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ORIGINAL ARTICLE

The sub-cellular localisation of the potato (*Solanum tuberosum* L.) carotenoid biosynthetic enzymes, CrtRb2 and PSY2

Stefania Pasare · Kathryn Wright · Raymond Campbell · Wayne Morris · Laurence Ducreux · Sean Chapman · Peter Bramley · Paul Fraser · Alison Roberts · Mark Taylor

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Abstract Carotenoids are isoprenoids with important biological roles both for plants and animals. The yellow flesh colour of potato (*Solanum tuberosum* L.) tubers is a quality trait dependent on the types and levels of carotenoids that accumulate. The carotenoid biosynthetic pathway is well characterised, facilitating the successful engineering of carotenoid content in numerous crops including potato. However, a clear understanding concerning the factors regulating carotenoid accumulation and localisation in plant storage organs, such as tubers, is lacking. In the present study, the localisation of key carotenoid biosynthetic enzymes was investigated, as one of the unexplored factors that could influence the accumulation of carotenoids in potato tubers. Stable transgenic potato plants were generated by over-expressing *β-CAROTENE HYDROXYLASE 2* (*CrtRb2*) and *PHYTOENE SYNTHASE 2* (*PSY2*) genes, fused to red fluorescent protein (RFP). Gene expression and carotenoid levels were both significantly increased, confirming functionality of the fluorescently tagged proteins. Confocal microscopy studies revealed different sub-organellar localisations of CrtRb2-RFP and PSY2-RFP within amyloplasts. CrtRb2 was detected in small vesicular structures, inside amyloplasts, whereas PSY2 was localised in the stroma of amyloplasts. We conclude that it is important to consider the location of biosynthetic enzymes when engineering the carotenoid metabolic pathway in storage organs such as tubers.

Keywords Amyloplast · Carotenoid · Localisation · Plastid · Potato · Tuber

Abbreviations

CrtRb	β-Carotene hydroxylase
$DiOC_6$	3,3′-Dihexyloxacarbocyanine iodide
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography
PG	Plastoglobuli
PSY	*PHYTOENE SYNTHASE*
RFP	Red fluorescent protein

Introduction

Carotenoids are one of the most diverse classes of natural compounds, with over 600 types known to occur in nature (Fraser and Bramley 2004). Plant carotenoids are derived from isopentenyl diphosphate and are generally composed of a C_{40} isoprenoid skeleton with or without epoxy, hydroxyl and keto groups. Hydrocarbon carotenoids are known as carotenes, whilst oxygenated carotenoids are known as xanthophylls (Britton 1995). Carotenoids play fundamental roles in human nutrition as antioxidants and vitamin A precursors and their consumption is increasingly associated with protection from a range of diseases. Commercially, they are used as safe food, feed and cosmetic colourants, whereas in planta, carotenoids protect plants from photo-oxidative stress (reviewed in Cazzonelli 2011).

Most of the fundamental reactions involved in carotenoid biosynthesis have been established (reviewed in Ruiz-Sola

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S. Pasare · K. Wright · R. Campbell · W. Morris · L. Ducreux · S. Chapman · A. Roberts · M. Taylor (✉)
Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK
e-mail: mark.taylor@hutton.ac.uk

S. Pasare · P. Bramley · P. Fraser
School of Biological Sciences, Royal Holloway, University of London, Egham Hill, Egham, Surrey TW20 OEX, UK

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and Rodriguez-Concepcion 2012). In order to enhance the carotenoid content of plant-derived food, the carotenoid biosynthetic pathway has been subject to genetic engineering over the past decade (Fraser et al. 2009), with rudimentary rate-limiting enzymes amplified, multiple steps in pathways engineered (Diretto et al. 2007), new products or pathways introduced (Morris et al. 2006) and precursors diverted (Kovacs et al. 2007). Although notable successes in metabolic engineering have been achieved (reviewed in Giuliano et al. 2008), our understanding of the regulation of carotenoid biosynthesis and accumulation is far from complete. Carotenoid formation, catabolism and its regulation is complex and subject to developmental and physiological signals.

The plant carotenoid biosynthetic proteins are encoded in the nucleus and require import into the plastids (Hirschberg 2001). Catalysing the conversion of GGPP into phytoene in the first committed step of carotenoid biosynthesis, *PHYTOENE SYNTHASE* (*PSY*) is a nuclear-encoded small gene family consisting of *PSY1*, *PSY2* and the recently discovered *PSY3*, shown to exist in grasses (Li et al. 2008). Phytoene is enzymatically converted into all-*trans* lycopene, the major branch point precursor for downstream carotenoids that contain ε- or β-rings at each terminus of the lycopene molecule. Ring-specific hydroxylases mediate the formation of xanthophylls such as lutein and zeaxanthin (Tian and DellaPenna 2004; Quinlan et al. 2012). There are two types of carotenoid hydroxylases found in plants. One type is the non-heme di-iron BCH or HYD class, resembling the bacteria crtZ and the cyanobacteria CrtR-B enzymes that catalyse the hydroxylation of β rings. The second is the cytochrome P450 CYP97 type, which catalyses the hydroxylation of both β- and ε-rings (Ruiz-Sola and Rodriguez-Concepcion 2012). The import of proteins is mediated by a specific translocation complex in which folding, oligomeric assembly, membrane insertion and, in chloroplasts, further translocation take place (reviewed in Li and Chiu 2010). How the biosynthetic enzymes interact and form metabolons (enzyme complexes) is an open question that awaits further studies and elucidation. This was recently highlighted by the discovery that different PSY isozymes have different sub-organellar localisations within the plastid (Shumskaya et al. 2012).

In plants, carotenoids are usually synthesised in differentiated plastids of leaves, roots, flowers, fruits and seeds (reviewed in Cazzonelli and Pogson 2010). Generally, they accumulate in chloroplasts (green, photosynthetically active plastids) or chromoplasts, plastids that confer bright yellow, orange or red colours to many flowers and fruits. These plastids accumulate high levels of carotenoid pigments, but usually no chlorophyll, in various structures, such as crystalloids, fibrils or membranes. These structures serve as deposition sinks to sequester excess carotenoids and may also prevent the end products of the carotenoid biosynthetic pathway from inhibiting carotenoid biosynthesis (Rabbani et al. 1998; Al-Babili et al. 1999).

Besides pathway engineering, the manipulation of the plastid organelle either by increasing its total compartment area in the cell (Howitt and Pogson 2006) or altering the type of plastid has demonstrated the potential to dramatically increase the yields of plastid-derived isoprenoids (Cookson et al. 2003; Galpaz et al. 2008). In another example, a mutation in the cauliflower (*Brassica oleracea*) orange-curd (*Or*) gene results in the transformation of normally white-coloured cauliflower curd to orange colour due to the accumulation of β-carotene (Li et al. 2001). The mutant *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that appears to act as a switch that triggers the differentiation of plastids into chromoplasts. The mutant form of the Or protein, when expressed transgenically in potato tubers, also results in a large increase in β-carotene accumulation and possibly the differentiation of tuber plastids into chromoplasts (Lopez et al. 2008).

