Plant Biotechnology Journal

Plant Biotechnology Journal (2015), pp. 1–13 doi: 10.1111/pbi.12365

Product stability and sequestration mechanisms in *Solanum tuberosum* engineered to biosynthesize high value ketocarotenoids

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Received 23 November 2014;
revised 8 February 2015;
accepted 22 February 2015.
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Accession Numbers for genes discussed in the article: Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AB377271, AB377272 and X17295.

Keywords: ketocarotenoid, carotenoid, sequestration, metabolic engineering, *Solanum tuberosum*, starch.

Summary

To produce commercially valuable ketocarotenoids in *Solanum tuberosum*, the 4, 4′ β-oxygenase (*crtW*) and 3, 3′ β-hydroxylase (*crtZ*) genes from *Brevundimonas* spp. have been expressed in the plant host under constitutive transcriptional control. The CRTW and CRTZ enzymes are capable of modifying endogenous plant carotenoids to form a range of hydroxylated and ketolated derivatives. The host (cv. Désirée) produced significant levels of nonendogenous carotenoid products in all tissues, but at the apparent expense of the economically critical metabolite, starch. Carotenoid levels increased in both wild-type and transgenic tubers following cold storage; however, stability during heat processing varied between compounds. Subcellular fractionation of leaf tissues revealed the presence of ketocarotenoids in thylakoid membranes, but not predominantly in the photosynthetic complexes. A dramatic increase in the carotenoid content of plastoglobuli was determined. These findings were corroborated by microscopic analysis of chloroplasts. In tuber tissues, esterified carotenoids, representing 13% of the total pigment found in wild-type extracts, were sequestered in plastoglobuli. In the transgenic tubers, this proportion increased to 45%, with esterified nonendogenous carotenoids in place of endogenous compounds. Conversely, nonesterified carotenoids in both wild-type and transgenic tuber tissues were associated with amyloplast membranes and starch granules.

Introduction

Potatoes represent one of the most widely consumed vegetables across the globe and are a staple food crop. In addition, tuber material is often utilized as animal feed and as an industrial source of starch. The development of new potato varieties that command higher commercial value is a current goal for both growers and industry. In addition, the commercial cultivation of potato generates large quantities of waste tuber and vegetative material which offer potential as renewable feedstocks for use in biorefining cascades.

Ketocarotenoids, such as canthaxanthin and astaxanthin, are high-value pigments used in the food, feed and health sectors (Breithaupt, 2007) and are presently produced by chemical synthesis, using precursors derived from the petro-chemical industry (Ausich, 1997). Several algal species can act as natural sources, but production requires highlight, controlled nutrient depletion and extended growth periods, which increase susceptibility to contamination (Lorenz and Cysewski, 2000). With the exception of several marine bacteria (Misawa *et al.*, 1995) and the fungus *Xanthophyllomyces dendrohous* (Park *et al.*, 2009), microbes capable of synthesizing ketocarotenoids are rare. On a hectare basis, plant-based sources are more economically favourable (Ausich, 1997) and offer the least environmental impact. Unfortunately, *in planta* the presence of high value ketocarotenoids such as astaxanthin has only ever been reported in the flowers of the *Adonis* species (Cunningham and Gantt, 2005). This plant is not readily amenable to agricultural production. Although the *Adonis* plant and other microbial sources may not be the suitable production hosts, their biosynthetic genes involved in the formation of ketocarotenoids have been isolated facilitating the metabolic engineering in heterologous hosts (Choi *et al.*, 2005). Ketocarotenoid formation has been engineered into a number of crop plants, giving rise to a myriad of keto/hydroxylated carotenoids with varying qualitative and quantitative profiles. The gene products used, promoters, crops and levels are summarized in Table S1.

In this study, the 4,4′ carotenoid oxygenase (*crtW*) and 3, 3′ carotenoid hydroxylase (*crtZ*) from *Brevundimonas* have been utilized (Choi *et al.*, 2005). The gene products are highly effective in converting monohydroxylated carotenoids to ketocarotenoids. CRTW catalyses the introduction of keto groups at the 4 and 4′ positions of the carotenoid β-ionone rings, in the absence or presence of hydroxylation at the 3, 3′ position (Figure 1). Thus, CRTW can act directly on the endogenous β-carotene, or zeaxanthin pools as precursors. CRTZ from *Brevundimonas*

Please cite this article as: Mortimer, C.L., Misawa, N, Ducreux, L., Campbell, R., Bramley, P.M., Taylor, M. and Fraser, P.D. (2015) Product stability and sequestration mechanisms in *Solanum tuberosum* engineered to biosynthesize high value ketocarotenoids. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12365

catalyses the incorporation of hydroxyl moieties at the 3, 3′ positions on the β-ionone rings and acts on previously ketolated β-ionone ring carotenoids at the 4, 4′ positions. The potential products from the actions of CRTZ and W on endogenous precursors are illustrated in Figure 1. Here, the astaxanthin biosynthetic pathway has been engineered into potato plants. The products formed have been characterized, both their stability and the mechanisms utilized to sequester these new products elucidated, and the implications discussed.

Results

Generation and phenotypic analysis of *S. tuberosum* transformants expressing *crt*Z and *W*

Agrobacterium-mediated transformation was used to introduce vector p*crtZW* into *S. tuberosum* cv. Désirée. The vector contained chimeric genes of interest, consisting of chemically synthesized forms of *crt*Z and *W*, codon optimized for expression in plants. Each gene product was targeted to the plastid with the RuBisCo small subunit signal peptide. Constitutive expression was achieved with independent CaMV35S promoters. Following antibiotic selection, plants confirmed to contain both transgenes by PCR were regenerated. Independent transformed lines (10 in total) were cultivated in the glasshouse. Nontransgenic wild-type (WT) and 'empty vector' (vector minus the *crtZ/W* transgenes) controls were regenerated concurrently. No difference in plant vigour or tuber yield between transgenic plants and controls was observed (Figure S1). However, all plants expressing *CrtZ/W* possessed brown/green leaves compared to lighter green leaves observed in the WT (Figure 2a and c). Colour-metre readings confirmed these changes, with ΔE**ab* values showing a 10%–40% difference, compared to the controls.

Identification and quantification of pigments present in leaves and tubers of *S. tuberosum* transformants

To rapidly visualize the presence of new nonendogenous carotenoids, thin-layer-chromatography (TLC) analysis was performed. The presence of unique carotenoid components in the extracts was evident when compared to the WT (Figure 3). On the basis of co-chromatography (Rf values) with authentic standards and colour, the WT pigments in leaf extracts separating on TLC were chlorophyll, lutein and β-carotene. All extracts from *crtZ/W* lines contained astaxanthin, phenicoxanthin, canthaxanthin and echinenone. Lutein, violaxanthin and antheraxanthin were

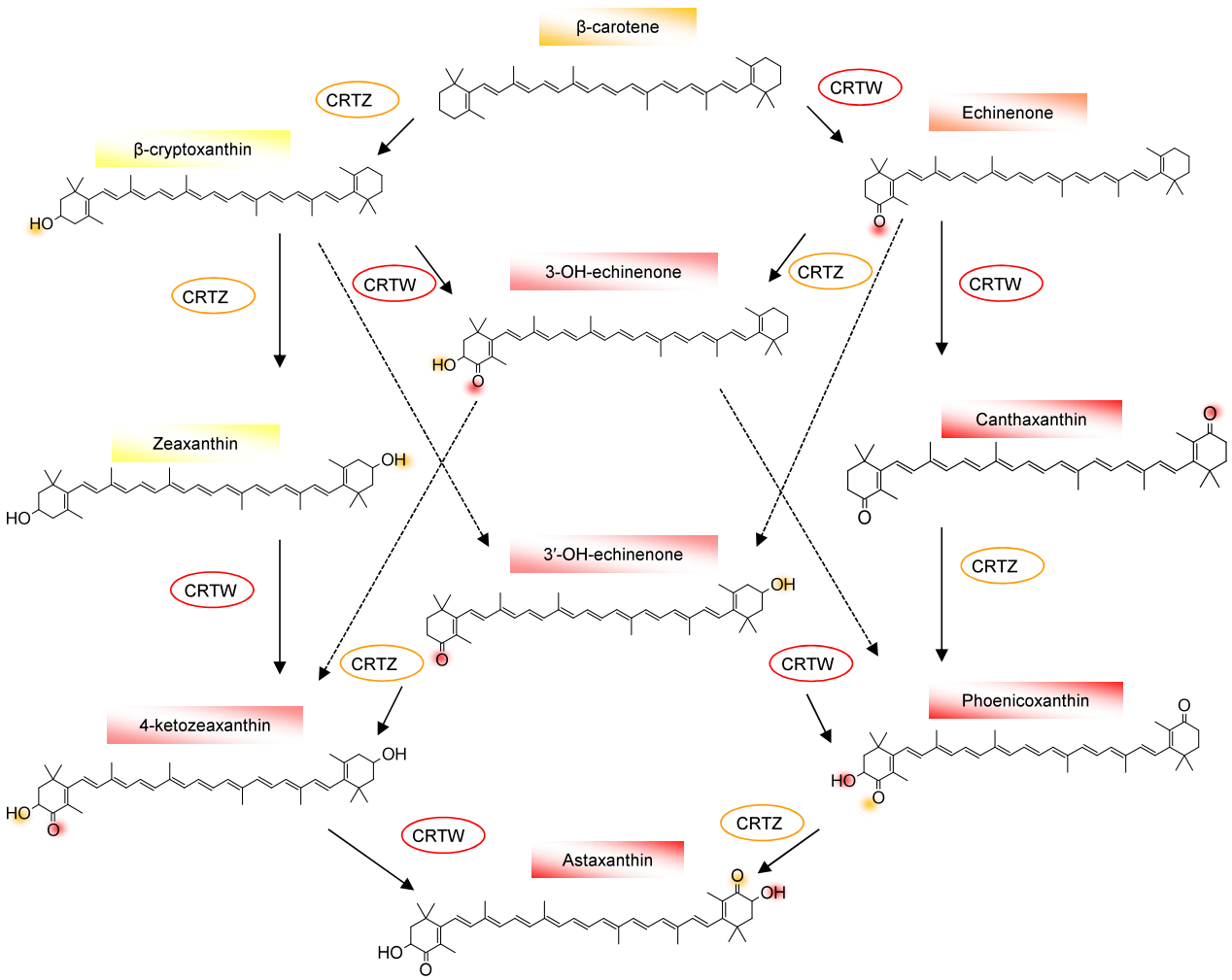

Figure 1 Schematic illustration of the biosynthesis of astaxanthin from β-carotene as catalysed by from *Brevundimonas* sp. CRTW and CRTZ. Oxygen/hydroxy moieties introduced into the carotenoid skeleton are shaded in red and yellow, respectively. Enzymes are indicated by their gene assignment symbols: CRTW, β-carotene ketolase; CRTZ, 3, 3′ hydroxylase.

