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RESEARCH PAPER

Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein

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Abstract

In order to enhance the carotenoid content of potato tubers, transgenic potato plants have been produced expressing an *Erwinia uredovora crtB* gene encoding phytoene synthase, specifically in the tuber of *Solanum tuberosum* L. cultivar Désirée which normally produces tubers containing *c.* 5.6 μg carotenoid g^{-1} DW and also in *Solanum phureja* L. cv. Mayan Gold which has a tuber carotenoid content of typically 20 μg carotenoid g^{-1} DW. In developing tubers of transgenic *crtB* Désirée lines, carotenoid levels reached 35 μg carotenoid g^{-1} DW and the balance of carotenoids changed radically compared with controls: β-carotene levels in the transgenic tubers reached *c.* 11 μg g^{-1} DW, whereas control tubers contained negligible amounts and lutein accumulated to a level 19-fold higher than empty-vector transformed controls. The *crtB* gene was also transformed into *S. phureja* (cv. Mayan Gold), again resulting in an increase in total carotenoid content to 78 μg carotenoid g^{-1} DW in the most affected transgenic line. In these tubers, the major carotenoids were violaxanthin, lutein, antheraxanthin, and β-carotene. No increases in expression levels of the major carotenoid biosynthetic genes could be detected in the transgenic tubers, despite the large increase in carotenoid accumulation. Microarray analysis was used to identify a number of genes that were consistently up- or down-regulated in transgenic *crtB* tubers compared with empty vector controls. The implications of these data from a nutritional standpoint and for further modifications of tuber carotenoid content are discussed.

Key words: β-Carotene, carotenoids, lutein, microarray, potato, transgenic.

Introduction

Carotenoid pigments are essential for photosynthesis and function as attractants in some higher plant organs (Demmig-Adams *et al.*, 1996). In humans and animals, the health benefits of dietary carotenoids are becoming increasingly apparent (Van den Berg *et al.*, 2000). Evidence is rapidly accruing that different carotenoids have 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; Hadley *et al.*, 2002). Lutein and zeaxanthin intake appear to provide protection against age-related macular degeneration (Krinsky *et al.*, 2003). The widespread occurrence of vitamin A deficiency is well documented and emphasizes the requirement for staple foods enhanced in carotenoids exhibiting provitamin A activity (Ye *et al.*, 2000, and references therein).

Many of the genes encoding the enzymes of the carotenoid biosynthetic pathway have been cloned from both plant and microbial sources over the past decade (reviewed in Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). Despite the rapid increase in understanding of the structural genes of the carotenoid biosynthetic pathway, its regulation is not well understood. Consequently, the rules that govern which carotenoids accumulate and to what levels, remain to be elucidated in many cases. This is of particular importance in plant storage organs that form major parts of our diet. Previous reports describe modifications of the amounts and types of carotenoid that accumulate in experimental and

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food plants by transgenic manipulation of the carotenoid biosynthetic pathway (reviewed in Fraser and Bramley, 2004). The overexpression of phytoene synthase has a particularly potent effect on storage organ carotenoid levels and results in increases in total carotenoid content in carrot roots (Hauptmann *et al.*, 1997), tomato fruit (Fraser *et al.*, 2002), canola seed (Shewmaker *et al.*, 1999), and *Arabidopsis* seed (Lindgren *et al.*, 2003). The extent of the increase varied between 1.6-fold in tomato fruit to 50-fold in canola seed, although the carotenoid level in non-transformed tomato was much greater than in control canola seeds. In all cases of phytoene synthase over-expression, β-carotene levels increased substantially, however, there is more variation in the effects on other carotenoids and isoprenoids. For example, in tomato fruit, lycopene levels increase broadly in parallel with the increase of β-carotene and total carotenoids. In canola, however, α-carotene and phytoene also increase on over-expression of a bacterial phytoene synthase. In *Arabidopsis* seeds, lutein levels increase significantly on over-expression of an endogenous phytoene synthase. The different effects of phytoene synthase over-expression may reflect the differences in origin of the over-expressed gene. The bacterial and plant encoded proteins may differ in their ability to form protein–protein complexes leading to different balances of products. Superimposed on this effect is a decrease in the flux control coefficient of phytoene synthase on over-expression. This causes other steps in the pathway to become rate-limiting (Fraser *et al.*, 2002). The metabolic bottleneck thus created may not be the same in different plants and organs, leading to the accumulation of different carotenoids.

In this report, the focus is on carotenogenesis in potato tubers. As the world's fourth most important source of calories, any nutritional enhancement of the potato tuber is of great significance. In most white-fleshed tuber cultivars, carotenoid content is low (38–62 μg 100 g^{-1} FW; Breithaupt and Bamedi, 2002), however, in diploid progeny of crosses of accessions of *S. stenotomum* and *S. phureja*, relatively high levels of carotenoids can accumulate giving rise to an orange tuber flesh colour, high in either zeaxanthin or lutein-5,6-epoxide and lutein depending on the parental material (Lu *et al.*, 2001). In the tubers from some of these crosses, total carotenoid content reached up to 1435 μg 100 g^{-1} FW compared with typical carotenoid levels of *c.* 10 000 μg 100 g^{-1} FW in carrot taproots (Simon and Wolff, 1987). Down-regulation of zeaxanthin epoxidase in tubers of *S. tuberosum* led, in some transgenic lines, to a dramatic increase in the zeaxanthin content and the total tuber carotenoid content increased by up to 5.7-fold (Römer *et al.*, 2002). Thus the whole of the carotenoid biosynthetic pathway appeared to be up-regulated in some transgenic lines. The expression level of zeaxanthin epoxidase was determined in a range of potato germplasm and an inverse correlation between zeaxanthin epoxide-specific transcript level in tubers and tuber carotenoid content was indicated (Morris *et al.*, 2004). These data highlight the need for a greater understanding of the factors that regulate carotenogenesis both at the biochemical and gene expression levels. In this study, a bacterial phytoene synthase gene has been specifically expressed in the tuber of *S. tuberosum* and the yellow–orange-fleshed tuber of *S. phureja.* The effects on tuber carotenoid profiles are described and associated changes in the expression of the pathway genes were monitored. Global changes in gene expression that occur as a result of the transgenic modification were also studied using microarray analysis of transcript profiles.

