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# Optimising ketocarotenoid production in potato tubers: Effect of genetic background, transgene combinations and environment

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ABSTRACT

Astaxanthin is a high value carotenoid produced by some bacteria, a few green algae, several fungi but only a limited number of plants from the genus *Adonis*. Astaxanthin has been industrially exploited as a feed supplement in poultry farming and aquaculture. Consumption of ketocarotenoids, most notably astaxanthin, is also increasingly associated with a wide range of health benefits, as demonstrated in numerous clinical studies. Currently astaxanthin is produced commercially by chemical synthesis or from algal production systems. Several studies have used a metabolic engineering approach to produce astaxanthin in transgenic plants. Previous attempts to produce transgenic potato tubers biofortified with astaxanthin have met with limited success. In this study we have investigated approaches to optimising tuber astaxanthin content. It is demonstrated that the selection of appropriate parental genotype for transgenic approaches and stacking carotenoid biosynthetic pathway genes with the cauliflower *Or* gene result in enhanced astaxanthin content, to give six-fold higher tuber astaxanthin content than has been achieved previously. Additionally we demonstrate the effects of growth environment on tuber carotenoid content in both wild type and astaxanthin-producing transgenic lines and describe the associated transcriptome and metabolome restructuring.



## 1. Introduction

Carotenoids are a large group of over 750 naturally occurring coloured pigments synthesised by plants, algae and many bacteria [1]. Commercially, they are used as safe food, feed and cosmetic colourants, whereas *in planta*, carotenoids protect plants from photo-oxidative stress (reviewed in [2]). Humans benefit in a number of ways from dietary carotenoids present in green leaf tissue and many fruits, seeds, roots and tubers (reviewed in [3,4]). Carotenoids with a β-ring end group are required for the synthesis of vitamin A, and deficiency for this vitamin remains a major health problem in some parts of the world [5]. Evidence is rapidly accruing that different carotenoids have other different beneficial effects, although the mechanisms of action remain unclear. For example, lycopene appears to have a protective effect against prostate cancer [6]. Lutein and zeaxanthin intake appear to provide protection against age-related macular degeneration [7,8]. Consumption of ketocarotenoids, most notably astaxanthin, is also increasingly associated with a wide range of health benefits, tested in over 65 clinical studies (reviewed in [9]) Examples include involvement in the antioxidation of low-density lipoprotein [10], anticancer activities [11], singlet oxygen-quenching activity [12] and enhancement of immune responses [13].

In nature, astaxanthin is produced by some bacteria, a few green algae, several fungi and by members of the *Adonis* genus in plants [14–16]. Accumulated through dietary intake, astaxanthin is found in salmon, trout, krill, shrimp, crayfish, crustaceans, and the feathers of some birds, resulting in the characteristic red/pink colouration of flesh/carapace/feathers. Its use as a feed additive in salmon farming is one of the main costs of aquaculture [17].

Currently, for economic reasons, the vast majority of astaxanthin is produced by chemical synthesis. However, the synthesised pigment is composed of only 25% of the desired naturally occurring stereoisomer (3*S*,3′S), with the majority 3*S*,3′R and 3*R*,3′R stereoisomers which are thought to effect the bioactivity of natural astaxanthin. In addition, contamination of synthetic astaxanthin

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with reaction intermediates or by-products restricts its use to a feed supplement in aquaculture [18]. Astaxanthin suitable for human consumption is produced from commercially grown unicellular green algae *Haematococcus pluvialis* which accumulates up to 4–5% dry weight ketocarotenoid [19]. However, this organism is slow to grow and requires high light intensities and therefore expensive to produce. Consequently, the metabolic engineering of plants which produce commercial quantities of ketocarotenoids is an attractive approach.

In plants, astaxanthin is synthesised from β-carotene by the introduction of hydroxyl and keto moieties at the 3,3′ and 4,4′ positions of the β-ionone rings catalysed by a 3,3′ hydroxylase (β-carotene hydroxylase) and 4,4′-ketolase (β-carotene ketolase) via intermediate carotenoids (Fig. 1). A recent study by Cunningham and Gantt has shown that *Adonis aestivalis* has a distinctly different biosynthetic pathway for the conversion of carotenoid β-rings into the 3-hydroxy-4-keto-β-rings present in astaxanthin, where the conversion is catalysed in a three step pathway by a carotenoid 4-hydroxy-β-ring 4-dehydrogenase (CBFD) and a 4-hydroxy-β-ring 4-dehydrogenase(HBFD) [20]. Whilst β-carotene hydroxylases are ubiquitous, all plants, with the exception of members of the *Adonis* genus, lack the necessary ketolase gene. Functional ketolase genes have been isolated from a variety of bacterial and algal sources, such as the marine bacterium *Paracoccus* sp. N81106, designated *crtW* [21], *crtO* from the cyanobacterium, *Synechocystis* sp. PCC6803 [22] and *bkt1*, *bkt2*, *bkt3* from *H. pluvalis* [23,24]. The *crtW* gene from the marine bacterium *Brevundimonas* sp., strain SD212 encodes an enzyme demonstrated to accept both β-carotene and zeaxanthin as substrates [25]. This enzyme also catalysed the conversion of adonixanthin to astaxanthin in *Escherichia coli* more efficiently than Paracoccus CrtW [26]. Co-expression with the CrtZ enzyme isolated from the same species resulted in efficient conversion of β-carotene to astaxanthin [25,27].