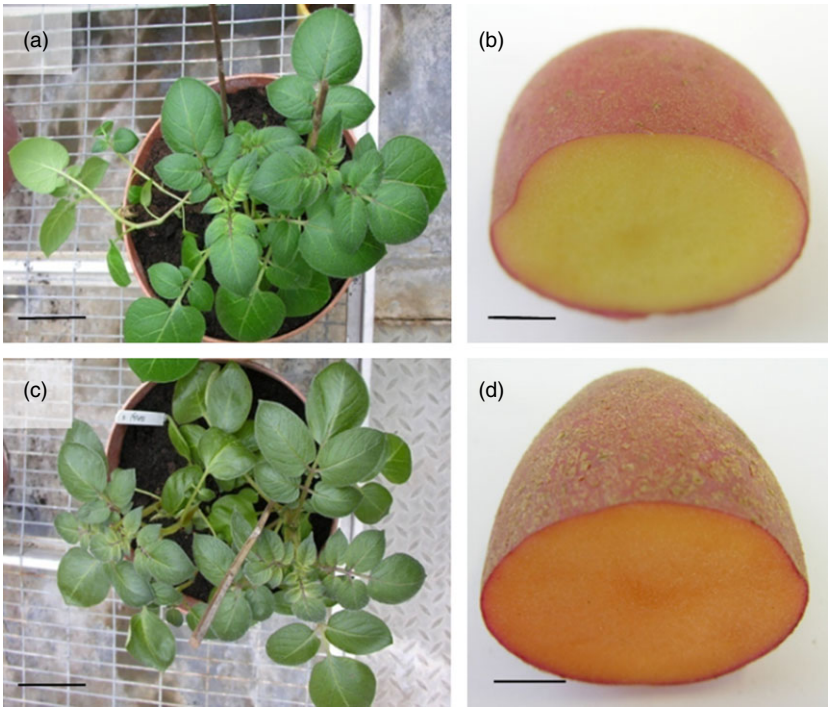

Figure 2 Colour changes in leaf, flower and tuber tissue of transgenic potato line E1P1(1) expressing *Brevundimonas* sp. *crtW* and *crtZ*. (a–b) WT: (a) Leaf. (b) Tuber. (c–d) Line E1P1(1): (c) Leaf. (d) Tuber. The WT phenotype is also representative of EV plants. The phenotype of line E1P1(1) is representative of all *crtZ*/*crtW* transgenic potato lines produced. Scale bars are 10 cm in leaf images and 1 cm in tuber images.

identified in chromatograms of WT tuber extracts, along with yellow diffuse banding in the Rf region from 0.6 to 0.8. These components were found to co-chromatograph with carotenoid esters derived from marigold flowers. Diffuse banding was also observed in this region in transgenic extracts, but was of an intense orange pigmentation. These components were found to co-chromatograph with ketocarotenoids esters derived from *Haematoccous* cysts or *Adonis* flowers. Intensely coloured components, identified from Rf values as astaxanthin, 4-ketozeaxanthin and 4-ketolutein, were detected in all transgenic tuber extracts. Based upon the intensity of TLC components, three high ketocarotenoid-containing lines (E1P1(1), E1P1(4) and E1P1(10)) were selected for detailed pigment analysis by high-performance liquid chromatography with photo-diode-array detection (HPLC-PDA).

Transgene expression of both *crt*W and *crt*Z in recombinant plants was confirmed by quantitative RT-PCR in leaf and tuber tissue from lines E1P1(1), E6P1(4) and E1P1(10). There was no significant difference ($P > 0.05$) in the expression of either transgene, between any of the transgenic lines in leaf or tuber tissue (Figure S2). The chromatographic and spectral properties of the carotenoids in WT and GM lines are provided in Table S2, listed by component number (C#). Figure 4a illustrates a typical carotenoid profile from a WT leaf extract, with 13 pigment components, neoxanthin (C#1), violaxanthin isomers (C#2–5), antheraxanthin (C#6), chlorophyll-b (C#7), lutein (C#8), zeaxanthin (C#9), chlorophyll-a (C#10), pheophytin (C#11), β-carotene (C#12) and its geometric isomer (C#13). Figure 4b illustrates a profile resulting from the presence of CRTZ/W, the unique peaks being 4-ketolutein (C#14), astaxanthin (C#15), phenicoxanthin (C#16), canthaxanthin (C#17) and echinenone (C#18). The content of individual carotenoids within transgenic lines is provided in Table 1. In summary, the proportion of unique ketocarotenoids in leaf extracts varied from 20% to 34% of the total carotenoids. In WT tubers, violaxanthin isomers, antheraxanthin, lutein and zeaxanthin were present (Figure 5a).

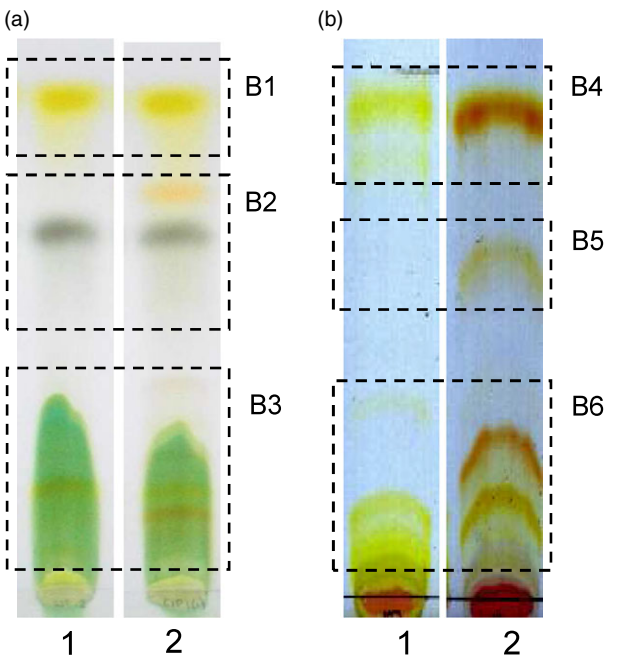

Figure 3 TLC separation of pigment extracts from leaf and tuber tissue of WT and *crtZ*/*crtW* transgenic potato. (a) Analysis of pigment extracts from leaf material. Lane 1 WT. Lane 2 *crtW*/*crtZ* line E1P1(1). Region B1 contains β-carotene; B2 contains echinenone and chlorophyll; B3 contains canthaxanthin, astaxanthin, lutein and chlorophyll. (b) Analysis of pigment extracts from tuber tissue. Lane 1 WT. Lane 2 line E1P1(1). Region B4 contains carotenoid esters; B5 contains 4-ketozeaxanthin; B6 contains lutein, violaxanthin, astaxanthin and 4-ketolutein. See Table S2 for spectral and chromatographic properties.

In addition, a number of carotenoid esters were detected (components labelled CE in Figure 5a). The endogenous carotenoids diminished when CRTZ and W were present (Figure 5b),

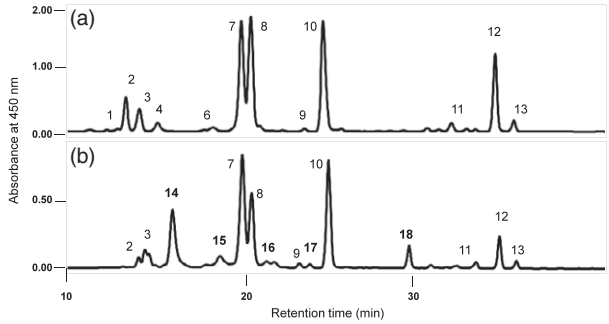

Figure 4 HPLC-PDA profiles of carotenoids present in leaf tissue from WT and *crtZ/crtW* transgenic potato plants. (a) WT leaf. (b) *crtZ/crtW* transgenic leaf. Each chromatogram component is labelled. Those labelled 14–18 (numbered in bold) were unique to transgenic plants. See Table S2 for spectral and chromatographic properties.

resulting in the appearance of 4-ketolutein, astaxanthin, 4-ketozeaxanthin (C#19) and a number of ketocarotenoid esters (components labelled KE in Figure 5b). These esters did not display persistence or inflexions in their UV/Vis spectra typical of endogenous carotenoids. Instead, a classic keto 'bell'-shaped spectra were evident, with a spectral maxima between 465 and 477 nm. Therefore, we presume that these were a mixture of astaxanthin and 4-ketozeaxanthin esters.

Sequestration mechanisms in leaf and tuber tissues

Following the determination that the CRTZ/W enzymes could form nonendogenous carotenoids in both leaf and tuber tissues, the mechanisms associated with the storage of these compounds were investigated. As a representative example of the *crtZ/W* transgenic phenotype, line E1P1(1) was used for these studies and compared to its WT counterpart.