Materials and methods

Growth of tubers

Solanum tuberosum L. (cv. Désirée) and *Solanum phureja* (cv. Mayan Gold) were grown from seed tubers or *in vitro*-propagated tissue culture plants in 10 cm diameter pots containing compost. Plants were raised in a greenhouse maintained at a day/night temperature of 20/15 °C. The maximum irradiance was approximately 10 500 μmol m^{-2} s^{-1} and the mean day length was 16 h. Tubers were harvested from plants after flowering but prior to senescence and were designated 'developing tubers' and also from fully senesced plants, designated 'mature' tubers.

Binary vector construction and transformation of potato

The phytoene synthase gene (*crtB*) from *Erwinia uredovora* was obtained from N Misawa, Kirin Brewery Co. Ltd., Kanagawa, Japan. The open reading frame of the *crtB* gene was cloned in-frame behind the pea *rbc*S transit peptide in vector pJIT117 (Guerineau *et al.*, 1988) in order to target the *crtB* protein to the plastid. The resulting fusion had the following amino acid sequence surrounding the transit peptide cleavage site (–Val–Lys–Cys–Met–Pro–Ala–Ala–Asn–Asn–Pro–Ser–). For tuber-specific expression, the fusion was cloned behind a patatin promoter contained in a cassette (pBI240.7; Bevan *et al.*, 1983) and transferred to the multiple cloning site of pBIN19.

The LBA4404 strain of *Agrobacterium tumefaciens* was transformed with this pBIN19 derivative vector by electroporation. Transformed *Agrobacterium* cells were selected by their resistance to kanamycin (100 μg ml^{-1}) and rifampicin (100 μg ml^{-1}). Potato transformation (cv. Désirée) using the *crtB* construct and pBIN19 as an 'empty-vector' control was carried out using the *Agrobacterium*-mediated transformation method, essentially as described by Millam (2004). Approximately 30 independent lines for the *crtB* construct were generated. For transformation of *S. phureja* (cv. Mayan Gold) a modified transformation protocol was developed (LJM Ducreux, WL Morris, MA Taylor, S Millam, unpublished data). Critically, the time of exposure of the explants to *Agrobacterium* was reduced from 45 min to 5–10 min due to problems in bacterial overgrowth in this species.

Analysis of potato tuber carotenoids

Peeled whole-tuber samples (pooled samples of three tubers) were freeze-dried and stored at −80 °C prior to analysis. Total potato tuber carotenoids were extracted and analysed by reverse phase HPLC as detailed in Morris *et al.* (2004) on triplicate samples.

Analysis of tuber ABA and α-tocopherol content

For ABA analysis, 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. Approximately 0.2 g of developing tuber material was analysed for each replicate. Samples were extracted in 2 ml 80%

acetone and were treated according to Artsaenko *et al.* (1995). ABA was quantified using a Phytodetek-ABA-kit (Agdia, Elkhart, IN), using extracts diluted 1:10 in TRIS-buffered saline. For analysis of α-tocopherol from fresh potato tuber, sample extractions were carried out in Pyrex test tubes. Tuber sampling procedures have been described elsewhere (Griffiths and Dale, 2001), with tubers being cut into eighths and the two opposite eighths collected and extracted. This method allows for the presence of longitudinal and radial concentration gradients within the tissue. The cut tuber was immediately placed in liquid nitrogen and ground to a fine powder. The nitrogen was allowed to boil off and the frozen tuber was then subject to sequential extraction by shaking (5 min each at 1500 rpm, ambient temperature) with methanol (3 ml) containing methyl nonadecanoate internal standard (200 µl, 10 mg 50 ml^{-1} of methanol) followed by chloroform (6 ml). Finally, water (1.5 ml) was added, the mixture was shaken by hand (30 s) and then separated into two layers following centrifugation (10 min at 1000 rpm, ambient temperature). The bottom chloroform layer was removed by pipette, washed with water (1 ml) and, following further centrifugation, the bottom layer was isolated, dried overnight over anhydrous sodium sulphate at −20 °C, and then reduced to a small volume under dry nitrogen before being transferred to a 1 ml glass reaction vial. The remaining chloroform was evaporated and the resulting non-polar tuber extract dissolved in 100 µl isohexane containing 50 ppm 2,6-di-*tert*-butyl-4-methylphenol (BHT). Samples were stored at −20 °C prior to analysis by gas chromatography-mass spectrometry (GC-MS). Samples were derivatized by silylation and analysed by GC-MS as described by Shepherd *et al.* (1999). Quantification was based on the use of integrated total ion chromatogram peak areas for α-tocopherol and methyl nonadecanoate to calculate the ratio of α-tocopherol/internal standard. Reference samples containing 0.04 mg of internal standard and 0.04, 0.002, and 0.004 mg, respectively of α-tocopherol were used to calibrate the analysis.

