires the concerted action of both a carotene 4,4′ oxygenase and a 3,3′ hydroxylase, this may indicate a high activity of the 3,3′ hydroxylase in potato tubers. Consistent with this viewpoint, potato tubers generally contain no or very low levels of β-carotene except when transgenically manipulated (Ducreux et al., 2005a), but do contain proportionately high levels of xanthophylls (Morris et al., 2004). The much lower levels of ketocarotenoid found in the Desiree transgenic lines expressing *bkt1* is not accompanied by a lower transcript level of β-carotene hydroxylase. In fact the β-carotene hydroxylase transcript level was approximately 2-fold higher in Desiree tubers than in those from Mayan Gold, although this does not exclude the possibility that enzyme activity is lower in Desiree tubers. Additionally potato tubers expressing *bkt1* also contain a high proportion of ketolutein, but no detectable α-carotene-one, indicating a high activity of α-carotene-hydroxylase. In previous attempts to engineer ketocarotenoid biosynthesis in plants, ketolutein has only been found in *Arabidopsis* and not in tobacco or tomato. An approach to increase the total level of tuber astaxanthin implied by these results would be to down-regulate lycopene ε-cyclase expression in the tuber, effectively blocking the route to lutein and ketolutein biosynthesis.

In Desiree tubers, *bkt1* transformation either on its own or in combination with *crtB*, results in only small changes in the levels of the endogenous phytoene synthase and β-carotene hydroxylase transcripts. In the Mayan Gold background however, the levels of both these transcripts decrease compared to wild-type values, with the phytoene synthase transcript decreasing by up to 4-fold. This highlights the regulatory mechanisms that operate in the carotenoid biosynthetic pathway that are not yet understood.

In previous transgenic studies in tobacco, a much higher proportion of ketocarotenoid was stored in an esterified form. For example in tobacco nectaries, 99% of the astaxanthin was esterified (Mann et al., 2000; Ralley et al., 2004). Although it was difficult to determine the proportion of xanthophyll esters that were derived from ketocarotenoid in this study, at least 70% of the ketocarotenoid present was not esterified. This may reflect that potato tubers do not generally store high levels of carotenoid possibly indicating that an efficient carotenoid storage mechanism is lacking in this tissue. It has been suggested that carotenoid storage mechanisms are rate limiting for storage organ carotenoid accumulation in some plant tissues such as cauliflower (Li et al., 2001) and the mechanisms that regulate carotenoid esterification and storage remain to be clarified.

The *bkt1* steady-state transcript level and BKT1 protein level are both higher in Mayan Gold *bkt1* line 2 than in *bkt1* line 1 (Figs. 2 and 3) and yet the ketocarotenoid levels in tubers are comparable. This finding implies that other factors are limiting ketocarotenoid accumulation above a

threshold value. Such factors could include the capacity for ketocarotenoid storage which may become rate-limiting. In the case of ketolutein accumulation, which also requires the action of an ε-ring hydroxylase, this activity could also be involved in defining a maximum value of ketocarotenoid accumulation. Another possibility is that phytoene synthase activity becomes rate limiting in the *bkt1* Mayan Gold tubers, as there is an approximate 4-fold decrease in transcript level in the transgenic lines compared with controls. Although *crtB* transcript and protein could be detected in transgenic lines by "semi-quantitative" northern and western analyses in some transgenic lines (Fig. 5), there was no apparent correlation between the levels of transcript, protein and final tuber carotenoid content. This may reflect varying degrees to which CRTB is targeted, processed or assembled into a functional complex. A similar phenomenon was noted in our previous study (Ducreux et al., 2005a, b) and also when *crtB* was expressed in tomato fruit (Fraser et al., 2001). As is commonly found in transgenic experiments, there was no correlation between transgene copy number and transcript level.

In an attempt to increase tuber ketocarotenoid levels over those measured on transformation with *bkt1*, the combined effects of *bkt1* and *crtB* over-expression were investigated. Bacterial *crtB* genes encode phytoene synthase and over-expression in potato tubers leads to an increased level of carotenoid accumulation (up to 6-fold) and also to an accumulation of β-carotene, a carotenoid not normally found in potato tubers (Ducreux et al., 2005a). In order to introduce both transgenes simultaneously, a co-transformation approach was used. This procedure simply requires the co-cultivation of explants with a mixture of *Agrobacterium* cultures each harbouring a different transgene construct. Approximately 10% of the transgenic lines generated were co-transformed with both transgenes, and expression of the transgenes could be detected by northern and western analysis. Overall, the frequency of co-transformation was 4 co-transformants per 300 explants (1.3%), similar to the frequency of 1.6% previously obtained in potato co-transformation experiments (Chang et al., 2002). The co-transformation approach has not been widely used for potato, however it does provide a powerful means of rapidly stacking transgenes. Additionally, as the same selectable marker can be used for each transgene, the method allows combinations of transgenes to be analysed without the requirement of engineering new constructs. A direct comparison of the single and double transgenics is difficult because the transgenes will be located in different loci in the genome in single and double transformants, potentially affecting their expression levels. However, the ketocarotenoid levels in tubers from the *crtB*/*bkt1* transformant were not enhanced in any of the lines compared with the Desiree tubers transformed with *bkt1* alone even when both transgenes were expressed and the levels were still much lower than those measured in the *S. phureja* transformed with *bkt1*. As some *bkt1*/*crtB* transgenic tubers accumulated β-carotene, this substrate was not utilised efficiently by the ketolase enzyme in the transgenic tuber. This may indicate that β-carotene is stored in a form inaccessible to the ketolase or that the ketolase does not readily use β-carotene as a substrate. In contrast, astaxanthin accumulation in the *S. phureja* transgenics appears to be at the expense of zeaxanthin and ketolutein accumulation is at the expense of lutein accumulation. Previous work has demonstrated that down-regulation of zeaxanthin epoxidase results in a large accumulation of zeaxanthin in tubers from some transgenic lines (Römer et al., 2002) and so over-expression of *bkt1* in this background may enhance astaxanthin levels further.

In summary, we demonstrate that the potato tuber can be engineered to store astaxanthin, which in contrast to other plant tissues investigated to date, accumulates in the absence of ketocarotenoid intermediates. Although the level of tuber astaxanthin accumulation is relatively low (up to $13.9\,\mu g\,g^{-1}$ DW) in comparison to some algal systems, the potato is an important staple and is consumed frequently in relatively large amounts by many populations. In addition to any nutritional benefit, the level of astaxanthin in *bkt1* expressing tubers was sufficient to affect tuber colour, an important determinant of consumer preference.

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