Potato tubers generally contain low levels of carotenoids, although there is a wide variation between white- and yellow-fleshed genotypes (Iwanzik et al. 1983; Breithaupt and Bamedi 2002). Metabolic engineering approaches have been successful in elevating potato tuber carotenoid levels to nutritionally significant levels (reviewed in Cazzonelli 2011). Interestingly, in the study of Ducreux et al. (2005), transgenic potato lines that expressed the highest levels of the bacterial gene *PHYTOENE SYNTHASE* (*CrtB*) in the tuber, both at the transcript and protein level, did not accumulate the highest levels of tuber carotenoid. These data suggest that complex mechanisms regulate the level of carotenoid product. In view of the potential importance of the type of plastid in which carotenoids can accumulate, a significant gap in our knowledge is the location of carotenoid accumulation in potato tubers. Unlike fruit and root tissues that accumulate carotenoids, no obvious chromoplasts can be seen in potato tubers in electron micrographs of yellow-fleshed tubers (Taylor et al. unpublished). In order to study the site of carotenoid synthesis, the approach adopted in this study was to localise two key enzymes of carotenoid biosynthesis in the potato tuber.

Recent gene expression data (Ducreux et al. 2008) have shown elevated expression of both *PSY2* and *CrtRb2* genes in carotenoid-rich *Solanum tuberosum* group *Phureja* tubers compared with white-fleshed *tuberosum* types. This confirmed previous reports (Fraser et al. 1994; Santos et al. 2005; D'Ambrosio et al. 2004) of the importance of these two key genes in the carotenoid biosynthetic pathway. In a study comparing the expression patterns of major genes encoding the enzymes of the carotenoid pathway, Morris et al. (2004) cloned the cDNAs encoding *PSY2* and the *CrtRb2* genes from a tuber library. The sequences were found to be 96 and 97 % identical to tomato *PSY2* and

CrtRB2, respectively (Fraser et al. 1994). These cDNAs were selected for sub-cellular localisation studies, as localising these key enzymes is likely to determine the site of synthesis of carotenoids in potato tubers.

Fusions of CrtRb2 and PSY2 to green (GFP) and red (RFP) fluorescent proteins were engineered and transiently expressed in leaves and stably expressed in tubers. Non-invasive confocal microscopy enabled the localisation of the RFP-tagged biosynthetic enzymes within leaves and tubers to be determined.

Materials and methods

Plasmid generation

The coding sequences of potato *PSY2* and *CrtRb2*, with stop codons removed, were PCR amplified from cDNA clones using primers with *Sal*I and *Xho*I restriction sites engineered at the 3′ and 5′ termini (Table 1). The PfuUltra II enzyme was employed, following the manufacturer's instructions (http://www.genomics.agilent.com/). The purified PCR products were cloned into the *Sal*I and *Xho*I sites of pGENTR1a, a Gateway Entry Vector (Invitrogen, UK). The entry clones obtained, P-pGEN (containing the *PSY2* gene) and B-pGEN (containing the *CrtRb2* gene), were sequenced to confirm correct insertion of the start codon and fluorescence tag. The *CrtRb2* and *PSY2* sequences were cloned into the pK7FWG2 (11,880 bp) and pK7RWG2 (11,866 bp) binary vectors (Karimi et al. 2002) via Gateway recombination (Invitrogen, UK), giving rise to fluorescently tagged enzymes at the C-terminal and under the control of the 35S Cauliflower Mosaic Virus promoter (constructs termed P-pK7R and B-pK7R). For stable transformation, constructs were modified by replacing the 35SCaMV promoter with the tuber-specific B33 patatin promoter. The 1.7-kb sequence of this promoter was amplified in a PCR reaction from the B33-pBIN19 plasmid kindly provided by Dr. Sophia Sonnewald (University of Erlangen-Nuremberg, Germany), using primers with *Spe*I and *Pme*I restriction sites at the 5′ and 3′ termini. The resulting PCR product was T/A cloned into pGEM-T Easy (Invitrogen) and sequenced to verify accuracy of the sequence and correct insertion of the restriction sites. The B33-patatin fragment was then used to replace the CaMV35S promoter in the P-pK7R and B-pK7R binary vectors via the *Spe*I and *Pme*I restriction sites to generate expression clones containing the *PSY2* and *CrtRb2* transgenes under the B33-patatin promoter, designated P-B33-pK7R and B-B33-pK7R.

Plant material

Seed of *Nicotiana benthamiana* Domin and seed tubers of potato cv. Desiree were obtained from stocks maintained at The James Hutton Institute, UK. The potato tubers were planted in compost in 30-cm-diameter pots. The *N. benthamiana* plants were sown in compost in 10-cm-diameter pots. Plants were grown in a glasshouse under a 20/15 °C day/night temperature regime. The daily maximum light intensity varied between 400 and 1,000 μmol m^{-2} s^{-1} with a mean day length of 16 h.

Transient and stable expression systems

For transient expression of the genes of interest, leaves from *N. benthamiana* Domin and potato cv. Desiree were used in *Agrobacterium tumefaciens*-mediated transformation (Bhaskar et al. 2009). The expression vectors were introduced by electroporation into *Agrobacterium* AGL1 cells, with the *virG* expression enhancer (Cho and Winans 2005). Transformed *Agrobacterium* were routinely tested by PCR to ensure they contained the respective *PSY2*- and *CrtRb2*-based plasmids. Cultivation of bacteria cells and inoculation into leaves were performed as described by Brandizzi et al. (2002). Stable expression was carried out by *A. tumefaciens*-mediated transformation as described by Ducreux et al. (2005).

Quantitative RT-PCR analysis

For gene expression analysis, qRT-PCR was carried out as described by Ducreux et al. (2008) using the Universal Probe Library (UPL) System (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). Tuber tissue was freeze dried, followed by total RNA extraction (Ducreux et al. 2005). The sequences of the UPL primers StEF1alpha_fwd, StEF1alpha_rev, StPSY2_fwd, StPSY2_rev, StCrtRb2_fwd, StCrtRb2_rev and probes PSY2 136 and CrtRb2 150 are shown in Table 2.