DNA extraction and Southern analysis

Plant genomic DNA was extracted from leaves as described previously (Draper *et al.*, 1988). Ten micrograms of DNA were digested with *Bam*HI or *Kpn*I as these enzymes cut once within the T-DNA insert, and resolved by electrophoresis on 0.8% agarose gels. DNA was transferred to nylon membranes (Hybond-N^+, Amersham). Filters were hybridized with *crtB* cDNA labelled to high specific activity (*c.* 1×10^9 cpm μg^{-1}) with [α-^{32}P] dCTP using random primers (HiPrime, Boehringer). Following hybridization, filters were washed at low stringency (0.5× SSC, 0.1% SDS at 45 °C) and exposed to X-ray film for 24 h at −70 °C with intensifying screens.

Northern blot analysis

Northern blots were performed using total RNA extracted from tuber samples using an RNA isolation kit (Qiagen GmbH, Hilden, Germany). Ten µg 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-Mannheim) α-^{32}P dCTP-labelled DNA for 16 h at 42 °C in NorthernMax^{TM} ultrahyb buffer (Ambion Inc., Austin, Texas). A potato 18S ribosomal RNA probe was used as a control. Filters were washed in low and high stringency buffer at 42 °C (according to Ambion protocol) until an acceptable signal-to-noise ratio was achieved. Relative gene expression was determined by autoradiography.

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). 60 µg 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 PBS containing 3% (w/v) BSA, and 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 at a dilution of 1:1000 in PBS. Secondary antibody alkaline phosphatase conjugates was used at 1:7000 dilution. Western blots were developed using an alkaline phosphatase system according to the manufacturer's instructions (Bio-Rad).

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, California, USA), to generate a first strand cDNA template. Potato ubiquitin primers were used as a control. Samples were amplified 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 ΔΔCT method (http://www.appliedbiosystems.com) using data obtained with the ubiquitin-specific primers as an internal control.

Microarray processing and analysis

Total RNA was extracted from individual developing tubers of transgenic and control (empty-vector) lines using the RNeasy kit (Qiagen). Methods for fluorescent target RNA labelling, array hybridization, and washing were based on methods described by TIGR (http://www.tigr.org/tdb/potato/images/SGED_SOP_5.2.1.pdf). Indirect aminoallyl labelling of target RNA was performed as follows: a mix of total RNA (20 µg) and oligo $d(T)_{18}$ (2 µg) was denatured (10 min at 70 °C), chilled on ice, and a reverse transcription reaction was performed in a 45 µl volume containing 10 mM DTT, 0.5 mM each of dATP, dCTP, and dGTP, 0.2 mM dTTP, 0.3 mM aadUTP, and 400 U Superscript II (Invitrogen), for 3 h at 42 °C. RNA was degraded through the addition of 15 µl each of 1 M NaOH and 0.5 M EDTA, prior to neutralization with 15 µl of 1 M HCl. cDNA was purified using the MinElute PCR kit (Qiagen) as recommended and eluted twice with 10 µl phosphate elution buffer (4 mM phosphate buffer pH 8.6). Cy3 or Cy5 monofunctional dye ester (Amersham Biosciences) was dissolved in 10 µl DMSO and 1 µl added to purified cDNA adjusted to 0.2 M $NaHCO_3$ (pH 9.0). Cy dyes were coupled to cDNA in the dark at room temperature for 1 h prior to stopping the reaction with 3 µl 4 M hydroxylamine hydrochloride and incubating for a further 30 min. Labelled cDNAs were combined as appropriate and purified with the MinElute PCR kit (Qiagen).

Microarrays were supplied by TIGR (10K feature spotted cDNA potato arrays: http://www.tigr.org/tdb/potato/microarray2.shtml). Slides were prehybridized for 1 h at 50 °C in prehybridization buffer (5× SSC, 0.1% SDS, and 1% BSA) and rinsed in filtered water (×2) prior to centrifuge drying. For all hybridizations, cDNA derived from one replicate of a control (empty vector) line (Cy3) was hybridized with one replicate of a transgenic line (Cy5). Purified target cDNAs were denatured along with polyA DNA (2 µg; Amersham) for 5 min at 95 °C and combined with 3 vols SlideHyb Buffer 1 (Ambion), before being applied to the array surface beneath a HybriSlip (Grace Bio-Labs). Arrays were incubated in a humid chamber at 50 °C for 16 h and the arrays subjected to a series of 5 min washes (1× SSC, 0.1% SDS at 50 °C; 0.1× SSC at room temperature; 0.05× SSC at room

temperature), prior to rinsing in filtered water and isopropanol. Arrays were centrifuged dry and scanned with an ArrayWoRx Auto scanner (Applied Precision) with various exposure settings for Cy3 (595 nm) and Cy5 (685 nm) at 9.756 μm resolution, generating separate TIFF images.