Isolated leaf chloroplasts were separated into plastid membranes and plastoglobuli-enriched fractions. In both the WT and transgenic genotypes, the plastoglobuli contained a relatively small proportion (0.2%–1.3%, respectively) of the total carotenoids found in the plastid (Table 2). However, the transgenic genotype contained a 5-fold increase in the carotenoid content of the plastoglobule. The endogenous carotenoids found in the plastoglobule fraction were phytoene, β-carotene, lutein and

Table 1 Carotenoid content in (a) leaves and (b) tubers of WT and *crtZ/crtW* transgenic potato plants

	WT	EV	E1P1(1)	E6P1(4)	E1P1(10)
(a) Leaf tissue (μg per mg DW)					
Lutein	7.58 ± 0.19 (49)	7.36 ± 0.19 (44)	5.92 ± 0.37* (40)	4.93 ± 0.34* (41)	5.57 ± 0.08* (45)
β-Carotene	2.10 ± 0.06 (14)	2.40 ± 0.07 (14)	1.41 ± 0.03* (10)	1.23 ± 0.11* (10)	0.96 ± 0.08* (8)
cis-β-Carotene	0.35 ± 0.03 (2)	0.32 ± 0.01 (2)	0.21 ± 0.00* (1)	0.19 ± 0.01* (2)	0.15 ± 0.01* (1)
Zeaxanthin	0.09 ± 0.01 (1)	0.15 ± 0.07 (1)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
Antheraxanthin	0.43 ± 0.12 (3)	0.47 ± 0.02 (3)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
Violaxanthin	4.65 ± 0.68 (30)	5.69 ± 0.58 (34)	2.96 ± 0.16* (20)	2.27 ± 0.34* (19)	1.41 ± 0.34* (11)
Neoxanthin	0.13 ± 0.03 (1)	0.27 ± 0.04 (2)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
4-Ketolutein	†	†	3.06 ± 0.27† (21)	1.90 ± 0.37† (16)	2.92 ± 0.19† (24)
Echinenone	†	†	0.74 ± 0.02† (5)	0.67 ± 0.03† (6)	0.78 ± 0.01† (6)
Canthaxanthin	†	†	0.06 ± 0.00† (<1)	0.08 ± 0.01† (1)	0.09 ± 0.03† (1)
Phoenicoxanthin	†	†	0.09 ± 0.00† (1)	0.37 ± 0.11† (3)	0.16 ± 0.01† (1)
Astaxanthin	†	†	0.31 ± 0.03† (2)	0.51 ± 0.07† (4)	0.30 ± 0.02† (2)
Total Carotenoid	15.33 ± 0.92 (100)	16.66 ± 0.82 (100)	10.50 ± 0.35* (71)	8.62 ± 0.38* (71)	8.08 ± 0.49* (66)
Total Ketocarotenoid	†	†	4.26 ± 0.02† (29)	3.53 ± 0.31† (20)	4.24 ± 0.21† (34)
Total	15.33 ± 0.92 (100)	16.66 ± 0.82 (100)	14.76 ± 0.22 (100)	12.15 ± 0.54* (100)	12.32 ± 0.30* (100)
Total Chlorophyll	19.29 ± 0.57	17.15 ± 0.90	20.46 ± 1.95	22.63 ± 0.94*	14.97 ± 0.88*
Ratio Chl*a*:Chl*b*	3.96 ± 0.9	4.28 ± 0.03	3.87 ± 0.06	4.10 ± 0.07	4.19 ± 0.03
Ratio Chlorophyll: Carotenoid	1.36 ± 0.04	1.07 ± 0.03	1.53 ± 0.02	1.93 ± 0.03	1.43 ± 0.06
(b) Tuber Tissue (μg per g DW)					
Lutein	4.72 ± 1.04 (17)	4.54 ± 0.04 (16)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
Zeaxanthin	1.10 ± 0.21 (4)	2.66 ± 0.15* (9)	0.86 ± 0.21 (3)	0.44 ± 0.03* (2)	1.31 ± 0.11* (4)
Antheraxanthin	4.75 ± 0.69 (17)	2.49 ± 1.61 (9)	0.95 ± 0.11* (3)	1.24 ± 0.07* (5)	0.84 ± 0.02* (2)
Violaxanthin	7.92 ± 1.30 (29)	10.68 ± 1.51 (37)	4.24 ± 2.38 (13)	3.85 ± 0.19* (15)	4.33 ± 0.51* (13)
4-Ketolutein	†	†	13.70 ± 2.36† (42)	8.78 ± 1.05† (35)	11.68 ± 1.08† (35)
4-Ketozeaxanthin	†	†	0.53 ± 0.09† (2)	0.55 ± 0.10† (2)	1.76 ± 0.62† (5)
Astaxanthin	†	†	1.38 ± 0.13† (4)	1.29 ± 0.13† (5)	1.70 ± 0.04† (5)
Non-ketolated Esters	9.07 ± 1.14 (33)	8.29 ± 1.26 (29)	3.17 ± 0.79* (10)	0.90 ± 0.29* (4)	0.93 ± 0.29* (3)
Ketocarotenoid Esters	†	†	7.46 ± 1.19† (23)	8.36 ± 1.32† (33)	11.16 ± 1.91† (33)
Total	27.57 ± 2.94 (100)	28.68 ± 1.72 (100)	32.30 ± 4.70 (100)	25.43 ± 3.50 (100)	33.70 ± 3.62* (100)

Total carotenoid and ketocarotenoid contents were calculated as the sum of each carotenoid or ketocarotenoid, respectively, as determined by HPLC-PDA analysis. Values are the average of six measurements comprised of three biological replicates in duplicate, ±SEM. Keto; ketocarotenoid.

*Statistically significant difference to WT ($P < 0.05$).

†Pigment unique to crtZ/crtW transgenic plants.

Values in parenthesis are percentage compositions of accumulative carotenoid quantities

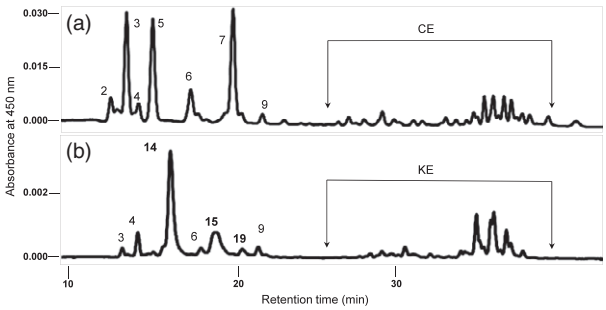

Figure 5 HPLC-PDA profiles of carotenoids present in tuber tissue from WT and *crtZ/crtW* transgenic potato plants. (a) WT tuber. (b) *crtZ/crtW* transgenic tuber. Each chromatogram component is labelled. Those labelled 14–19 (numbered in bold) and KE were unique to transgenic plants. CE, carotenoid esters; KE, ketocarotenoid esters. See Table S2 for spectral and chromatographic properties.

Table 2 Pigment analysis in plastid components from leaf tissue of wild-type and *crtZ/crtW* potato. (a) Pigment analysis in plastoglobuli. (b) Pigment analysis in plastid membranes (chloroplast and thylakoid)

	Pigment (μg/g-fresh weight)	
	WT	E1P1(1)
(a)		
Phytoene	0.01 ± 0.00 (8)	0.16 ± 0.01* (31)
Lutein	0.05 ± 0.00 (36)	0.15 ± 0.01* (29)
β-Carotene	0.03 ± 0.00 (24)	0.01 ± 0.01* (18)
cis-β Carotene	Trace	0.01 ± 0.00* (2)
Zeaxanthin	Trace	0.09 ± 0.00* (18)
Antheraxanthin	Trace	0.00 ± 0.00* (0)
Violaxanthin	0.02 ± 0.00 (7)	0.06 ± 0.01* (12)
Neoxanthin	0.03 ± 0.00 (21)	Trace†
4-Ketolutein	†	0.02 ± 0.00† (4)
Echinenone	†	0.01 ± 0.00† (1)
Canthaxanthin	†	Trace†
Phoenicoxanthin	†	Trace†
Astaxanthin	†	Trace†
Total Carotenoid	0.13 ± 0.01 (100)	0.49 ± 0.04* (94)
Total Ketocarotenoid	†	0.03 ± 0.00† (6)
Total	0.13 ± 0.01 (100)	0.52 ± 0.04* (100)
(b)		
Lutein	305.10 ± 1.12 (57)	171.14 ± 0.91* (40)
β-Carotene	139.76 ± 0.79 (26)	84.92 ± 0.62* (20)
cis-β- Carotene	9.65 ± 0.03 (1)	11.05 ± 0.07 (3)
Antheraxanthin	0.00 ± 0.00 (0)	3.33 ± 0.15* (1)
Violaxanthin	72.48 ± 0.26 (14)	52.33 ± 0.31* (12)
Neoxanthin	7.92 ± 0.06 (1)	3.23 ± 1.98 (1)
4-Ketolutein	†	73.68 ± 0.29† (17)
Echinenone	†	20.04 ± 0.29† (5)
Canthaxanthin	†	1.32 ± 0.02† (0)
Phoenicoxanthin	†	1.33 ± 0.01† (0)
Astaxanthin	†	4.23 ± 0.07† (1)
Total Carotenoid	534.91 ± 2.18 (100)	326.01 ± 2.68* (76)
Total Ketocarotenoid	†	100.61 ± 0.37+9† (24)
Total	534.91 ± 2.18 (100)	426.62 ± 2.80* (100)

†Pigment unique to crtZ/crtW transgenic plants.

*Statistically significant difference to WT ($P < 0.05$).

Values in parenthesis are percentage compositions of accumulative carotenoid quantities.

violaxanthin. In the transgenic genotype, the content of these endogenous carotenoids increased dramatically in the plastoglobuli. For example, a 16-fold elevation of phytoene, representing 31% of the plastoglobule carotenoid content in the transgenic lines, was found. In total, newly formed ketocarotenoids represented 6% of the carotenoid content in the plastoglobule, of which 4-ketolutein was the most predominant. The data suggested that on a quantitative basis, most of the synthesized ketocarotenoids were sequestrated in plastid membranes.

To ascertain whether ketocarotenoids were incorporated into the photosynthetic pigment complexes, thylakoids were prepared from WT and transgenic leaf material and the photosynthetic complexes extracted and separated by electrophoresis, under partial denaturing conditions. The core-complex I (CCI), core-complex II (LHCII monomer) and trimeric light-harvesting complex II (LHCII trimer) were identified by comparison with reported mobilities (Figure 6). In the transgenic line E1P1(1), the amount, as judged by intensity, of LHCII trimer was significantly reduced by 40% ($P < 0.05$), while the LHCII monomer increased significantly (about 40%) relative to the WT. Carotenoid analysis was performed on the identified complexes (Table 3). To varying degrees, both echinenone and 4-ketolutein were found in all the photosynthetic complexes analysed. Comparison of the WT and transgenic CCI pigment composition indicted that the proportion of β-carotene remained constant, but a proportion of lutein was replaced by echinenone in the transgenic complexes, and that in CCII a proportion of β-carotene was replaced by 4-ketolutein. Little variation was observed in the pigment compositions of transgenic monomeric and trimeric LHCII, relative to WT counterparts. Having determined the presence of newly synthesized ketocarotenoids within the photosynthetic complexes of the transgenic *crt*W and *Z* genotypes, the impact of the perturbations on the photochemical efficiency of PSII (Fv/Fm) was investigated and the distribution of chlorophylls in transgenic plants analysed. In comparison with the WT, the transgenic line had an 8% reduction in Fv/Fm value. However, neither the chlorophyll content nor ratio of chlorophyll-a/b was significantly altered ($P > 0.05$).