Table 1 Primer sequences for cloning of full-length PSY2 and CrtRb2 into pGENTR1a

Primer name	Sequence
Primer forward CrtRb2	5′-AAA AGT CGA CAA AAT GGC GGC CGG AAT-3′
Primer reverse CrtRb2	5′-AAA ACT CGA GCC TAA TAA TCC CTT AGA AAT TTT A-3′
Primer forward PSY2	5′-AAA AGT CGA AAT GTC TGT TGC TTT GTT GTG-3′
Primer reverse PSY2	5′-AAA ACT CGA GCC TGT CTT TGC TAG TGG GGA A-3′

Table 2 Primer and probe sequences as designed in the Roche UPL assay online tool, used for qRT-PCR analysis

UPL primer/probe name	UPL primer/probe sequence
Primer forward Ef1-α	5′-CTTGACGCTCTTGACCAGATT-3′
Primer reverse Ef1-α	5′-GAAGACGGAGGGGTTTGTCT-3′
Primer forward CrtRb2	5′-TGAGATGAACGACGTTTTCG-3′
Primer reverse CrtRb2	5′-GAAGAAACCGTATGAAAGAAG AGC-3′
Primer forward PSY2	5′-TCTGGGCAATATATGTGTGGTG-3′
Primer reverse PSY2	5′-GATGCATTAGGGCCATCAAC-3′
Probe 117 Ef1-α	5′-AGCCCAAG-3′
Probe 150 CrtRb2	5′-TGCTGTTC-3′
Probe 136 PSY2	5′-TGATGAGC-3′

Microscopy

Localisation of the proteins of interest, tagged with GFP/RFP in transformed leaf or tuber material, was achieved by imaging with an upright confocal laser scanning microscope (CLSM). The microscopes used were a Leica SP2 (Leica Microsystems Ltd., Hemel Hempstead, UK) and a Zeiss LSM 710 (Zeiss Jena, Germany). Cells were imaged with 63× objectives. Transmitted light images (pseudo-bright field) were collected using transmitted light detectors on the confocal microscopes. Images of GFP or 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) were collected by excitation at 488 nm with emissions collected at 500–530 nm with the simultaneous collection of chlorophyll auto-fluorescence between 650 and 700 nm. RFP was excited at 561-nm excitation with emissions collected between 590 and 630 nm, with sequential imaging of GFP as necessary. Captured images were analysed with the LCS software (Leica Microsystems) or ZEN 2010 software (Zeiss), provided by the manufacturing companies and assigned false colouring: green for GFP and DiOC6 fluorescence, magenta for RFP, and blue for chlorophyll auto-fluorescence. Confocal and bright field images were processed using the Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA, USA).

Fluorescent staining of amyloplast membrane

The fluorescent dye $DiOC_6$ was used to stain transgenic and control tuber tissue slices. The dye was diluted to a stock of 1 to 2 mg ml^{-1} in dimethyl sulfoxide and used as a working solution of 1 to 2 μg ml^{-1} in water.

Total carotenoid extraction and HPLC analysis

Whole-tuber samples were frozen in liquid nitrogen and stored at −80 °C prior to analysis. The extraction of total potato tuber carotenoids and subsequent analysis by reversed-phase HPLC were carried out as described by Morris et al. (2004).

Results

Localisation studies in leaves

In silico analysis of the N-termini of the CrtRb2 and PSY2 proteins using the ChloroP program (Emanuelsson et al. 1999; http://www.cbs.dtu.dk) strongly suggested they would be targeted to chloroplasts (Fig. 1). The sequences of CrtRb2 and PSY2 were also analysed using the TargetP prediction programme (Emanuelsson et al. 2007). For CrtRb2, this analysis also suggests that the protein is plastid targeted. For PSY2, TargetP does not predict a chloroplast location, underlining gaps in our knowledge regarding targeting sequences. The ChloroP-predicted lengths of the N-terminal pre-sequences of CrtRb2 and PSY2 (59 and 48 amino acids, respectively), which are cleaved following import into plastids, are shown as bold amino acid residues in Fig. 1. Constructs carrying fusions of full-length cDNAs encoding PSY2 and CrtRb2 with RFP or GFP at the 3′ termini were transiently expressed in fully expanded *N.*

StCrtRb2

MAAGISASAGSRTICLRHNPFRGPKSASTAPPVLFFSPLTRNFGAILL
SRRKPRLAVCFVLKNEKLNSTIQSECEVIQDQIQVEINEEKSLAACWLA
EKLARKKSERFTYLVAAVMSSLGITSMAILSVYYRFSWQMEGGEVPFS
EMLATFTLSFGAAVGMEYWARWAHRALWHASLWHMetHESHHRPRE
GPFEMNDVFAITNAVPAIALLSYGFFHKGLVPGLCFGAGLGITVFGMA
YMFVHDGLVHKRFPVGPIANVPYFRRVAAAHQLHHSDKFDGVPYGLF
LGPKELEEVGGLEELEKEVNRRIKISKGLL

StPSY2

MSVALLWVVSPNSEVLNGTGFLDSVREGNRGLESSRFPSPNRNSMWKGR
FKKGGRQEWNFGFLNADLRYSCLGRSRTENGRSFSVQSSLVASPAGEMAVS
SEKKVYEVVLKQAALVKRHLISTEDIEVKPDIVVPGNLGLLSEAYDRCGEVCA
EYAKTFYLGTMLMTPDRRRAIWAIYVWCRRTDELVDGPNASHITPQALDR
WEARLERYFASGRPFDMLDAALSDTVSKFPVDIQPFRDMVEGMRMDLWK
SRYNNFDELYLYCYYVAGTVGLMSVPIMGIAPESKATTESVYNAALALGIAN
QLTNILRDVGEDARRGRVYLPQDELAQAGLSDEDIFAGRVTDKWRIFMKKQ
IQRARKFFDEAEKGVTELSSASRWPVLASLLLYRKILDEIEANDYNNFTRRAY
VSKPKKLLTLPIAYARSLVPPKSTSSPLAKT

Fig. 1 Amino acid sequences of potato PSY2 and CrtRb2 proteins. The transit peptide sequences, 48 amino acids in length for PSY2 and 59 for CrtRb2, as predicted by ChloroP, are shown in *bold*

benthamiana leaves using an agro-infiltration approach. Both GFP-tagged CrtRb2 and PSY-2 proteins (green signal) are localised in the chloroplasts (indicated by blue false-colouring of chlorophyll auto-fluorescence) of the leaf mesophyll cells, but each has a different sub-organellar localisation. The CrtRb2-GFP signal (Fig. 2a) is located throughout the stroma of the chloroplasts (as shown in Fig. 2b, marked by the chlorophyll auto-fluorescence). This is highlighted by the presence of a stromule, as seen in Fig. 2a and c. The PSY2-GFP signal (Fig. 2d) was detected as concentrated foci in the chloroplasts (Fig. 2e and f). The agro-inoculation of *N. benthamiana* leaves with both PSY-2-GFP (Fig. 2g) and CrtRb2-RFP (Fig. 2h) constructs showed their localisation was stable, even when they were co-expressed in the same chloroplast (Fig. 2i).