Data were acquired from images using SoftWoRx Tracker software (Applied Precision). Images were imported and aligned with clone position information (TIGR GAL file) using automated and manual grid alignment features. Median spot and individual median background (annulus setting) intensity values were extracted for each wavelength and imported into GeneSpring (v. 6.1; Silicon Genetics), whereby replicate spots in each array were averaged. Data sets for each array were normalized using the Lowess algorithm and filtered by expression level to remove unreliable data points. Data from biological replicates across transgenic lines was used to determine confidence levels for differential expression (*t*-test *P*-values) which was filtered at a *P* value >0.05. A subset of genes was selected from this filtered list, which showed greatest fold-change and/or replicated differential expression from independent probes.

Semi-quantitative RT-PCR

Total RNA was extracted from tuber tissues using the RNeasy kit from Qiagen. Following DNase treatment, RT-PCR was carried out essentially as described by Simpson and Brown (1995), using 4.0 μg total RNA following the Life Technologies Superscript reverse transcriptase protocol. Primer sequences were as follows: STMET49 forward, CTTAATGATAAGCTCTGAGG, reverse, GCAACTATAACATTTCTTAGG; STMGQ82 forward, CTAGATTACACTCGAATTGC, reverse, TCGCTGCCTTAATTTCTC, STMHT55 forward, GCCGGAAGTGGAGCAAATAA reverse, ATCCGCCAATTGCTTCTTCA; STMIG86 forward, CTTCTTAGATTTACTACGG, reverse, ATGAGTAAGATGCGTTGG; STMHA49 forward, AAACTCAACTCTCTCTCGTT, reverse, CCAGAAGAATTCAGTACCTC; STMCE90 forward, ATGAGGTTCTGAGTGATCC, reverse, TGATCATCTCACCAGTACC; STMHY52 forward, AGGAACAAGTACAGGAGG, reverse, AACAGTAGCAACACCACC.

Results

Tuber-specific expression of a bacterial phytoene synthase gene leads to major changes in carotenoid content

Constitutive over-expression of phytoene synthase can have pleiotropic effects on plant development (Fray *et al.*, 1995) and so a construct was engineered containing the tuber-specific patatin promoter in conjunction with a plastid targeting sequence from the small subunit of ribulose-1,5-*bis*phosphate carboxylase/oxygenase and the *crtB* gene from *Erwinia uredovora* (Sandmann and Misawa, 1992). The aim was to achieve tuber-specific over-expression with plastid targeting of the transgene product. *Agrobacterium tumefaciens*-mediated transformation was used to introduce the transgene into both *S. tuberosum* cv. Désirée and *S. phureja* cv. Mayan Gold. By observing the effects of transgene over-expression in both *S. tuberosum* cv. Désirée, that produces light yellow-fleshed tubers containing *c.* 8.6 μg carotenoid g^{-1} DW and high (typically 20 μg carotenoid g^{-1} DW) tuber carotenoid (*S. phureja* cv. Mayan Gold) germplasm, it was hoped to understand more fully the factors that limit carotenoid content and balance.

Approximately 30 independent *crtB* transgenic lines of *S. tuberosum* cv. Désirée were generated. Based on visual assessment of tuber colour, a range of lines was selected for detailed analysis. Southern analysis demonstrated that the *crtB* transgene copy number varied between one and four. There was no obvious correlation between copy number and carotenoid content (Table 1). In the most extreme case, developing tuber carotenoid content increased by a factor of approximately seven from 5.6 μg g^{-1} dry weight (DW) typically found in wild-type control tubers to 35.5 μg g^{-1} DW. Furthermore, the balance of tuber carotenoids in the *crtB* lines was radically different from that observed in controls. In transgenic line *crtB*9, for example, the major tuber carotenoids were lutein (31% of total) and β-carotene (29% of total) whereas in control tubers, β-carotene was present at only neglible levels and lutein was typically up to 19-fold lower constituting 13% of the much lower total carotenoid content. The levels of total carotenoids were generally lower in mature tubers (for example, the mean total level in *crtb*5 tubers was approximately 30% lower than in tubers harvested from developing plants; Table 1). This may reflect the activity of the patatin promoter, which may be greater in developing than in mature tubers, a conclusion that is consistent with the results of western blotting with CRTB-specific antibodies (Fig. 1A), as the level of CRTB decreases in mature tubers. Northern analysis confirmed the expression of *crtB* in tubers from

Table 1. *Carotenoid content of Désirée tubers (μg g^{-1}DW)*

Values shown are for developing tubers of all clones and mature tubers of transgenic line *crtB5* (crtB5*). Values are the means of three replicates (standard error of the mean shown for total carotenoid values). For individual carotenoid values standard errors were less than 10% of the mean. Transgene copy number was estimated by Southern analysis (data not shown).

Sample	Neo	Vio	Ant	Lut	Zea	β-Car	Esters	Total	Copy no.
Wt	0.56	2.18	0.39	0.73	0.11	0.00	1.62	5.6±0.3	
EV	0.41	3.74	0.34	0.48	0.07	0.00	1.77	6.8±0.5	
CrtB2	0.83	6.61	0.41	4.75	0.21	5.37	2.48	20.7±1.8	1
CrtB5	0.91	6.13	0.68	5.22	0.00	7.26	2.50	22.7±2.7	4
CrtB9	1.07	8.52	0.71	11.01	0.71	10.30	3.20	35.5±1.6	ND
CrtB11	0.77	1.50	0.18	1.14	0.12	1.80	0.06	6.0±0.4	4
CrtB5*	0.65	1.74	1.35	6.85	0.00	2.82	2.89	16.3±1.3	4