Transmission electron microscopy revealed significant ultrastructural changes to the plastids of GM lines (Figure 7). In comparison with the WT, chloroplasts of transgenic *crt*Z/W genotypes were reduced in area (approximately two-fold), although the number of chloroplasts per cell remained the same. There was a virtual absence of starch granules within plastids in transgenic lines. A comparison between plastid area minus the area occupied by the starch granules indicated that the chloroplast area between the WT and transgenic was comparable. More plastoglobuli per plastid were observed in the transgenic plastids, but were reduced in their area. Collectively, the total plastoglobuli area per plastid was similar (WT 0.14 μm^2 and transgenic 0.13 μm^2). The morphology of the transgenic-derived plastoglobuli appeared different, with more intense staining. No significant difference between the WT and transgenic genotypes was found in the thylakoid area per plastid, or number of grana per plastid.

It has been reported that in stable foods with high starch contents, such as potatoes, cereals and bananas, carotenoids are primarily biosynthesized and sequestered in amyloplast membranes (Pasare *et al.*, 2013). To determine whether this was also the case for ketocarotenoids in the GM lines, amyloplast envelopes from tubers were isolated. Fractions containing intact

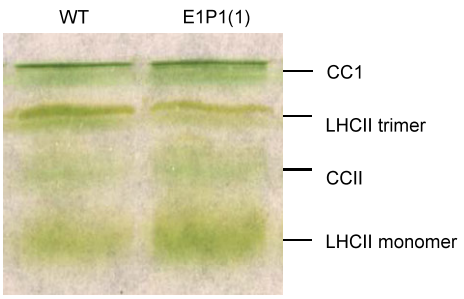

Figure 6 Partially denaturing 'green' gel, electrophoretic separation, of pigment–protein complexes from WT and transgenic plastid preparations. CCI, core-complex 1; CCII core-complex II; LHCII monomer, monomeric form of light-harvesting complex II; LHCII trimer, trimeric form of light-harvesting complex II; LHCI, light-harvesting complex I. The distribution of carotenoids isolated from photosynthetic complexes is indicated in Table 2.

amyloplasts and amyloplast membranes, from WT and *CrtZ/W* transgenic plants, were fractionated using a modified version of a protocol described previously for chloroplast subcellular fractionation (Nogueira *et al.*, 2013). Comparisons were made between the WT and the representative transgenic genotype E1P1(1). An immunodetection approach was used to probe the different fractions for known proteins, with well-characterized subplastidial locations. Unexpectedly, a distinct band was observed in the lowest density step of the sucrose gradient (Fractions (F)1-4, Figures S3 and S7), consistent with the enrichment of lipid bodies, such as micelles or plastoglobuli. These fractions reacted positively when probed with antisera raised against the plastoglobulin 35 (PGL35) protein. Distinct separations between the marker proteins from inner and outer plastidial membranes were not possible; instead, enrichments across fractions were achieved. For example, the chloroplast inner envelope membrane translocon complex protein (TIC40) was predominantly found in F8, while the chloroplast outer envelope membrane translocon complex protein (TOC75) was most pronounced in F9. RuBisCO (Rbcl), a stromal marker, was found in stromal fractions in addition to F1, and F7 to 16 of sucrose gradients. In all cases, the immunoreactive bands corresponded to the predicted molecular weight (MW) of the target proteins. At the bottom of each sucrose gradient, a white pellet, with a pigmented outline, was observed. The pellets tested positive for starch. Comparison between the WT and transgenic lines showed a consistent profile. Stromal preparations and fractions collected from sucrose gradients were used for carotenoid analyses. No carotenoids were isolated from either WT or transgenic stromal preparations. The carotenoid content of the WT plastoglobule fractions were entirely esterified and represented 13% of the total pigment found across the gradient. In the transgenic tubers, this proportion increased to 45%, with esterified ketocarotenoids in place of endogenous compounds found in the WT. Components with UV/Vis spectra consistent with tocopherols were also detected at 286 nm in the plastoglobule fractions, from both WT and transgenic gradients. Immunoreactive bands for the TOC75 and TIC40 proteins were most prominent in F 8–10. The carotenoid and ketocarotenoid contents of these fractions were predominantly unesterified in both the WT and transgenic lines. No notable changes in the percentage composition of the individual components within these fractions were observed. With the exception of the stromal Rbcl protein, no immunoreactive signals were detected in extracts from the pellets of WT or transgenic fractions. However, 5% and 13% of the respective carotenoid content from WT and transgenic gradients were extracted from the pellets. Consequently, we assessed whether solubilized starch was capable of binding carotenoids, to elucidate whether the carotenoids extracted from the pellets were bound with starch or being precipitated in another manner. Individual solutions containing either solubilized starch or the ketocarotenoid astaxanthin were separated by size exclusion chromatography. Subsequently, solutions containing both astaxanthin and soluble starch were separated to ascertain whether this altered the fraction in which either was eluted. When in solution alone, starch eluted early from the size exclusion matrix, while astaxanthin eluted late (Figure S4). When in solution together, however, astaxanthin eluted in the same fraction as starch, suggesting they were bound together.

Table 3 Distribution of carotenoids within photosynthetic complexes isolated from leaf plastids (percentage)

	Lutein	β-Carotene	Neo and Viola	Echinenone	4-Ketolutein	Total pigment (μg)
CCI						
WT	51 ± 2	49 ± 0	0 ± 0	0 ± 0	0 ± 0	0.815 ± 0.023
ZW	36 ± 2	50 ± 0	0 ± 0	14 ± 1	0 ± 0	1.579 ± 0.233
LHCII trimer						
WT	76 ± 1	2 ± 1	22 ± 1	0 ± 0	0 ± 0	5.142 ± 0.382
ZW	78 ± 3	2 ± 4	18 ± 4	1 ± 0	0 ± 0	5.694 ± 0.879
CCII						
WT	46 ± 1	24 ± 3	29 ± 3	0 ± 0	0 ± 0	0.892 ± 0.051
ZW	52 ± 3	11 ± 1	15 ± 1	4 ± 1	19 ± 1	3.199 ± 0.173
LHC mono						
WT	66 ± 5	2 ± 3	32 ± 3	0 ± 0	0 ± 0	2.180 ± 0.178
ZW	69 ± 3	2 ± 3	27 ± 3	2 ± 0	0 ± 0	5.042 ± 0.952

ZW, transgenic line E1P1(1); CCI, core-complex 1; CCII core-complex II; LHCII monomer, monomeric form of light-harvesting complex II; LHCII trimer, trimeric form of light-harvesting complex II; LHCI, light-harvesting complex I.

Percentage compositions were calculated from total carotenoids extracted from each gel band as determined by HPLC-PDA analysis. Values are the average of three measurements from separate plastid preparations ± SEM.

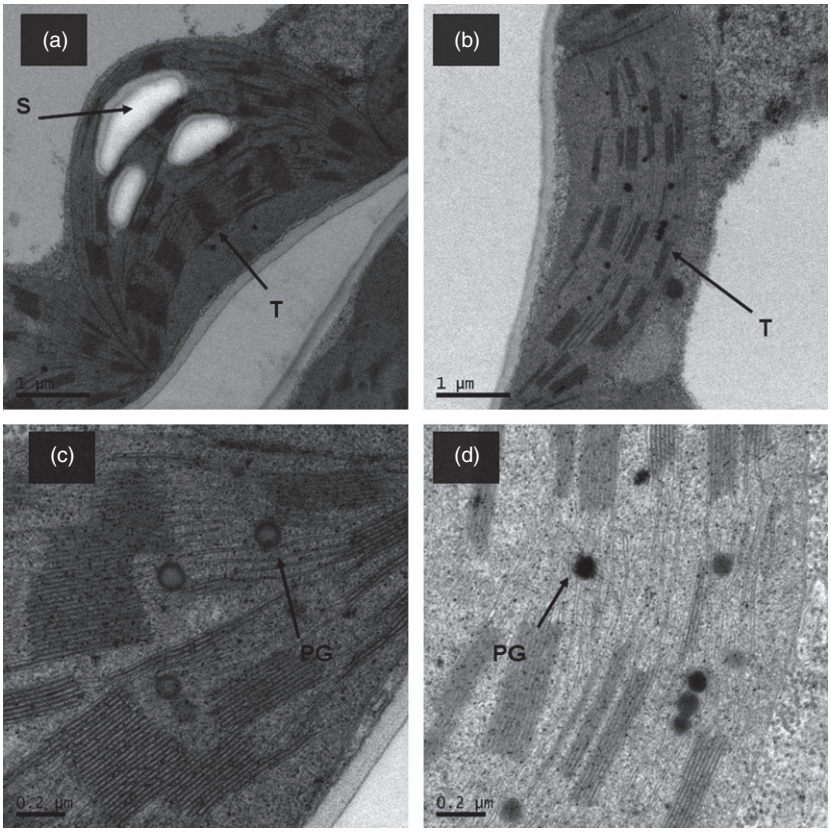

Figure 7 Changes in leaf plastid ultrastructure resulting from the expression of *Brevundimonas* sp. *crtW* and *crtZ*. (a) WT plastid. (b) E1P1(1) plastid. (c) WT plastoglobuli. (d) E1P1(1) plastoglobuli. S, starch granule; T, thylakoid membrane; PG, plastoglobule. Scale bars are indicated in each panel. Sections were prepared from transverse sections of three leaves; representative sections have been illustrated.

Carotenoid stability during tuber development, cold storage and processing

Three stages of tuber development were investigated: expanding (0.5–1 cm diameter), mature (>4 cm diameter) and cold stored (mature tubers stored in darkness at 4 °C for 6 months). Comparisons were made between the carotenoids of WT and a representative transgenic genotype E1P1(1). On a dry-weight (DW) basis, the total tuber carotenoid content increased significantly in both WT and transgenic tubers as development progressed from expanding to mature, and subsequently decreased following cold storage (Table 4). The level of violaxanthin and lutein in WT, and violaxanthin and 4-ketolutein in transgenic tubers, increased two-fold during following cold storage. In transgenic tubers, the levels of zeaxanthin, 4-ketozeaxanthin and astaxanthin also increased. Conversely, the level of esterified components decreased in both the WT and transgenic tubers following storage.