Probably due to potato leaf morphology, agro-infiltration of potato leaves does not generally lead to such high transient expression levels. However, by selecting appropriate potato genotypes and at a suitable size and developmental stage (potato leaves 7 to 10 cm in diameter from 6-week-old plants), good levels of transient expression were achieved in fully expanded potato leaves. These confirmed previous findings from *N. benthamiana* mesophyll cells, demonstrating the same differential localisation pattern of GFP-tagged CrtRb2 and PSY2, in potato cv. Desiree leaves (Fig. 3).

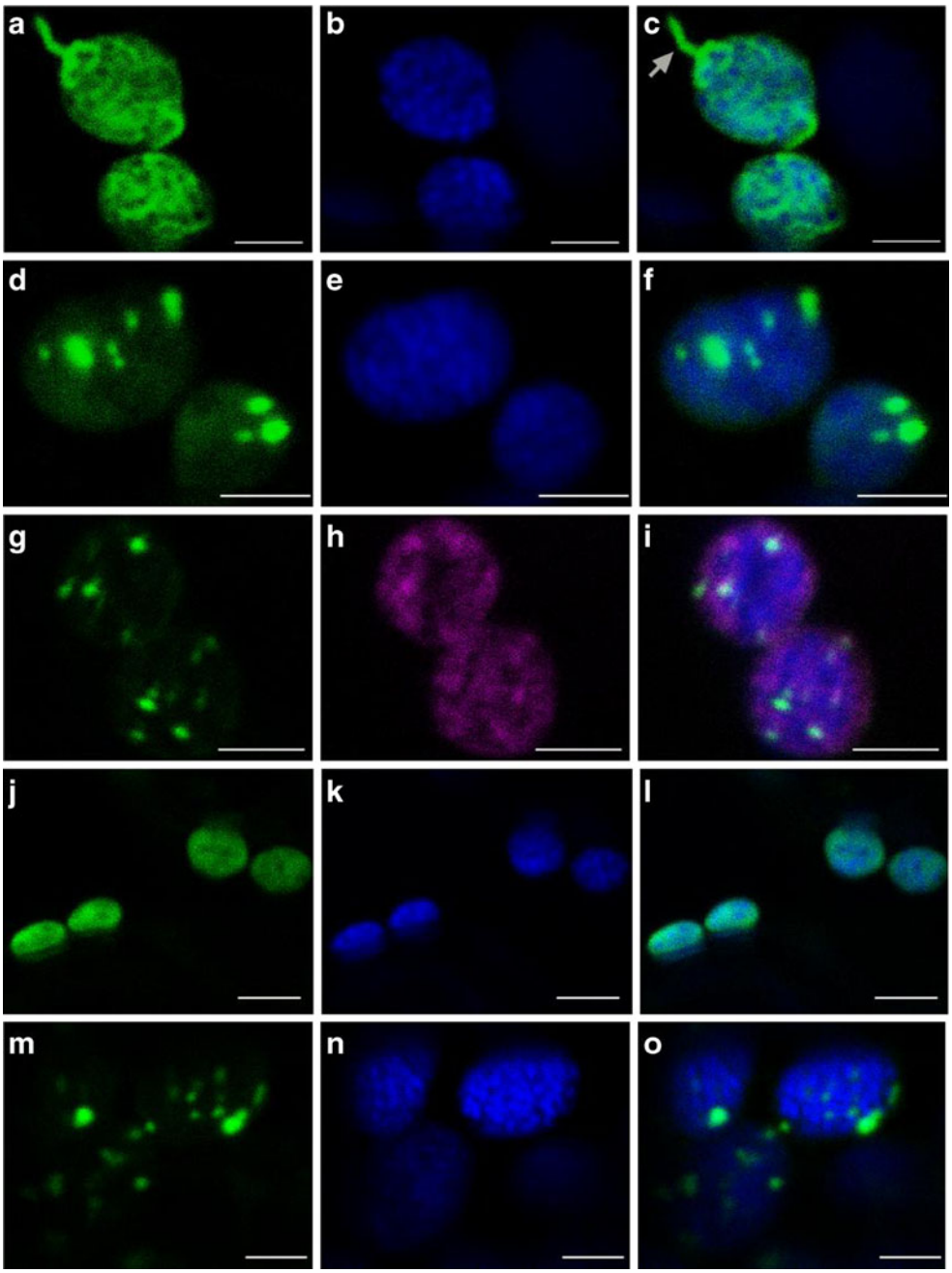

Fig. 2 Transient expression of CrtRb2 and PSY2 in *Nicotiana benthamiana* leaf chloroplasts at 3 dpi. The full-length sequence of CrtRb2-GFP **a** localised into the stroma of chloroplasts, but not the auto-fluorescent, chlorophyll-containing thylakoids (**b**), being particularly noticeable in a stromule (*arrow*; **c**). In contrast, the PSY2-GFP (**d**) fusion protein was detected as concentrated foci in the chloroplasts (**e**–**f**). Co-infection of the mesophyll cells with PSY2-GFP (**g**) and CrtRb2-RFP (**h**) did not alter their localisation pattern in chloroplasts (**i**). Expression of the N-terminal 60 amino acids of CrtRb2 and PSY2, GFP tagged, confirmed previous observations made when using the full-length sequences, as the truncated CrtRb2-GFP (**j**–**l**) localised in the stroma and the truncated PSY2-GFP (**m**–**o**) fusion protein was found as concentrated foci in chloroplasts. Bars=5 μm (**a**–**i**; **m**–**o**); 7 μm (**j**–**l**)