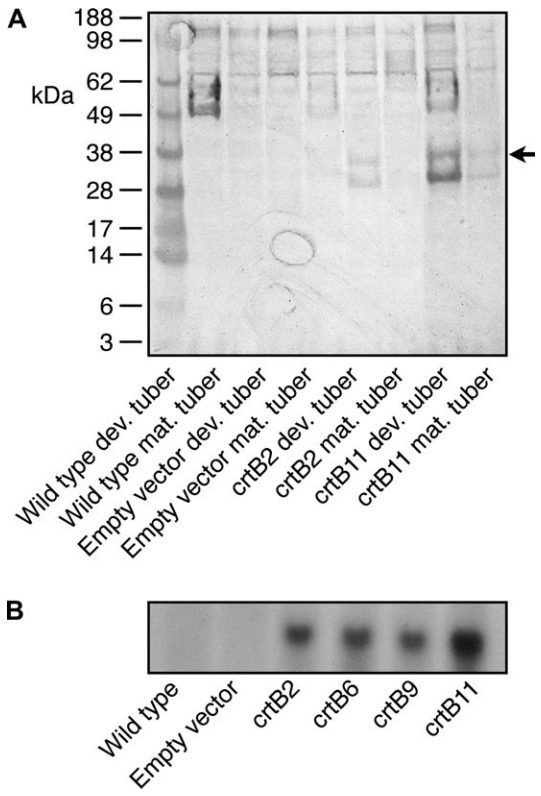

Fig. 1. (A) Western analysis of Désirée tubers (an empty-vector control and two transgenic lines, *crtB2* and *crtB11*) at the developing and mature stage using an anti-CRTB polyclonal antibody. Approximately 60 μg total protein was loaded in each lane. An immunoreactive band of molecular weight 35 kDa can be detected in the *crtB* lines (indicated with arrow). (B) Northern analysis of developing Désirée tubers; *crtB* lines (2, 6, 9, 11) compared with wild-type (WT) and empty-vector (EV) lines. The transcript size was estimated to be 1600 nucleotides.

the transgenic lines (Fig. 1B) and western analysis demonstrated the presence of the bacterial phytoene synthase in transgenic tubers by the presence of an immunoreactive CRTB protein with a molecular weight of *c.* 35 kDa, absent in the wild type and empty vector controls (Fig. 1A). An additional immunoreactive CRTB protein of molecular weight *c.* 30 kDa was also detected in *crtB* transgenic lines and this may be a degradation product as also observed in canola transformed with *crtB* (Ravanello *et al.*, 2003; Shewmaker *et al.*, 1999). Although *crtB* transcript and protein could be detected in transgenic lines by 'semi-quantitative' northern and western analyses, there was no apparent correlation between the levels of transcript, protein, and final tuber carotenoid content. This may reflect that in some transgenic lines, although containing CRTB at high levels, the protein is not correctly targeted, processed or assembled into a fully functional complex.

As some *S. phureja* accessions produce tubers of relatively high carotenoid content it was of interest to determine whether it was possible to increase carotenoid levels further or whether tuber carotenoid levels would reach a maximum value, limited by other factors. Attempts were made to transform several high tuber carotenoid *S. phureja* accessions, however, most were recalcitrant to transformation. It was possible to transform *S. phureja* cv. Mayan Gold, however, by making several critical modifications of the standard *S. tuberosum* transformation protocol (LJM Ducreux, WL Morris, MA Taylor, S Millam, unpublished results). Six independent *crtB* transgenic lines of *S. phureja* cv. Mayan Gold were selected to give a range of tuber carotenoid levels and were analysed in detail. Southern analysis demonstrated that transgene copy number varied from one to five (Table 2). As with the *S. tuberosum crtB* transgenics, there was no strong correlation between the apparent *crtB* expression level (determined by northern and western analyses) and final tuber carotenoid content (data not shown). Carotenoid contents in developing tubers were compared in transgenic lines with controls. The total tuber carotenoid level was maximally 78 μg g^{-1} DW (Table 2). The balance of carotenoids also changed in the *crtB* transgenics. Violaxanthin, antheraxanthin, lutein, and β-carotene were the major carotenoids that accumulated in the transgenics, whereas controls contained only neglible amounts of β-carotene and a much greater (*c.* 50% compared with 37%) proportion of violaxanthin.

Analysis of ABA and α-tocopherol levels in tubers

In some cases, over-expression of *crtB* in plant tissues leads to effects on the levels of isoprenoids other than carotenoids (Shewmaker *et al.*, 1999; Lindgren *et al.*, 2003). The levels of ABA and α-tocopherol were determined in developing tubers from Désirée *crtB* transgenic lines and empty-vector controls to investigate whether similar effects could be observed in potato tubers. The developing tuber ABA levels for two transgenic lines fell within the range of values measured in tubers from an empty-vector control line (Table 3). Values were similar to those previously published for potato tubers (Biemelt *et al.*, 2000). The level of α-tocopherol increased by approximately 70% in developing tubers of two *crtB* transgenic lines (Table 3).

The protein content of *crtB* tubers remained unchanged compared with empty-vector controls and no effects on tuber dormancy, were observed in lines *crtB5* and *crtB6* compared with empty-vector and wild-type tubers. For greenhouse-grown tubers from these lines, more than 90% of the tubers produced sprouts of at least 2 mm after 8 weeks of storage at 4 °C.