Table 4 Carotenoid and starch content of developing WT and *crtZ/crtW* transgenic tubers (μg/g DW)

	WT expanding	WT mature	WT stored	E1P1(1) expanding	E1P1(1) mature	E1P1(1) stored
Lutein	0.55 ± 0.24 (40)	4.72 ± 1.04 (17)	9.06 ± 1.68 (32)	1.50 ± 0.56 (31)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
Zeaxanthin	0.04 ± 0.02 (3)	1.10 ± 0.21 (4)	0.24 ± 0.05 (1)	0.28 ± 0.08* (6)	0.86 ± 0.21 (3)	3.45 ± 0.60* (7)
Antheraxanthin	0.03 ± 0.01 (2)	4.75 ± 0.69 (17)	1.12 ± 0.32 (4)	0.00 ± 0.00 (0)	0.95 ± 0.11* (3)	0.00 ± 0.00* (0)
Violaxanthin	0.38 ± 0.21 (28)	7.92 ± 1.30 (29)	14.65 ± 3.31 (51)	0.25 ± 0.06 (5)	4.24 ± 2.38 (13)	9.40 ± 0.65 (18)
4-Ketolutein	†	†	†	0.39 ± 0.04† (8)	13.70 ± 2.36† (42)	27.65 ± 3.00† (52)
4-Ketozeaxanthin	†	†	†	0.39 ± 0.14† (8)	0.53 ± 0.09† (2)	7.31 ± 1.22† (14)
Astaxanthin	†	†	†	1.33 ± 0.23† (27)	1.38 ± 0.13† (4)	1.76 ± 0.21† (3)
Non-ketolated Esters	0.35 ± 0.21 (26)	9.07 ± 1.14 (33)	3.58 ± 0.21 (12)	0.00 ± 0.00 (0)	3.17 ± 0.79* (10)	0.00 ± 0.00* (0)
Ketocarotenoid Esters	†	†	†	0.77 ± 0.27† (16)	7.46 ± 1.19† (23)	3.35 ± 1.12† (6)
Total	1.37 ± 0.64 (100)	27.57 ± 2.94 (100)	28.65 ± 4.58 (100)	4.91 ± 1.56 (100)	32.30 ± 4.70 (100)	52.92 ± 6.28* (100)
Starch‡	n/d	659.94 ± 23.49*	522.19 ± 26.29*	n/d	550.12 ± 19.78*	490.70 ± 16.11*

Total carotenoid and ketocarotenoid contents were calculated as the sum of each carotenoid or ketocarotenoid, respectively, as determined by HPLC-PDA analysis. Values are the average of six measurements comprised of three biological replicates in duplicate, ±SEM. Keto; ketocarotenoid.

*Statistically significant difference to WT ($P < 0.05$).

†Pigment unique to *crtZ/crtW* transgenic plants. Values in parenthesis are percentage compositions of accumulative carotenoid quantities.

‡mg/g DW.

Mature tubers were subjected to two processing treatments; baked for 60 min at 200 °C, or boiled for 30 min, prior to carotenoid analysis (Table 5). On a DW basis, the total carotenoid content of WT tubers appeared stable following heat treatment. Conversely, the total content of transgenic tubers decreased (approximately two-fold) significantly ($P < 0.05$). Despite the apparent stability of carotenoids in WT tubers, analysis of the individual components revealed larger changes. An increase (approximately three-fold) in the level of lutein extracted from heat-treated WT tubers, relative to fresh, was observed. The level of 4-ketolutein in transgenic tubers, however, decreased following heat treatment, contributing most prominently to the overall decrease in carotenoids in these extracts. Violaxanthin, antheraxanthin and esterified components were reduced in both WT and transgenic extracts following heat treatment of the tubers.

Starch content of tubers metabolically engineered to produce ketocarotenoids

Due to the nutritional and commercial importance of starch of potato tubers, and the discovery that expression of *CrtZ* and *CrtW* led to a decreased level of starch granules in leaf plastids, the starch content of transgenic tubers was investigated. The starch content of fresh and cold-stored mature tubers of both WT and the representative transgenic genotype E1P1(1) was determined (Table 4). The levels in both fresh and cold-stored transgenic tubers were significantly lower (16% and 6%), than in the WT. A significant ($P < 0.05$) reduction in the total starch content following cold storage was observed in both WT (20%) and transgenic (10%) tubers.

Discussion

Biosynthetic routes to nonendogenous ketocarotenoids in *S. tuberosum*

Transgenic potato lines expressing *crtZ* and *W* contain ketocarotenoids that are not present in WT vegetative tissues and a decrease in hydroxylated carotenoids, such as zeaxanthin (Table 1). This demonstrates that both *Brevundimonas* CRTZ and W enzymes are functional and act to modify the endogenous carotenoids. However, the range of products suggests that CRTZ and W are not under coordinated regulation, but act in a random, promiscuous manner upon the surrogate precursors available. For example, 4-ketolutein is formed by the action of the monoketolation of lutein in photosynthetic and tuberous tissues. This correlates with *in vitro* enzyme data (Misawa *et al.*, 1995). The collective data from the transgenic tissues suggest no dramatic increase in the total carotenoid content. However, the reduction in β-carotene, in transgenic photosynthetic tissues, suggests an increase in flux to deliver precursors of the ketocarotenoids, through the β-ring-derived branch of the carotenoid pathway. The latter is disrupted most significantly, with reduced formation of violaxanthin and zeaxanthin converted into 4-ketozeaxanthin and astaxanthin in all mature transgenic tissues.

How does the plastid sequestrate newly formed, nonendogenous carotenoids?

Separation of subplastidial components, in conjunction with microscopy, was adopted to establish where nonendogenous carotenoids are stored in transgenic tissues. In photosynthetic tissues, the plastid membranes displayed no quantitative differences were observed between the WT and *crtZ/W* transgenic lines, but qualitatively, the level of lutein and violaxanthin present in transgenic membranes was reduced and displaced by ketocarotenoids. The increased presence of phytoene in the plastoglobules of the transgenic lines (Table 2) suggests that the pathway had been upregulated to produce precursors, either in response to increased end-product utilization (forward-feed mechanisms) or the removal of an initiator of negative feedback. It is likely that the additional phytoene sequestered within the plastoglobuli of transgenic photosynthetic *S. tuberosum* tissue, rather than in thylakoid membranes, prevents membrane instability. Such an accumulation and effect on plastid membranes was found in wheat treated with the bleaching herbicide SAN 9789 (Dahlin, 1986). The presence of phytone in plastoglobuli isolated from tomato fruit has also been reported (Nogueira *et al.*, 2013). Unlike xanthophylls, such as zeaxanthin, with polar head groups conferred by hydroxylation of the C3 and C3′ positions on the β-ionone rings, which act to stabilize membrane phospholipid bilayers (Havaux *et al.*, 2007), the hydrocarbon phytoene displays mobility within the membrane bilayer, which can lead to membrane instability (Havaux *et al.*, 2007). This supports the hypothesis that metabolites can be dynamically partitioned into

Table 5 Carotenoid stability in heat-treated WT and *crtZ/crtW* transgenic tubers (μg/g DW)

	WT fresh	WT baked	WT boiled	E1P1(1) fresh	E1P1(1) baked	E1P1(1) boiled
Lutein	5.90 ± 0.41 (28)	11.07 ± 0.65 (51)	14.80 ± 0.22 (64)	3.55 ± 0.23* (8)	3.74 ± 0.31* (22)	4.10 ± 0.05* (17)
Zeaxanthin	0.43 ± 0.03 (2)	4.73 ± 0.34 (22)	2.16 ± 0.04 (9)	1.98 ± 0.08* (4)	1.61 ± 0.16* (9)	1.39 ± 0.07* (6)
Antheraxanthin	0.52 ± 0.06 (2)	0.22 ± 0.02 (1)	0.14 ± 0.01 (1)	0.58 ± 0.13 (1)	0.00 ± 0.00* (0)	0.00 ± 0.00* (0)
Violaxanthin	9.27 ± 1.68 (44)	1.06 ± 0.12 (5)	2.14 ± 0.11 (9)	10.60 ± 1.45 (24)	1.08 ± 0.04 (6)	3.44 ± 0.09* (15)
4-Ketolutein	†	†	†	9.71 ± 2.18† (22)	0.21 ± 0.11† (1)	0.48 ± 0.00† (2)
4-Ketozeaxanthin	†	†	†	3.35 ± 0.38† (8)	4.65 ± 0.47† (27)	4.94 ± 0.49† (21)
Astaxanthin	†	†	†	1.63 ± 0.13† (4)	1.10 ± 0.11† (6)	1.71 ± 0.02† (7)
Non-ketolated	5.06 ± 0.58 (24)	4.48 ± 0.20 (21)	3.81 ± 0.37 (17)	5.39 ± 1.48 (12)	2.31 ± 0.41* (13)	4.22 ± 0.57 (18)
Ketocarotenoid Esters	†	†	†	7.64 ± 0.59† (17)	2.52 ± 0.68† (15)	3.24 ± 0.28† (14)
Total	21.21 ± 1.7 (100)	21.57 ± 1.21 (100)	23.06 ± 0.53 (100)	44.41 ± 4.83*(100)	17.25 ± 1.68*(100)	23.56 ± 0.74*(100)

Total carotenoid and ketocarotenoid contents were calculated as the sum of each carotenoid or ketocarotenoid, respectively, as determined by HPLC-PDA analysis. Values are the average of six measurements comprised of three biological replicates in duplicate, ±SEM. Keto; ketocarotenoid.

*denotes a statistically significant difference to WT ($P < 0.05$).

†Pigment unique to *crtZ/crtW* transgenic plants. Values in parenthesis are percentage compositions of accumulative carotenoid quantities.