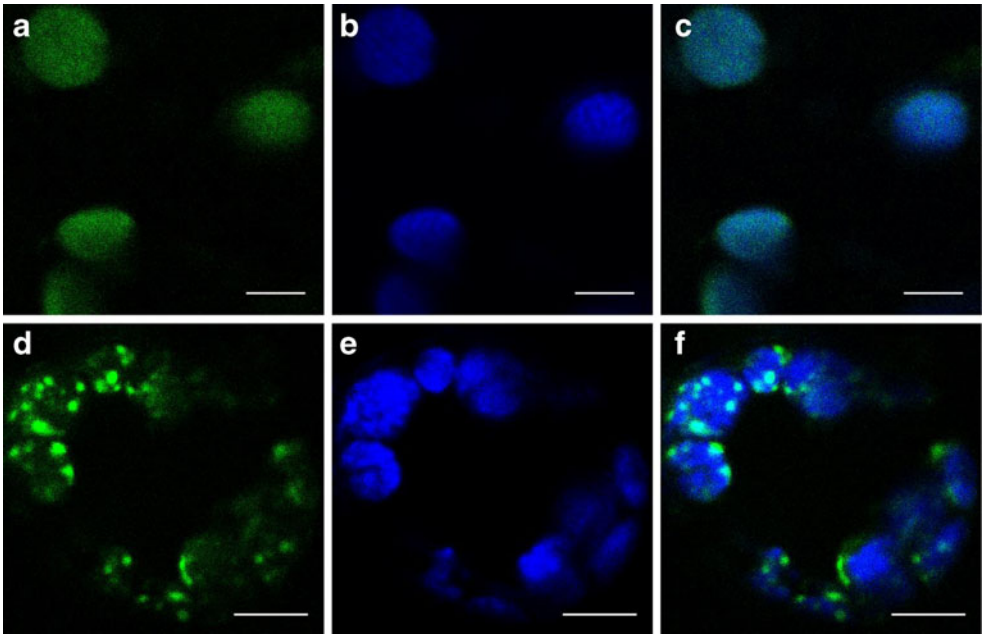

Fig. 3 Transient expression of CrtRb2-GFP and PSY2-GFP fusion proteins in potato cv. Desiree leaf chloroplasts at 3 dpi. Confirming observations of these fusion proteins in *Nicotiana benthamina*, CrtRb2-GFP (**a**) was detected in the chloroplasts (**b**) of potato mesophyll cells, as a stromal signal (**c**), whereas PSY2-GFP (**d**) localised as concentrated foci into the chloroplasts (**e**–**f**). Bars=5 μm (**a**–**c**) and 10 μm (**d**–**f**)

To confirm that the observed localisation patterns of the mature proteins were not perturbed by over-expression of active enzyme (possibly resulting in changes in carotenoid accumulation), truncated sequences of CrtRb2 and PSY2 were fused to the N-terminus of GFP and RFP and transiently expressed in mesophyll cells of tobacco leaves. Based on Bionda et al. (2010), the required length for efficient translocation of proteins into chloroplast is 60 amino acids. Hence, the first 60 amino acids from potato CrtRb2 and PSY2 proteins were used to generate fusions of these peptides with RFP or GFP at the 3′ terminus. For CrtRb2, the truncated construct is predicted to contain one amino acid of the mature protein, whereas for PSY2, 12 amino acids of the mature protein are predicted to be fused to the fluorescent reporter. The in vivo localisation patterns of the truncated CrtRb2 and PSY2 fusions were investigated by confocal microscopy and showed identical localisation patterns to those obtained with the full-length CrtRb2 and PSY2 fusions (Fig. 2j–o): the fluorescent signal from the truncated CrtRb2 (Fig. 2j–l) was localised throughout the stroma of the chloroplasts, whereas the truncated PSY2-derived signal (Fig. 2m–o) was detected as concentrated foci in the chloroplasts. As transit peptide cleavage is thought to occur in the stroma, the localisation of CrtRb2 was to be expected. For PSY2, this experiment indicates that the signal required for sub-plastidial localisation resides in the N-terminal portion of the mature protein.

Stable expression of tagged PSY2 and CrtRb2 in potato tubers

It was considered timely to investigate the site of sub-cellular localisation of key carotenoid-producing enzymes in potato tubers, as it could aide in elucidating the many factors regulating carotenoid accumulation in storage organs. Although extending the agro-infiltration technique from leaves to some other tissues such as tomato fruits has proved possible (Orzaez et al. 2006), our attempts to use agro-infiltration in potato tubers were unsuccessful. Thus, *Agrobacterium*-mediated transformation was used to generate stable transgenic potato lines. This has the added benefit that expression levels tend to be lower and more physiologically relevant in stable transgenics than in transient expression systems. For these experiments, expression of *CrtRb2* and *PSY2-RFP* fusions was driven by the tuber-specific B33-patatin promoter (Rochasosa et al. 1989) in order to minimise developmental effects of the transgene that have been previously reported for carotenoid pathway genes (Fray et al. 1995).

Tubers from several of the 19 independent transgenic lines over-expressing the *PSY2-RFP* fusion had yellow flesh, indicating an elevated carotenoid content in the transformants (Fig. 4). Total tuber carotenoid contents of both *CrtRb2-RFP* and *PSY2-RFP* over-expressing potato were up to 3.5- and fivefold, respectively, higher than in control tubers (Fig. 5).

The transcript levels of *CrtRb2* and *PSY2* were measured in the mature transgenic tubers using qRT-PCR assays. Expression levels of the target genes were elevated by 10-fold for *CrtRb2* transgenic line K1 and by sixfold in tubers from *PSY2* over-expressing potato line R5 (Fig. 6a–b).

Sub-cellular localisation of tagged CrtRb2 and PSY2 in potato tuber parenchyma

Confocal fluorescence microscopy was employed to determine the localisation of the RFP-tagged carotenoid biosynthetic enzymes CrtRb2 and PSY2 in potato tubers over-

Fig. 4 Yellow tuber flesh of the transgenic potato lines over-expressing PSY2 compared with Desiree, the control tuber tissue (**a**). Bar=4 cm

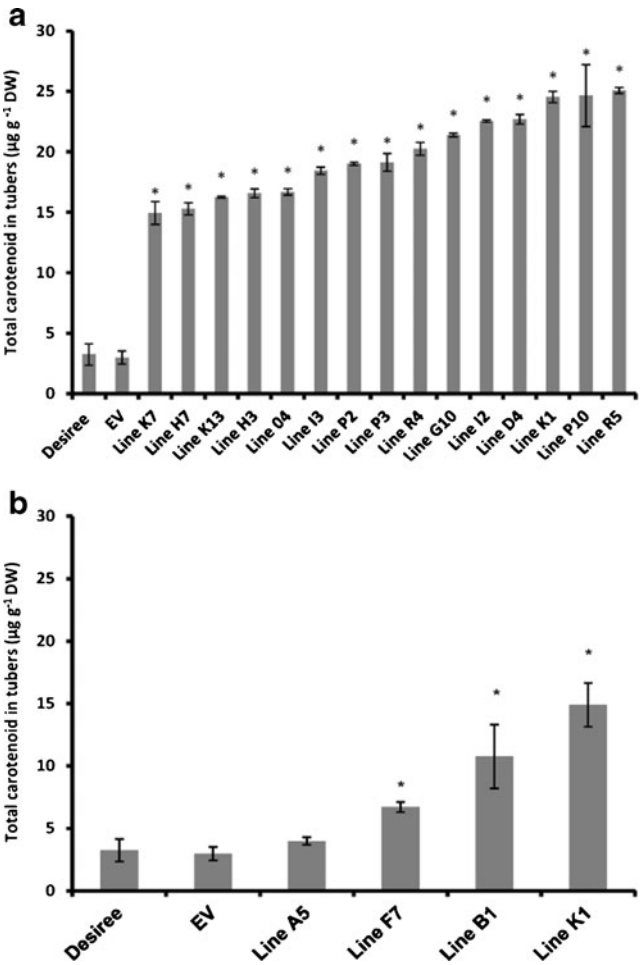

Fig. 5 Total carotenoid levels in tuber tissue of transgenic potato lines, over-expressing PSY2 (**a**) and CrtRb2 (**b**). Values are based on analysis of tissue from 90-day-old (mature) plants grown from tubers, as described in "Materials and methods", carried out in duplicate, where $n=1$. *Error bars* indicate±SE. Statistical differences from the wild type control were calculated with Student's one-tailed *t* test, assuming unequal variance. An *asterisk* was added where there was a significant difference between the calculated ($P<0.05$) values for transgenic lines and controls