Quantitative RT-PCR of pathway genes

The transcript levels of the genes encoding the major carotenogenic structural genes were determined by quantitative RT-PCR relative to the ubiquitin expression level, used as an internal control. Expression levels were compared in developing tubers from *S. tuberosum* cv. Désirée empty-vector and wild-type lines and three *crtB* transgenic lines. The data shown are for three replicates of each

Table 2. *Carotenoid content of developing tubers from* S. phureja *(cv. Mayan Gold) transformed with* crtB *and controls*

Values are means of the determinations from three tubers from each line (standard error of the mean shown for total carotenoid values). For individual carotenoid values standard errors were less than 10% of the mean except for neoxanthin where the standard error approached 20% for some lines. The *crtB* transgene copy number, determined by Southern analysis is also shown.

Sample	Neo	Vio	Ant	Lut	Zea	β-Car	Esters	Total	Copy no.
Wt	1.14	11.71	4.23	2.36	0.73	0.00	3.99	24.4±1.5	
Ev4	0.90	7.21	5.53	1.48	1.29	0.00	2.90	19.3±0.8	
CrtB1	1.56	29.16	12.50	18.48	4.17	6.51	5.73	78.1±9.1	4
CrtB2	1.56	23.34	14.52	16.86	4.14	5.71	11.67	77.8±3.5	1
CrtB3	1.44	17.49	12.62	11.54	3.97	3.07	3.97	54.1±1.89	ND
CrtB4	0.81	10.80	11.48	9.05	3.92	1.76	2.70	40.5±2.5	1
CrtB5	1.33	15.90	11.09	11.60	2.98	3.15	3.64	49.7±4.3	5
CrtB6	1.19	15.07	16.46	13.09	6.35	3.17	4.76	59.5±5.0	1

Table 3. *Levels of ABA (pmol* g^{-1} *DW), α-tocopherol (μg* g^{-1} *FW) and total protein (mg* $^{-1}$ *DW) in developing tubers from Désirée* crtB *lines compared with an empty-vector control line (EV)*

Values are shown ±standard error of the mean for three replicate measurements.

Transgenic line	ABA content	α-Tocopherol content	Total protein content
EV	2518±266	4.47±1.68	33.3±1.6
CrtB5	2663±214	7.71±1.14	34.9±1.0
CrtB6	2182±255	7.55±0.14	ND
CrtB11	ND	ND	34.4±0.7

sample. Surprisingly few clear-cut trends could be seen on analysis of these data (Fig. 2). The most significant effect was the decrease in phytoene desaturase transcript level consistently observed in the *crtB* transgenics compared with controls, reflected in the larger ΔΔCT values in the transgenic tubers. Smaller decreases in the expression levels of zeaxanthin epoxidase and neoxanthin epoxidase were also observed for some transgenic lines, particularly lines *crtB*6 and *crtB*9, which contain elevated levels of carotenoid (Fig. 2).

Microarray analysis of crtB *transgenics*

In order to determine global effects on the potato tuber transcriptome, on *crtB* over-expression, a microarray approach was used. Tuber expression patterns in six biological replicates of *crtB* expressing developing tubers (three individual tubers from line *crtB*6 and three individual tubers from *crtB*10) were compared with six replicates from empty-vector control developing tubers. Following data processing, several genes were shown to be consistently, significantly up-regulated and others down-regulated in transgenic tubers compared with controls (Table 4). The filtering strategy selected changes on the basis of low *P* value (less than 0.05), combined with the fold change in expression, for signals that were significantly greater than the background noise. The results of the microarray experiment were confirmed by semi-quantitative RT-PCR for a number of the most significant changes identified

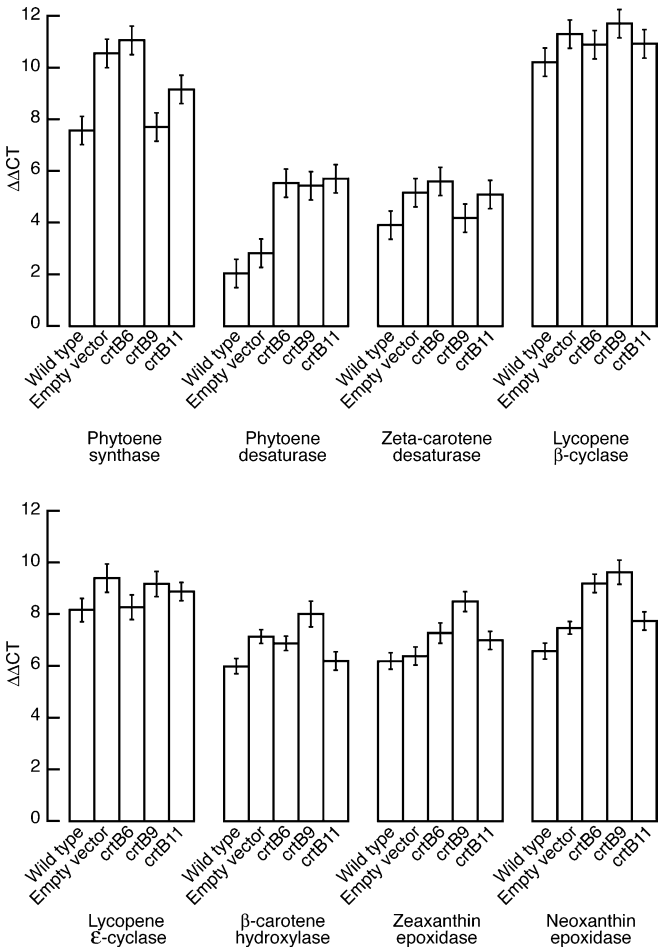

Fig. 2. Quantitative RT-PCR analysis of RNA extracted from Désirée *crtB* lines 6, 9 and 11 and controls. ΔΔCT values were calculated using ubiquitin expression level as a control. Error bars show the standard error of the mean for three replicate measurements. Primer sequences were as in Morris *et al.* (2004).