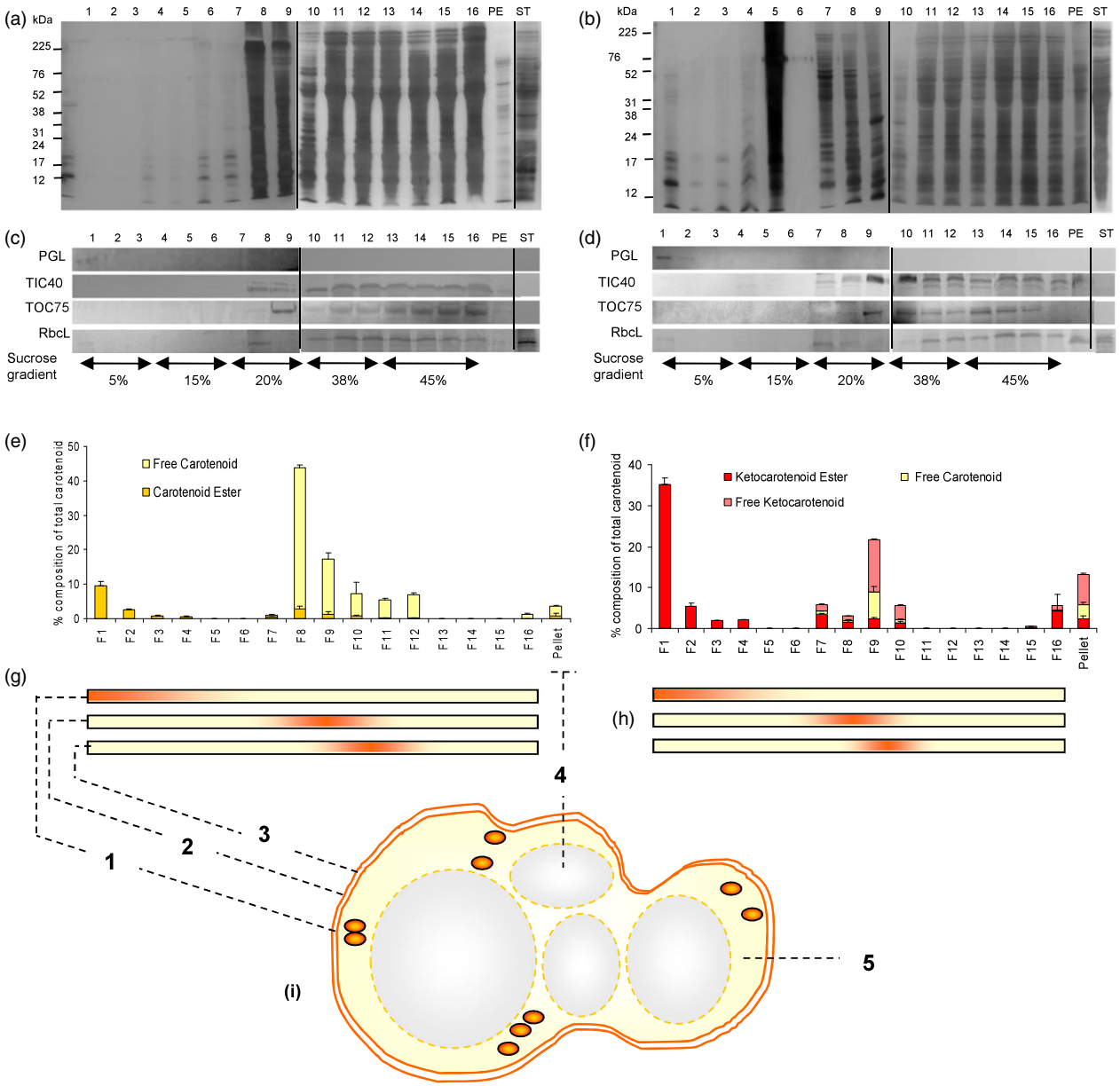

Figure 8 SDS-PAGE separation and immunoblot analysis of WT and *crtZ/crtW* transgenic, amyloplast fractions. (a–b) SDS-PAGE separation of WT (a) and E1P1(1) (b) amyloplast fractions. Total membranes from isolated amyloplasts were separated by flotation on a discontinuous sucrose gradient. Gradients were prepared from three separate plastid preparations. Fractions from the three separate gradients were pooled. Fractions 1 to 16 in addition to pellet (PE) and stromal preparations (ST) are indicated. (c–d) Immunoblot analysis of WT (c) and E1P1(1) (d) amyloplast fractions. Immunoblot analysis was performed using marker antibodies (Agrisera, Vännäs, Sweden). PVDF membranes were probed successively with antisera raised against plastoglobulin 35 (PGL35, 35 kDa); chloroplast inner envelope membrane translocon complex protein (TIC40, 40 kDa); chloroplast outer envelope membrane translocon complex protein (TOC75, 75 kDa); and the stromal large subunit of RuBisCO (RbcL, 52 kDa). (e–h) HPLC-PDA analysis of WT (e–f) and E1P1(1) (g-h) amyloplast fractions. Values are the average of three replicates, from pooled fractions of three independent discontinuous sucrose gradients. Error bars indicate +SEM. (i) Schematic representation of the abundance of amyloplast ultrastructures throughout sucrose gradients. An increase in colour intensity in each bar represents an increase in the abundance of the indicated structure, respective to the fractions in the panels above (e and h). (1) plastoglobuli; (2) inner chloroplast envelope; (3) outer chloroplast envelope; (4) starch granule; (5) stroma.

different suborganelle components (Austin *et al.*, 2006; Nogueira *et al.*, 2013). Whether the accumulated phytoene is metabolically inert or can be utilized as a substrate for phytoene desaturase is yet to be ascertained. Analysis of plastid ultrastructure revealed an increased number of plastoglobuli in the transgenic plastids in transgenic *crtZ/W* leaf tissue compared to the WT (Figure 7). However, the plastoglobuli were smaller in size and exhibited denser staining, suggesting that the composition of the plastoglobule is altered in the transgenic lines. This could be a direct consequence of the increased quantity and nonendogenous carotenoids present or effects on the other lipids associated with plastoglobule. It is possible that the reduced plastoglobule size results from the disruption of plastoglobule development, with the increased number of globules being a compensation

mechanism to maintain the same volume in the cell. In tuber tissues, no dramatic quantitative differences were observed in total carotenoid accumulation, between WT and *crtZ/W* transgenic lines. Qualitatively, however, the majority (70%) of endogenous carotenoids were reduced and displaced by ketocarotenoids. The profile of carotenoids in plastidial fractions indicated an accumulation of nonesterified carotenoids in both the WT and transgenic tuber tissues where TIC75 and TOC40 predominated, and also in association with a starch pellet. Unexpectedly, the esterified carotenoids in both WT and transgenic tubers were almost entirely isolated from low density fractions which reacted positively when probed with antisera raised against a plastoglobulin (fibrillin), consistent with the enrichment of plastoglobule, as suggested in Figure 8. Thus, it would appear that the carotenoid present in the membrane are nonesterified in nature but esterification act as a mechanism to sequestrate these nonendogenous compounds.

Are carotenoids stable during tuber development, cold storage and processing?

Relative to freshly harvested, the total carotenoid levels increased in cold-stored WT and *crtW/crtZ* transgenic tubers. The level of lutein in WT tubers, and 4-ketolutein in transgenic tubers, increased two-fold after cold storage, as did the levels of violaxanthin in both WT and transgenic tubers. In transgenic tubers, the levels of zeaxanthin, 4-ketozeaxanthin and astaxanthin also increased. An increase in lutein in tubers (Table 4) after cold storage has previously been observed in WT *S. phureja* (Morris *et al.,* 2004) and WT *S. tuberosum* cv. Désirée tubers (Lopez *et al.* 2008). In this study, we demonstrated that heat treatment results in a similar increase in lutein extractable from WT Désirée tubers, relative to fresh, as was observed for stored tubers relative to fresh. 4-ketozeaxanthin in *crtW/crtZ* transgenic tubers and zeaxanthin in transgenic and WT tubers also increased after heat treatment (Table 4). In combination, these observations suggest that some carotenoids are sequestered or bound in a complex, which prevents their full extraction from fresh tissue, and that they are liberated from the complex/interaction both through the application of heat, or over time during cold storage. It is possible that this is a common oversight when extracting carotenoids from tissues with high starch contents, as carotenoid complexes may be masked by starch, giving the false appearance that all pigments have been successfully extracted. Potentially, starch itself may prevent the full extraction of carotenoids from tuber tissue. In potato tubers, carotenoids are produced in the amyloplast membrane (Alban *et al.*, 1988; Ohad *et al.*, 1971), and it is likely that carotenoids become associated with the surface of starch granules during tissue homogenization and pigment extraction. As shown in the present study, with the aid of size exclusion matrixes, starch is capable of binding carotenoids (Figure S4). However, the carotenoid was successfully re-isolated from the starch–carotenoid suspensions, with no compound loss. This implies that if starch does prevent complete extraction of carotenoids, the carotenoids might be sequestered loosely within the starch granules, rather than bound to the surface. Starch becomes soluble in water when heated, granules swell and burst, the semi-crystalline structure is lost, and the smaller amylose molecules start leaching out of the granule forming a network that holds water and increases the mixture's viscosity (starch gelatinization). If carotenoids are bound/sequestered within starch granules, it is possible that during this process through heat treatment, carotenoids are then liberated. Starch degradation, accompanied by increases in sugars, has been well documented when tubers are stored at low temperatures (cold-induced sweetening; Ohad *et al.*, 1971; Smith *et al.*, 2005). As reported here, the starch content of both WT and transgenic cold-stored tubers was reduced relative to fresh material. This could be responsible for the increases in carotenoids observed in cold-stored tissues, the carotenoids being liberated from within the granules as they are degraded. Alternatively, carotenoids inaccessible to solvent extraction in fresh tissue may be bound in a complex which is yet to be characterized.

Unlike cold storage, cooking (as defined in the Methods section) dramatically decreased the levels of 4-ketolutein in transgenic tubers and violaxanthin in both WT and *crtW/crtZ* transgenic tissue (Table 5). It is probable that the increase in these compounds observed post-cold storage was not observed post-cooking, as these carotenoids are not heat stable. As such, if cooking had liberated additional 4-ketolutein and violaxanthin, they would have been degraded by the heat treatment. Both ketocarotenoids and carotenoids appear to be stable through cold storage. Yet, when exposed to heat, stability appears to vary between compounds. This is important when considering the potential of extracting carotenoids as co-products from agricultural crops, as many processing methods, such as starch extraction, require heat treatment. Therefore, certain compounds, such as 4-ketozeaxanthin and astaxanthin, are more suitable for co-product isolation than, for example, 4-ketolutein.