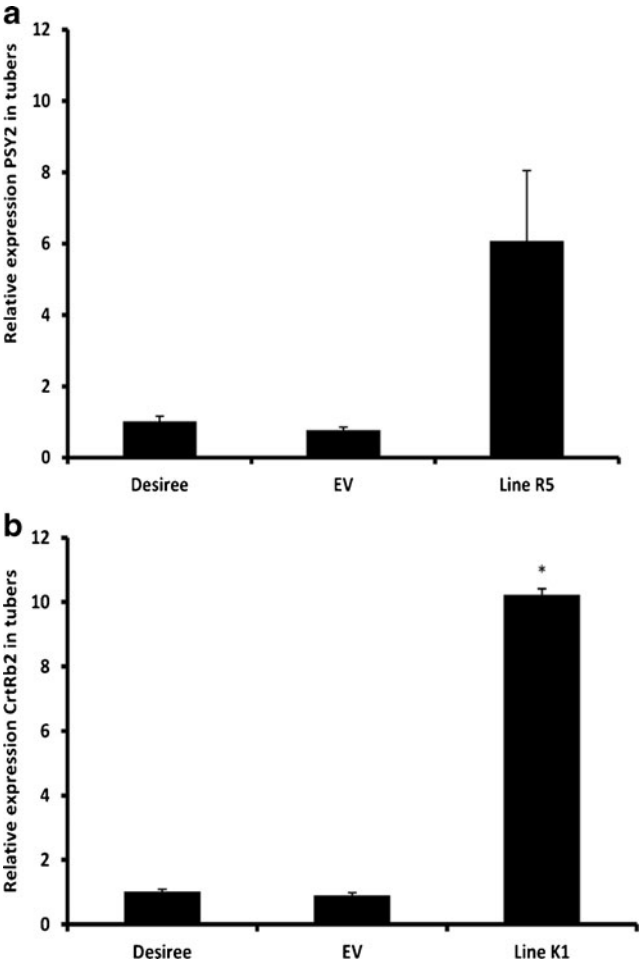

Fig. 6 Expression levels of *PSY2* (**a**) and *CrtRb2* (**b**) genes in mature tuber tissue of transgenic potato lines R5 and K1, over-expressing PSY2-RFP and CrtRb2-RFP respectively, compared to controls, wild type cv. Desiree and empty vector transformant (EV). Values are means of biological triplicate (that is, tubers from different plants of the same transgenic/control line) assays±SE, where $n=3$, expressed relative to the value in wild type samples. Statistical differences from the wild type control were calculated with Student's one-tailed *t* test, assuming unequal variance. An *asterisk* was added where there was a significant difference between the calculated ($P<0.05$) values for the transgenic lines and controls

expressing these proteins. The analysis was carried out on tubers from four selected transgenic lines, over three growing seasons. For each construct, reproducible localisation patterns were observed in the amyloplasts of parenchyma cells for all selected transgenic lines (data shown for the *CrtRb2* line K1 and *PSY2* line R5) and from tubers from all three growing seasons (Fig. 7). These results were verified by imaging tuber tissue of Desiree and empty vector controls at similar fluorescence settings as the transgenic tissue (Fig. 8).

To determine the location of the enzymes more precisely, sets of pseudo-bright-field images of amyloplasts from CrtRb2 and PSY2 transgenic potato were obtained (Fig. 7a and b, respectively). To offer a context to the RFP fluorescence signal detected in the cells (Fig. 7c–d), a green-fluorescent, lipophilic