(Fig. 3). The expression patterns of this set of genes was also determined in developing tubers of the *S. phureja* 337 *crtB* transgenics, compared with controls. Some of the genes up-regulated in the Désirée transgenics were also up-regulated in *S. phureja crtB* transgenics (STMIG86, encoding a protein kinase, STMET49, encoding a glycine-rich protein)

Table 4. *Clones that are consistently expressed at higher or lower levels in* crtB*-expressing tubers than in empty-vector controls*

Clone numbers are the TIGR potato gene index reference numbers.

Clone number	Fold ±expression	*t*-test *P*-value	Description
STMET49	3.6	5.0E-04	TC49049: unnamed protein product (potato)
STMGQ82	3.5	2.3E-02	TC49958: heat-shock protein 18p (common tobacco)
STMHT55	3.1	5.1E-03	TC45368: fibrillin homologue CDSP34 precursor chloroplast (potato)
STMIG86	3.0	3.3E-03	TC44733: cell wall-associated protein kinase (*Arabidopsis*)
STMHA49	2.7	6.3E-04	BQ509132: no match
STMCE90	0.5	1.9E-02	TC48985: dnaJ protein homologue (potato)
STMHY52	0.4	1.4E-02	TC53315: class I patatin (potato)

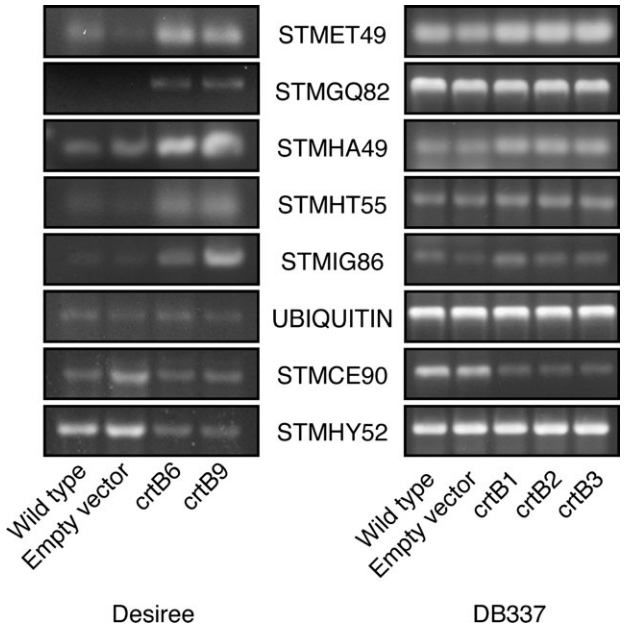

Fig. 3. Semi-quantitative RT-PCR analysis of expression levels of candidate genes identified by microarray analysis in developing tubers from Désirée and Mayan Gold *crtB* transgenic lines and controls. TIGR potato gene index clone identity numbers are given.

whereas for others the expression levels appeared to remain unchanged (STMHT55 encoding fibrillin, STMGQ82 encoding HS18) or showed only moderate changes (STMHA49). The expression levels of some genes decreased in the Désirée *crtB* transgenics compared with controls (genes encoding patatin and a dnaJ protein). The gene encoding the dnaJ protein was also down-regulated in the *S. phureja* background, although no changes in patatin transcript level were detected. Although patatin is the major tuber storage protein, no effects on total tuber protein content could be detected (Table 3) suggesting that this change is either compensated for by other increases in protein level, or that the change in transcript level is not reflected by the level of protein.

Discussion

Although it may be predicted that phytoene synthase overexpression will influence storage organ carotenoid content, the extent of the effect is highly variable in different plant species (Shewmaker *et al.*, 1999; Fraser *et al.*, 2002; Lindgren *et al.*, 2003). In light-yellow-fleshed potato tubers of *S. tuberosum* cv. Désirée, there was a maximal increase in total carotenoid content of 6.3-fold, raising total levels to those seen previously in tubers of some high carotenoid potato diploid germplasm. Interestingly, the carotenoid profile seen in the Désirée *crtB* lines was entirely different from profiles seen previously in high carotenoid diploid germplasm. Although high lutein- and zeaxanthin-containing tubers have been described in potato germplasm (Lu *et al.*, 2001) or as a result of transgenic modification (Römer *et al.*, 2002), a significant level of tuber β-carotene in any potato germplasm has not previously been reported. Some potato breeding lines do contain β-carotene, but at levels 60-fold lower than in the *crtB* transgenics reported here (Nesterenko and Sink, 2003).