Ketocarotenoid formation affects core plant processes and metabolite composition

Analysis of photosynthetic complexes revealed that the nonendogenous ketocarotenoids were predominantly membrane components, with only low levels associated with the photosynthetic complexes (Table 3). Carotenoids, especially xanthophylls, are vital components of the photosynthetic apparatus in higher plants (Frank and Cogdell, 1993) and are essential for the stable assembly of pigment–protein complexes (Green and Durnford, 1996). Previously, a lack of WT carotenoid composition in xanthophyll biosynthetic mutants of *A. thaliana* (Gilmore, 2001), and an α-ring carotenoid-free mutant of *Scenedesmus obliquus* (Bishop, 1996), has led to an instability of trimeric LHCII and a reduction in PSII efficiency, as was observed here for transgenic *S. tuberosum* (Table 3). The reduction in PSII efficiency did not appear to affect the transgenic potato plants vigour or tuber yield. Ketocarotenoid formation did, however, affect the accumulation of starch. Levels were reduced by 20% and 25%, respectively, in tubers of transgenic tissues (Table 4), relative to the WT, and starch granules were virtually absent in leaf plastids, as observed by TEM imaging (Figure 7). This could be due to a reduction in zeaxanthin in transgenic plants and subsequent impacts on the blue light-stimulated opening of stomata. Alternatively, if carotenoids are sequestered within starch granules, and more carotenoids are deposited in this manner in transgenic tubers, this could account for both the higher increase in carotenoids observed in cold-stored transgenic tubers, relative to WT, and also provide an explanation for the lower starch content in transgenic tissues; namely, the higher deposition of carotenoids in transgenic starch granules may displace starch. In addition to being grown as a vegetable crop, potatoes are independently grown for the production of industrial commodities, such as starch, which represents the most important carbohydrate used for food and feed purposes (Regierer *et al.*, 2002). The reduction in starch content in transgenic tubers would

be of significant impact to the crop; thus, the production of ketocarotenoids would unlikely be accepted as a compromise for the reduction in starch content. Leaf ketocarotenoid accumulation in transgenic lines evidently perturbs carbon metabolism, and the reduced starch content of tubers is likely a manifestation of these effects on source–sink relationships. The use of tuber-specific promoters may overcome this limitation. However, the utilization of foliage for high-value co-products is a key driver in the industry.

Implications for the production of valuable secondary metabolites *in planta*

Significant levels of valuable ketocarotenoids have been produced in this study by transcriptional and translationally enhanced *Brevundimonas CrtZ* and *W*, in both photosynthetic and tuber tissues. In addition to astaxanthin and canthaxanthin, other rare keto/hydroxylated carotenoids have been produced. Although the levels present are not as high as those found with transplastomic tobacco varieties (Hasunuma *et al.*, 2008), the present study extends the range of hosts amenable to ketocarotenoid production. However, in potato, ketocarotenoid production appears to come at the expense of the economically critical metabolite, starch. It would be interesting to see whether potato tubers engineered to produce more β-carotene as described in Diretto *et al.* (2007) also have reduced starch. This highlights the importance of investigating the effect that metabolic engineering has on economically important parameters in different host platforms and has implications on the utilization of such feedstocks as multipurpose crops for biorefining. The present study provides valuable insights into fundamental aspects of how plant cells sequestrate both endogenous carotenoids, and novel products generated from engineered biosynthetic capabilities. Interestingly, besides starch production, the most obvious perturbations appear to be at the structural level, with the alterations in subplastidial components (Figure 7), suggesting that the cell reacts to changes in chemical composition by adopting or adjusting its cellular structures, and that these are changes at the cellular level, arising from perturbations in chemical composition, not predefined cellular structures waiting to be packaged with defined metabolites.

Experimental procedures

Biological materials and growth conditions

Plasmid pCrtZW(pZH2B), as reported in Fujisawa *et al.* (2009), was transformed into *A. tumefaciens* strain LBA4404 by electroporation. *Agrobacterium*-mediated transformation of *S. tuberosum* cv. Désirée was carried out as described previously (Ducreux *et al.*, 2005). Plants were glasshouse-grown in 30-cm-diameter pots in M2 professional growing medium (Scotts Levington, Bramford, Suffolk, UK), with a daytime temperature of 20–25 °C and nocturnal temperature of 15 °C, in a 16-h supplementary light and 8-h darkness cycle. All tubers were harvested after 80 days to obtain varying developmental stages. Small developing (0.5–1 cm diameter) and mature tubers (>4 cm diameter) were collected. Four developing and three mature tubers, from each plant analysed, were flash-frozen in liquid nitrogen. Three mature tubers from each plant were 'cold-stored', that is stored in darkness at 4 °C for 6 months, prior to freezing. Three tubers from selected plants were processed: one-third of each tuber remained untreated, one-third was baked for 60 min at 200 °C, and one-third was boiled for 30 min. Samples from each treatment were pooled and flash-frozen. Leaf samples were flash-frozen upon harvest and freeze-dried.

Pigment extraction and analysis

Freeze-dried tissue was grounded to a fine powder with a Tissuelyser (Qiagen, Manchester, UK) at 30 Hz. Extractions were made from 10 to 20 mg samples of leaf or 200 mg samples of tuber tissue. Several organic solvents in different ratios to aqueous partitioning (methanol:buffer, 50 mM Tris pH7.5) solutions were assessed for carotenoid extraction. Chloroform was the best solvent in a 2 : 1 ratio, with methanol:buffer compared to acetone, ethyl acetate and hexane. It was estimated that chloroform extraction recovered 50% more of the total carotenoid content, with limits of detection (LOD), limits of quantification (LOQ) and limits of identification (LOI) in the range of 0.003, 0.005 and 0.01 μg/mg, respectively. Accordingly, carotenoids and chlorophylls were extracted using chloroform and methanol. Extracts were stored for 60 min on ice and 1 vol of water added. The organic hypophase was removed and the aqueous phase re-extracted with chloroform (2 by vol). Pooled extracts were dried under nitrogen gas. The residue was re-suspended in ethyl acetate (50 μL). HPLC separations were performed using C30 reverse-phase columns (YMC, Kyoto, Japan) mobile phases used were methanol (A), water/methanol (50/50 by volume), containing 0.2% ammonium acetate (B), and tert-methyl butyl ether (C). The gradient was 95% A: 5%B, isocratically for 6 min, stepped to 80% A: 5% B: 15% C, from which a linear gradient to 30% A: 5% B: 65% C over 42 min was performed. A Water Alliance (Water Alliance, Milford, Massachusetts) HPLC system was used (600S controller, 610 pump, 996 photodiode array detector and 717plus autosampler). Detection was performed continuously from 220 to 700 nm with an online PDA detector. Identification was performed by co-chromatography and comparison of spectral properties and retention times with authentic standards and reference spectra (Fraser *et al.* (2000) Britton *et al.*, 2004). Carotenoid esters were identified as compounds with carotenoid or ketocarotenoid-like spectra with delayed retention times. Quantitative determination of carotenoids was performed by comparison with individual dose–response curves (0.2 to 1.5 μg) constructed from authentic standards.

RNA isolation and quantitative RT-PCR analysis

RNA was extracted using the Qiagen RNeasy reagents and protocol, including on-column DNaseI digestion. The Rotor-gene SYBR Green qRT-PCR Kit was used to determine gene expression levels. Per reaction (25 μl), 25 ng of RNA, was used and primers added to provide a final concentration of 2 μm. Reactions were performed on a Rotor-Gene 3000 thermocycler (Qiagen). Thermocycling conditions were 10 min at 55 °C for reverse transcription, 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C. Melt curve analysis verified specificity. Quantification and calibration curves were run simultaneously with experimental samples, and Ct calculations were performed by the Rotor-Gene software (Qiagen). *Actin* and *elongation factor-1 alpha (ef1-a)* genes served as references for normalization in leaf and tuber tissue, respectively. All reactions were run in triplicate. Primers used are detailed in Table S4.

Analysis of leaf and tuber tissue

ΔE^*ab as a measure of leaf or tuber colour on the Hunter Lab colour scale was calculated from the colour parameters *L, b* and *a*

as measured with a MiniScan® XE plus (Hunter Labs, Reston, VA). *In vivo* chlorophyll fluorescence was determined using a pocket PEA chlorophyll fluorimeter (Hansatech Instruments, Norfolk, UK). Measurements were recorded with attached leaves. Fluorescence parameters are according to Vankooten and Snel (1990). $F_v/F_m = (F_m - F_0)/F_m$ is the maximum photochemical efficiency of PS II, in the dark-adapted state. Enzymatic quantification of starch content of tuber was performed according to the protocol described by Smith and Zeeman (2006). Transmission electron microscopy (TEM) imaging service was provided by the Microscopy and Imaging Facility at the University of Kent (University of Kent, UK). Subsequent images were analysed with ImageJ software (National Institutes of Health, Bethesda, MD). Material used for TEM was harvested at identical times.

Chloroplasts were isolated from WT and transgenic leaves, as detailed in Anderson *et al.* (1982). Partially denaturing 'green' gel electrophoresis of pigment–protein complexes was performed as detailed in Dormann *et al.* (1995). For comparison of band intensities, samples of WT and transgenic solubilized pigment–protein complexes representing 10 μg total chlorophyll were separated side by side on the same gels. Gels were scanned with a UMAX image scanner (GE Healthcare, Buckinghamshire, UK), and images analysed with ImageJ software to calculate relative band intensities. To enable sufficient pigment extraction from 'green' gels for detailed HPLC-PDA analysis, solubilized pigment–protein complexes representing 250 μg total chlorophyll from their respective tissues were separated. Pigment was extracted from isolated bands, as detailed in Aizawa *et al.* (1997). Separation of chloroplast components, plastoglobuli/plastid/thylakoid membrane, was performed as detailed in Ytterberg *et al.* (2006). Components from three separate chloroplast preparations were pooled. Carotenoids were extracted from pooled fractions using chloroform and methanol (2 : 1); following removal of the organic hypophase, a volume of methanol, equal to the organic phase removed, was added to concentrate proteins.