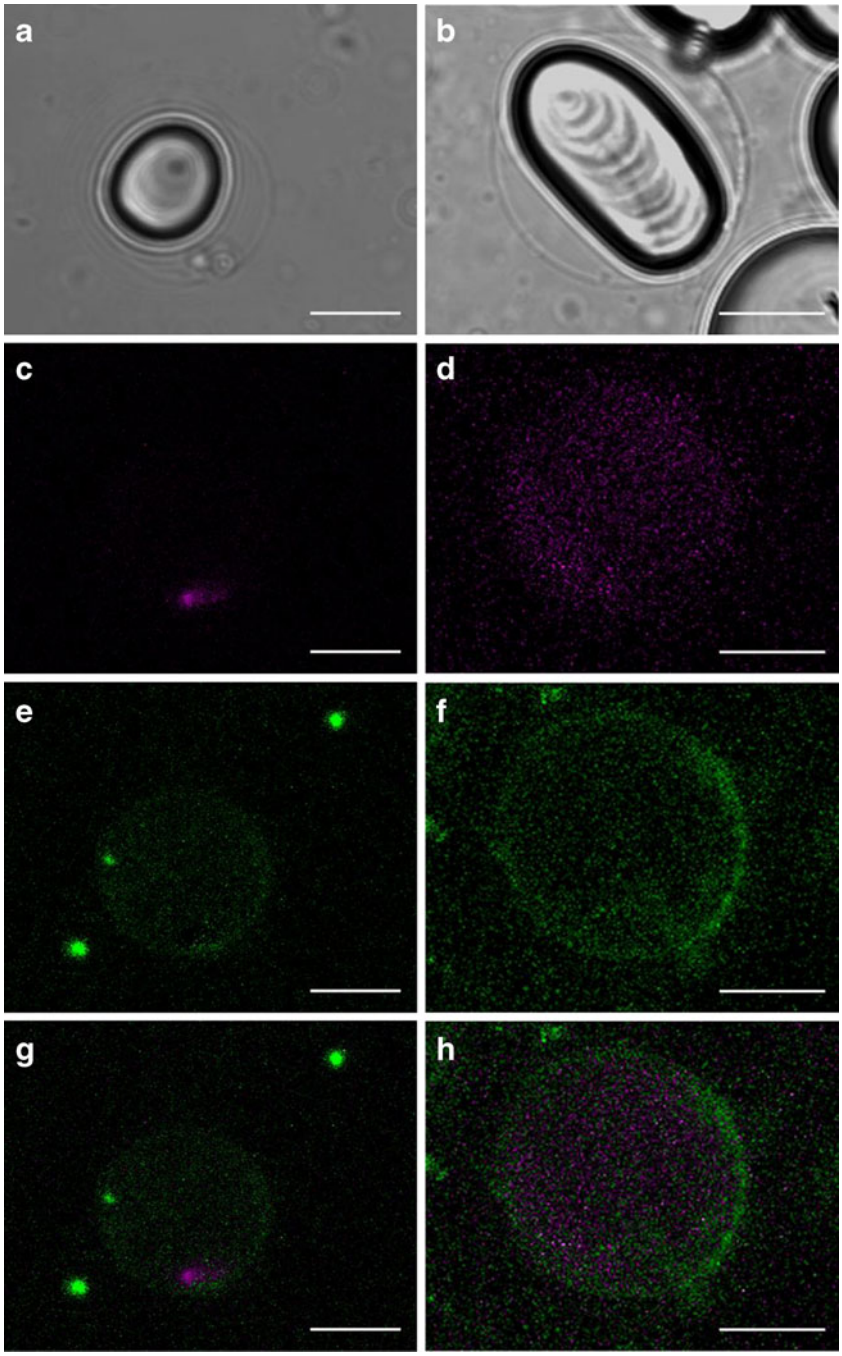

Fig. 7 Stable expression of CrtRb2-RFP and PSY2-RFP fusion proteins in potato transgenic lines. Free-floating intact amyloplasts from transgenic potato lines over-expressing CrtRb2 (**a**, **c**, **e**, **g**) and PSY2 (**b**, **d**, **f**, **h**) are presented. Pseudo-bright-field images show a starch grain surrounded by the amyloplast membrane (**a**, **b**). The CrtRb2-RFP fusion protein was localised to a punctate spot (**c**) that appears in the pseudo-bright-field image to be membrane bound (**a**), located within the amyloplast membrane indicated by staining with $DiOC_6$ (**e**). PSY2-RFP fusion protein was stromal (**d**), again bounded by a $DiOC_6$-stained membrane (**f**). Images **c**–**h** are single confocal sections with the overlay images illustrated in **g** and **h**. Bars=20 μm (**a**, **c**, **e**, **g**) and 40 μm (**b**, **d**, **f**, **h**)

dye, $DiOC_6$, was utilised. Originally designed for mitochondrial staining (Pringle et al. 1989; Weisman et al. 1990), $DiOC_6$ is known to label the nuclear envelope and endoplasmic reticulum if the dye concentration is increased (Koning et al. 1993) and, due to its lipophilic properties, can label additional cellular membranes including the monolayer membrane of isolated oil bodies (Acevedo et al. 2012). In time, $DiOC_6$ fluorescence was detected in the amyloplast membrane of potato tuber cells (Fig. 7e–f). The images of CrtRb2 and PSY2 in potato tubers were in marked contrast to the results obtained from leaf tissue. Hence, within the amyloplasts, the CrtRb2-RFP signal appeared in punctate foci or speckles (approximately 2–4 μM in diameter) (Fig. 7g), which, on the basis of transmission, images appear to be membrane bound, whilst PSY2-RFP was distributed throughout the stroma (Fig. 7h).

Discussion

Localisation of CrtRb2 and PSY2 in leaves and tubers

Very recently, it has been shown that in maize (*Zea mays*) leaves, different PSY isozymes target different sub-organellar localisations (Shumskaya et al. 2012). Furthermore, small

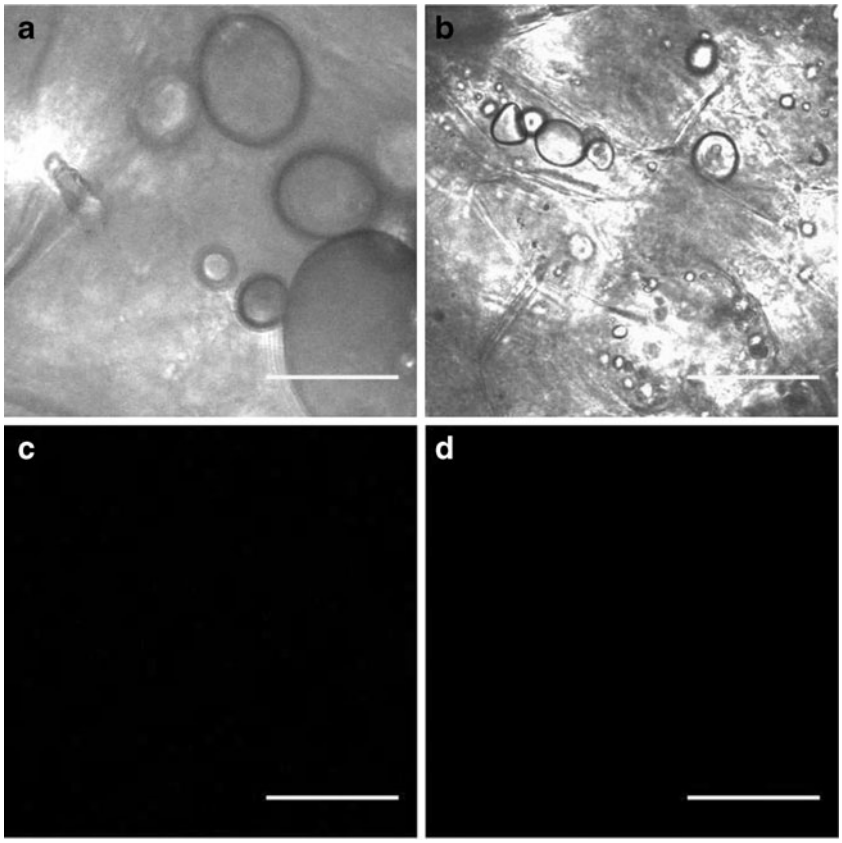

Fig. 8 Control images of Desiree and empty vector tuber tissue. Pseudo-bright-field and fluorescent images showing amyloplasts imaged in tissue of Desiree (**a**, **c**) and empty vector control tuber tissue (**b**, **d**). **c** and **d** show fluorescent images taken at a setting similar to the one used for detection of the RPF-tagged proteins PSY2 and CrtRb. Bars=25 μm (**a**, **c**) and 100 μm (**b**, **d**)

variations in PSY sequence and activity are sufficient to influence the different localisations. In the maize study, for PSY1, amino acid variation at amino acid 257 from a threonine to proline residue resulted in a change in localisation from the plastoglobuli (PG) to the stroma. However, little is known about the sub-organellar localisation of the carotenoid biosynthetic pathway in potato tubers. In this study, we addressed this issue by using confocal microscopy of fluorescently tagged CrtRb2 and PSY2 proteins. This analysis has revealed new insights about the localisation of these biosynthetic carotenoid enzymes in plastids in both leaves and tubers. At the sub-organellar level, distinct differences in the localisation patterns of these two proteins were observed. In leaves, the CrtRb2 signal was stromal, whereas PSY2 was detected as concentrated foci, apparently associated with the thylakoid membrane. In tubers, the tagged proteins localised in the amyloplasts, but in an opposite localisation pattern to that observed in the chloroplasts. Thus, the CrtRb2 signal was detected as concentrated foci apparently membrane bound, contained inside the amyloplasts, whereas the PSY2 signal was evenly distributed, corresponding to a stromal localisation.