The bioavailability of carotenoids is a complex issue and depends on many factors (reviewed in Fraser and Bramley, 2004). The conversion factor for β-carotene into retinol is currently being reviewed and varies between individuals in the population (Wang *et al.*, 2004). Consequently, without bioavailability studies it is difficult to predict the proportion of the RDA for vitamin A that could be provided by the *crtB* tubers. If the equivalence of β-carotene to retinol on a weight basis were 2 (as it is for β-carotene in oil), 300 μg retinol equivalents (the RDA for a 1–3-year-old child) are contained within *c.* 55 g DW of Désirée *crtB* tubers. However, some field studies suggest that 21 μg of β-carotene have the same activity as 1 μg of retinol (West *et al.*, 2002) and so it is unlikely that these tubers would provide 100% of the vitamin A RDA although a reasonable intake could make a significant contribution. Similarly, dietary supplementation with milligram quantities of lutein has been shown to be effective in raising macular pigment density (Bone *et al.*, 2003) and so the high lutein levels in the *crtB* tubers (maximally *c.* 18 μg g^{-1} DW in a *S. phureja* line *crtB*1) is also potentially beneficial. The third major carotenoid to accumulate in *crtB* tubers is violaxanthin, accounting for *c.* 25% of the total carotenoid in the most strongly affected *crtB* lines. This represents a relative decrease in violaxanthin compared with controls, although, in absolute terms, violaxanthin content is increased 2–4-fold. A simple explanation for the accumulation of β-carotene in *crtB* tubers is that the activities of β-carotene hydroxylases become rate-limiting as the carotenogenic flux increases,

resulting in a metabolic bottleneck. An interesting test of this hypothesis would be to over-express β-carotene hydroxylase in the *crtB* expressing lines and to monitor the effects on carotenoid balance. Thus it may be possible rationally to design tuber carotenoid content for specific nutritional benefits. As lutein also accumulates, but not the lutein precursors, lycopene, δ-carotene or α-carotene, it would appear that enzyme activities on this branch of the pathway are not rate-limiting.

In *S. phureja* (cv. Mayan Gold), *crtB* over-expression also had a major effect on tuber carotenoid content, again resulting in accumulation of β-carotene and high lutein content. The highest level of total tuber carotenoid content reached a mean value of *c.* 78 μg g^{-1} DW, an exceptionally high value for potato germplasm. Proportionately, rather less β-carotene accumulated in these tubers than in the Désirée transgenics. As the levels of antheraxanthin and violaxanthin also increase, β-carotene hydroxylation may not be such a major metabolic bottleneck in these transgenics. These results suggest approaches for optimizing the accumulation of particular carotenoids, for β-carotene accumulation, down-regulation of lycopene ε-cyclase and β-carotene hydroxylase in combination with *crtB* expression may result in a greater accumulation of β-carotene.

In *Arabidopsis* seeds, over-expression of *crtB* also led to increases in ABA level and associated effects on seed dormancy (Lindgren *et al.*, 2003). At the whole tuber level, a *crtB*-dependent change in ABA content in two lines in which carotenoid levels changed significantly could not be detected. In addition, in these lines, tuber dormancy was not affected by *crtB* expression. These data are in agreement with the findings of Römer *et al.* (2002), who also reported no changes in ABA levels or developmental effects in tubers in which carotenogenesis had been stimulated. Also in agreement with the findings of Römer *et al.* (2002) was the increase in α-tocopherol content in tubers in which carotenogenesis was stimulated. In *Brassica napus* seeds however, *crtB* expression led to an approximate 2-fold decrease in total tocopherol content (Shewmaker *et al.*, 1999). These contrasting effects of *crtB* expression may illustrate that isoprenoid metabolism is regulated in a different manner in seeds and tubers.

The effects of *crtB* over-expression on other transcripts in the potato tuber were also investigated to identify functions that may regulate carotenoid biosynthesis and storage. The largest consistent difference in expression level was for the phytoene desaturase-specific transcript, which showed a decrease in all *crtB* transgenic lines compared with wild type and empty-vector controls. This indicates that phytoene desaturase transcript level is not rate-limiting under wild-type conditions and that a significant decrease in expression level still enables elevated levels of carotenoid accumulation. It would be interesting to determine whether the decrease in phytoene desaturase expression occurs as a response of the plant to limit carotenogenesis. In this case, over-expression of phytoene desaturase in conjunction with phytoene synthase may enable even higher levels of carotenoids to accumulate. The transcript levels specific for zeaxanthin epoxidase and a putative neoxanthin epoxidase are also decreased in some transgenic lines, particularly in tubers from Désirée *crtB*9 in which carotenoid levels were particularly high. The increase in zeaxanthin in the *crtB* transgenic lines, however, is much lower than that observed on zeaxanthin epoxidase down-regulation (Römer *et al.*, 2002). It has been suggested that zeaxanthin epoxidase transcript levels may be regulated in potato tubers in order to maintain ABA homeostasis (Morris *et al.*, 2004) and the effect observed in the *crtB* tubers may be a manifestation of this mechanism.

Global changes in transcription were analysed in *crtB* tubers compared with those from empty-vector control lines using the 10 000 gene microarrays available from TIGR. One of the up-regulated cDNAs encodes fibrillin, well-characterized as a protein involved in carotenoid storage (Rey *et al.*, 2000). Its up-regulation on enhanced carotenogenesis in Désirée, although not previously reported in potato tubers, is entirely consistent with its known role. In the *S. phureja* transgenics, however, no increase in fibrillin transcript level was detected possibly reflecting that in high-carotenoid germplasm, fibrillin expression is constitutively higher. The functions of the other up-regulated cDNAs are not well characterized. STMIG86 does contain a conserved protein kinase domain and thus may have a regulatory function. As this gene was up-regulated in the *crtB* transgenics in both the Désirée and Mayan Gold background, its enhanced expression may reflect a regulatory role in carotenogenesis.

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