Amyloplast extraction was undertaken at 4 °C. Mature potato cores were washed in iced water and transferred to extraction buffer (0.3 M dipotassium phosphate, 10 mM magnesium chloride, 10 mM EDTA, 5 mM sodium bisulphite, 12% (w/v) sucrose). Tubers were vacuum-infiltrated with buffer for 30 min and then grated with a coarse kitchen grater. Subcellular structures were filtered through four layers of muslin cloth. Large starch granules were allowed to settle down a discontinuous sucrose gradient (3 M dipotassium phosphate, 10 mM magnesium chloride, 10 mM EDTA, 5 mM sodium bisulphite, 15% (w/v) sucrose, layered upon extraction buffer), for two h, leaving a pigmented suspension at the top of the gradient. The pigmented suspension was collected and subcellular components further separated through a 4-step discontinuous sucrose gradient as used for chloroplast fractionation in Ytterberg *et al.* (2006), with the modification that each sucrose step was applied in equal quantity. Fractions (2 mL) were collected from the top of sucrose gradients prepared from three separate plastid preparations. Fractions from the three gradients were pooled. Independently, stromal fractions were obtained from the pigmented suspension by centrifugation for 3 h at 100 000 ***g*** and 4 °C. Stroma remained in solution following high-speed centrifugation. Carotenoids were extracted from all collected fractions using chloroform and methanol (2 : 1 by volume). Following removal of the organic hypophase, a volume of methanol, equal to the organic phase removed, was added to concentrate the proteins. Proteins were separated by SDS-PAGE and either stained by silver nitrate or blotted onto nitrocellulose membranes for immunodetection. Blots were probed with serum raised against PGL35, TIC40, TOC75 and RbcL proteins (Agrisera, Vännäs, Sweden), as directed by the manufacturer.

Separation of starch–carotenoid suspensions by size exclusion matrix

Starch–carotenoid suspensions containing soluble starch (Sigma-Aldrich, St. Louis, MO) and astaxanthin (authentic standard) were separated using PD-10 desalinating columns (GE Healthcare). Columns were equilibrated with running buffer (50 mM Tris, 20% (v/v) ethanol) prior to use. Three sample suspensions were analysed and 24 fractions (1 mL) per sample collected. Fractions were used for pigment quantification by spectrophotometry, using the published absorbance coefficient and λ_{max} of astaxanthin (Britton, 1995), and for starch quantification by Lugols assay. Soluble starch was used to create a calibration curve for starch quantification. Three suspensions of astaxanthin alone (0.1 μg/μL suspended in ethanol) were analysed in the same manner. All comparative starch determinations were performed on tissues harvested at identical times.

Statistical analysis

Student's *t*-tests determined significant differences between pairwise comparisons of WT and transgenic samples. Student's *t*-tests, means and standard error of the means (SEM) were calculated using Excel software (Microsoft, Redmond, WA). Significance was determined when *t*-tests returned a $P \leq 0.05$.

Acknowledgements

N.M. is grateful to Central Laboratories for Frontier Technology, Kirin Holdings Company, Limited, Japan, as this work was in part performed there, supported by the New Energy and Industrial Technology Development Organization (NEDO), Japan. The study was also funded by the European Commission FP6-2005-FOOD-4B program, grant # 036296 to P.D.F and P.M.B. We thank Dr Brown at the Microscopy and Imaging Facility at the University of Kent, UK, for carrying out the TEM.

References

Aizawa, K., Cunningham, F.X. and Gantt, E. (1997) Enhanced recovery of chlorophyll and carotenoids with dextran-polyacrylamide gel electrophoresis. *Anal. Sci.* **13**, 253–256.

Alban, C., Joyard, J. and Douce, R. (1988) Preparation and characterization of envelope membranes from nongreen plastids. *Plant Physiol.* **88**, 709–717.

Anderson, B., Anderson, J.M. and Ryrie, I.J. (1982) Transbilayer organization of the chlorophyll-proteins of spinach thylakoids. *Eur. J. Biochem.* **123**, 465–472.

Ausich, R.L. (1997) Commercial opportunities for carotenoid production by biotechnology. *Pure Appl. Chem.* **69**, 2169–2173.

Austin, J.R., Frost, E., Vidi, P.-A., Kessler, F. and Staehelin, L.A. (2006) Plastoglobules Are Lipoprotein Subcompartments of the Chloroplast That Are Permanently Coupled to Thylakoid Membranes and Contain Biosynthetic Enzymes. *The Plant Cell.* **18**(7), 1693–1703. doi:10.1105/tpc.105.039859.

Bishop, N.I. (1996) The β, ε-carotenoid, lutein, is specifically required for the formation of the oligomeric forms of the light harvesting complex in the green alga, *Scenedesmus obliquus*. *J. Photochem. Photobiol. B*, **36**, 279–283.

Breithaupt, D.E. (2007) Modern application of xanthophylls in animal feeding – a review. *Trends Food Sci. Technol.* **18**, 501–506.

Britton, G. (1995) Structure and properties of carotenoids in relation to function. *FASEB J.* **9**, 1551–1558.

Britton, G., LiaaenJensen, S. and Pfander, H. (2004) *Carotenoids Handbook*. London: Birkhauser Verlag.

Choi, S.K., Nishida, Y., Matsuda, S., Adachi, K., Kasai, H., Peng, X., Komemushi, S., Miki, W. and Misawa, N. (2005) Characterization of beta-carotene ketolases, *CrtW*, from marine bacteria by complementation analysis in *Escherichia coli*. *Mar. Biotechnol.* **7**, 515–522.

Cunningham, F.X. and Gantt, E. (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*. *Plant J.* **41**, 478–492.

Dahlin, C. and Ryberg, H. (1986) Accumulation of phytoene in plastoglobuli of SAN-9789 (Norflurazon)-treated dark-grown wheat. *Physiologia Plantarum*, **68**, 39–45. doi:10.1111/j.1399-3054.1986.tb06593.x.

Diretto, G., Al-Babili, S., Tavazza, R., Papacchioli, V., Beyer, P. and Giuliano, G. (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE*, **4**, e350.

Dormann, P., Hoffmann-Benning, S., Balbo, I. and Benning, C. (1995) Isolation and characterization of an *Arabidopsis* mutant deficient in the thylakoid lipid digalactosyl diacylglycerol. *Plant Cell*, **7**, 1801–1810.

Ducreux, L.J.M., Morris, W.L., Hedley, P.E., Shepherd, T., Davies, H.V., Millam, S. and Taylor, M.A. (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. *J. Exp. Bot.* **56**, 81–89.

Frank, H.A. and Cogdell, R.J. (1993) The phytochemistry and function of carotenoids in photosynthesis. In *Carotenoids in Photosynthesis* (Young, A.J. and Britton, G., eds), pp. 253–326. London: Champan & Hall.

Fraser, P.D., Pinto, M.E.S., Holloway, D.E. and Bramley, P.M. (2000) Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J.* **24**, 551–558.

Fujisawa, M., Takita, E., Harada, H., Sakurai, N., Suzuki, H., Ohyama, K., Shibata, D. and Misawa, N. (2009) Pathway engineering of *Brassica napus* seeds using multiple key enzyme genes involved in ketocarotenoid formation. *J. Exp. Bot.* **60**, 1319–1332.

Gilmore, A. (2001) Xanthophyll cycle-dependent nonphotochemical quenching in Photosystem II: Mechanistic insights gained from *Arabidopsis thaliana* L. mutants that lack violaxanthin deepoxidase activity and/or lutein. *Photosynth. Res.* **67**, 89–101.

Green, B.R. and Durnford, D.G. (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 685–714.

Hasunuma, T., Miyazawa, S.I., Yoshimura, S., Shinzaki, Y., Tomizawa, K.I., Shindo, K., Choi, S.K., Misawa, N. and Miyake, C. (2008) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. *Plant J.* **55**, 857–868.

Havaux, M., Dall'osto, L. and Bassi, R. (2007) Zeaxanthin has enhanced antioxidant capacity with respect to all other xanthophylls in Arabidopsis leaves and functions independent of binding to PSII antennae. *Plant Physiol.* **145**, 1506–1520.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* **59**, 213–223.

Lorenz, R.T. and Cysewski, G.R. (2000) Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **18**, 160–167.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T. and Miki, W. (1995) Structure and functional-analysis of a marine bacterial carotenoid biosynthesis gene-cluster and astaxanthin biosynthetic-pathway proposed at the gene level. *J. Bacteriol.* **177**, 6575–6584.

Morris, W.L., Ducreux, L., Griffiths, D.W., Stewart, D., Davies, H.V. and Taylor, M.A. (2004) Carotenogenesis during tuber development and storage in potato. *J Exp Bot.* **55**, 975–982.

Nogueira, M., Mora, L., Enfissi, E.M., Bramley, P.M. and Fraser, P.D. (2013) Sub-chromoplast sequestration of carotenoids impacts on pathway regulation in tomato lines expressing different carotenoid gene combinations. *Plant Cell*, **25**, 4560–4579.

Ohad, I., Friedber, I., Neeman, Z. and Schramm, M. (1971) Biogenesis and degradation of starch (1) Fate of amyloplast membranes during maturation and storage of potato tubers. *Plant Physiol.* **47**, 465–477.

Park, K.M., Song, M.W. and Lee, J.H. (2009) Determination of kinetic parameters of growth and carotenogenesis in the red yeast *Xanthophyllomyces dendrorhous*. *Biotechnol. Bioprocess Eng.* **14**, 414–418.

Pasare, S., Wright, K., Campbell, R., Morris, W., Ducreux, L., Chapman, S., Bramley, P., Fraser, P., Roberts, A. and Taylor, M. The sub-cellular localisation of the potato (Solanum tuberosum L.) carotenoid biosynthetic enzymes, CrtRb2 and PSY2. Protoplasma 2013.

Regierer, B., Fernie, A.R., Springer, F., Perez-Melis, A., Leisse, A., Koehl, K., Willmitzer, L., Geigenberger, P. and Kossmann, J. (2002) Starch content and yield increase as a result of altering adenylate pools in transgenic plants. *Nature Biotechnology*, **20**, 1256–1260.

Smith, A.M. and Zeeman, S.C. (2006) Quantification of starch in plant tissues. *Nature Protocols*, **1**, 1342–1345.

Smith, A.M., Zeeman, S.C. and Smith, S.M. (2005) Starch degradation. *Annu. Rev. Plant Biol.* **56**, 73–98.

Vankooten, O. and Snel, J.F.H. (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.* **25**, 147–150.

Ytterberg, A.J., Peltier, J.-B. and van Wijk, K.J. (2006) Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. *Plant Physiol.* **140**, 984–997.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Harvested tuber weights of representative potato plants.

Figure S2 qRTPCR analysis of transgene expression in recombinant potato leaf (a) and tuber (b) tissue.

Figure S3 Separation of amyloplast fractions from WT (a) and transgenic E1P1(1) (b) tubers.

Figure S4 Separation of solutions containing astaxanthin and soluble starch by size exclusion matrix.

Table S1 Ketocarotenoid biosynthesis in higher plants through metabolic engineering.

Table S2 Chromatographic and spectral properties of pigments identified in WT and crtZ/crtW transgenic potato plant tissues.

Table S3 Primers used for qRTPCR expression analysis.