The benefits of using fluorescently tagged enzymes to study localisation of carotenoid biosynthetic enzymes have been reviewed recently (Shumskaya et al. 2012). In particular, it is known that PSY accumulates to low levels and the sensitivity of confocal microscopy enables detection that could possibly be missed using proteomic approaches. However, over-expression of the pathway genes could conceivably perturb the system and thus, observations are from tissues that are already subject to metabolic engineering. In leaves of both *N. benthamiana* and potato, we observe identical localisations when we express the tagged full-length cDNA and the N-terminus 60 amino acids tagged with the fluorescent protein. Clearly, in the latter case, no increase in carotenoid accumulation could be accounting for the observed localisation patterns. For CrtRb2, the transit peptide is predicted to be 59 amino acids and so the truncated construct encodes only one amino acid of the mature protein. For both the full-length and truncated CrtRb2 constructs, fluorescence was detected in the stroma. For PSY2, the transit peptide is predicted to be 48 amino acids and so the truncated construct encodes 12 amino acids of the mature protein, assuming the ChloroP prediction to be correct. As the transit peptide is cleaved on peptide entry, this result implies that the 12 amino acids of the mature protein are sufficient to drive localisation to the observed discreet foci.

Whilst the amino acid sequence of the mature PSY1 protein does strongly influence sub-plastidial localisation, particularly at residue 257 (Shumskaya et al. 2012), there was no indication that the N-terminus residues of the mature protein were influential in this respect, as it appears to be the case from our data. Follow-up work will address more precisely the sequences required to obtain sub-plastidial localisation in potato tissues. In further controls in leaves,

co-expression studies demonstrated the same localisations in cells that over-express the two enzymes simultaneously, emphasising the consistency in the observations. In tubers, where it was not possible to use transient expression approaches, introduction of the tagged enzymes resulted in elevated carotenoid levels. Transgenic elevation of PSY activity has previously been demonstrated to result in enhanced potato tuber carotenoid content (Ducreux et al. 2005), and elevated *CrtRb2* transcript level is associated with higher levels of tuber carotenoid (Zhou et al. 2011). Whilst this demonstrates that the over-expressed enzymes were functional, it is not possible to rule out that the observed localisations are in response to the increased throughput in the carotenoid pathway. Indeed, increased levels of the rate-limiting enzymes of carotenoid biosynthetic enzymes might drive plastid structural changes needed to provide a sink for the hydrophobic pathway products, as suggested by Shumskaya et al. (2012). However, the observed localisations in tubers were consistently observed in samples from four independent transgenic lines, grown through three seasons of vegetative growth.

Do potato carotenoid biosynthetic enzymes accumulate in plastoglobuli?

Previous studies have investigated the localisation of carotenoid biosynthetic enzymes, highlighting the importance of their association with membranes for activity and proposing new roles for PG as the active site of carotenoid conversion in chromoplasts. Chloroplast PGs are associated with the thylakoid membranes (Rey et al. 2000). They contain carotenoids, plastoquinones, tocopherols and various proteins and are surrounded by a lipid monolayer that is contiguous with the outer layer of the thylakoid lipid bilayer (Kessler et al. 1999).

Previous studies have reported PG to vary in size from 30 nm to several micrometers (Thomson and Platt 1973; Steinmüller and Tevini 1985), depending on the stage of development and environmental conditions. For example, it has been reported that PG can enlarge and proliferate in response to abiotic stress (for example, Zhang et al. 2010).

Recently, an *Arabidopsis* zinc finger protein, VAR3 (At5g17790), proved to interact with CCD4, was localised in the chloroplast as punctuated foci (Naested et al. 2004), very similar to the pattern of localisation of PSY2 in mesophyll cells, as observed in our study. Based on the identification of CCD4 in the proteome of chloroplast PG, Ytterberg et al. (2006) claimed these spots could represent PG. Moreover, Shumskaya et al. (2012) recently found that maize and rice (*Oryza sativa* L.) PSY2, PSY3 and *Arabidopsis* PSY localise as speckles in maize protoplasts and, based on co-localisation with maize plastoglobulin-2 (Zm-PG2), suggested these enzymes are targeted to the PG.

In non-photosynthetic plastids, details of PG associations with membranes remain to be fully resolved. Previous studies have used proteomic approaches to localise carotenoid biosynthetic enzymes. For example, Siddique et al. (2006) identified CrtRb2 in the proteome of pepper chromoplasts. In the same year, Ytterberg et al. (2006) found two CrtRb proteins, together with another two carotenoid biosynthetic enzymes in the proteome of pepper PG.

In tubers, CrtRb2 appears to accumulate in small, membrane-bound structures within the amyloplast. There is little information regarding the sub-structure of potato tuber amyloplasts and, therefore, it is difficult to speculate whether these are PG or some other structure. However, based on the studies from chloroplasts, they would be in the upper size range for PG.

Implications for metabolic engineering

It is possible that the sub-plastidial localisation of introduced transgenes may influence the effectiveness of a metabolic engineering strategy. For example, there was a major increase in β-carotene accumulation in Golden Rice 2 (Paine et al. 2005) compared with the earlier version (Ye et al. 2000). Golden Rice 2 utilised maize PSY1, whereas the original Golden Rice used a PSY gene from daffodil. The threonine$_{257}$ version of maize PSY1 appears as a soluble protein in plastids (Shumskaya et al. 2012) and this was the particular variant expressed in Golden Rice 2 endosperm. As discussed in Shumskaya and Wurtzel (2013), most other PSY variants localise in PGs and so it would be interesting to explore whether the localisation of PSY1 was a key factor in the improved engineering of Golden Rice 2.

In potato, both CrtRb2 and PSY2 are key enzymes in carotenoid biosynthesis as revealed by both genetic and transgenic studies (Brown et al. 2006; Ducreux et al. 2005). Metabolic engineering approaches have been used to produce potato tubers containing nutritionally significant levels of the provitamin A carotenoid, β-carotene (Diretto et al. 2007). However, efforts to build on these achievements are likely to benefit from enhanced understanding of pathway regulation, so that better targeted interventions with more predictable outcomes can be made.

The tissue specificity of the localisations of CrtRb2 and PSY2 adds a new element of complexity to the issue of metabolon formation for carotenoid biosynthesis. Currently, it is only possible to speculate about the reasons for, and implications of, these localisations. The present study suggests that the sub-organellar compartmentation of carotenoid biosynthetic enzymes may provide another tier for the control of carotenoid production and that different constraints may operate in different tissues, as observed by the localisation patterns of these proteins in leaves and tubers of potato. The differences in localisation between tissues also highlight the utmost importance of studying protein localisation in the exact tissue of interest (i.e., potato tubers) and not simply inferring

localisation results from a model plant (*N. benthamiana*) or a more facile experimental tissue (leaves). Taking this into account, such factors could benefit future attempts to optimise the transgenic production of carotenoids and other plastid-synthesised isoprenoids in plant storage organs.

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Conflict of interest None.

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