Several studies have used the transgenic expression of ketolase genes to produce ketocarotenoids in plants, with varying success in tomato [28,29], tobacco [18,24,30,31], Arabidopsis [32,33] and carrot [34,35].

Two previous studies report engineered ketocarotenoids in potato [36,37]. Gerjets and Sandmann [37] expressed a *crtO* gene isolated from the cyanobacterium, *Synechocystis* under a constitutive promoter. The amount of ketocarotenoids formed in the leaves and tubers represented ~10–12% of the total carotenoids with only a small amount of astaxanthin produced (~1.8% of the total). Morris et al. [36] expressed the *bkt1* gene from *H. pluvialis* under the tuber specific patatin promoter in a potato genotype that produces yellow-fleshed tubers (*Solanum tuberosum* Group Phureja cv. Mayan Gold). Whilst a significant proportion of the tuber carotenoid produced was astaxanthin, other ketocarotenoids such as keto-lutein were also produced.

The aim of the current study was to develop improved strategies for producing elevated astaxanthin levels in potato tubers. Towards this aim, we investigated the effects of using a potato genotype that produced tubers containing high levels of the astaxanthin precursor, zeaxanthin, and low levels of carotenoids that are not directly converted to astaxanthin such as lutein as parental material for transformation. We also employed codon optimised *Brevundimonas* SD212 *crtZ* and *crtW* genes under the CaMV 35S promoter [37]. The cauliflower *Or* mutant gene, thought to encode a DnaJ cysteine-rich domain-containing protein, triggers the differentiation of non-coloured plastids into chromoplasts providing a metabolic sink for the sequestration of carotenoids [38]. The *Or* gene has successfully been expressed in potato resulting in enhanced tuber carotenoid levels [38,39] by stabilising phytoene synthase following harvest and during cold storage [40].

The potential for using the *Or* transgene in combination with other genes known to impact on carotenoid biosynthesis to enhance astaxanthin accumulation was explored and finally we investigated the effects of environmental conditions on tuber carotenoid accumulation.

## 2. Materials and methods

### 2.1. crtZW and Or constructs

The *crtW* and *crtZ* genes, encoding the CrtW and CrtZ proteins from Brevundimonas sp. SD212, respectively, were chemically synthesised according to the codon usage of rape (GenBank accession no. AB377271 and AB377272, respectively). The construct pCrtZW-pZK3B has been described in [37].

The pBI-GBSS-OR construct described by Lopez et al. [39] containing the GBSS promoter (GenBank Accession No. A23740) and the cauliflower *Or* gene (GenBank Accession No. DQ482460) was kindly provided by Dr. Li Li, USDA-ARS, Plant, Soil and Nutrition Laboratory, Cornell University, Ithaca, NY 14853, USA.

Plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformed *Agrobacterium* cells containing the *crtZW* construct were selected by their resistance to hygromycin (100 μg ml$^{-1}$) and rifampicin (100 μg ml$^{-1}$), cells containing the *Or* construct were selected by their resistance to kanamycin (100 μg ml$^{-1}$) and rifampicin (100 μg ml$^{-1}$). Potato transformation (*S. tuberosum* Group Phureja cv. Mayan Gold) was carried out as described previously [41].

### 2.2. Growth conditions

Plants were grown over the summer months from seed tubers (May to late August) in a glasshouse maintained at a daytime temperature of 20 °C and a night-time temperature of 15 °C. The light intensity (photosynthetic photon flux density) ranged from 400 to 1000 μmol m$^{-2}$ s$^{-1}$ and the mean day length was 16 h.

Winter conditioned plants were grown between October and February in a glasshouse supplemented with high pressure sodium lighting providing a mean light intensity of 300 μmol m$^{-2}$ s$^{-1}$ at canopy level. The day/night temperature was 20/15 °C and the day length was 16 h.

Cabinet grown plants were raised in the glasshouse for four weeks following emergence then moved to growth cabinets under conditions of 16 h light (80% humidity), 20 °C at a light intensity of 300 μmol m$^{-2}$ s$^{-1}$ and 8 h dark (70% humidity), 15 °C for an additional 3 months growth.

### 2.3. Cold storage

Mature tubers from summer grown individual plants were stored in paper bags in the dark at 4 °C in a cold room. Following storage for 0, 3 and 6 months, three tubers from each individual plant were washed, peeled and cut into slices. Pooled samples were flash frozen in liquid nitrogen, freeze dried then stored at −80 °C prior to analysis.

### 2.4. Analysis of carotenoids

Three uniform sized peeled whole-tuber samples from three independent plants of each line were harvested and immediately flash frozen using liquid nitrogen. For leaf and stem samples, tissues from the terminal node and internodal sections of three independent plants were harvested. Samples were immediately frozen, freeze dried and stored at −80 °C prior to analysis. Total potato tuber, leaf and stem carotenoids were extracted and analysed by reverse phase HPLC as detailed in [36].

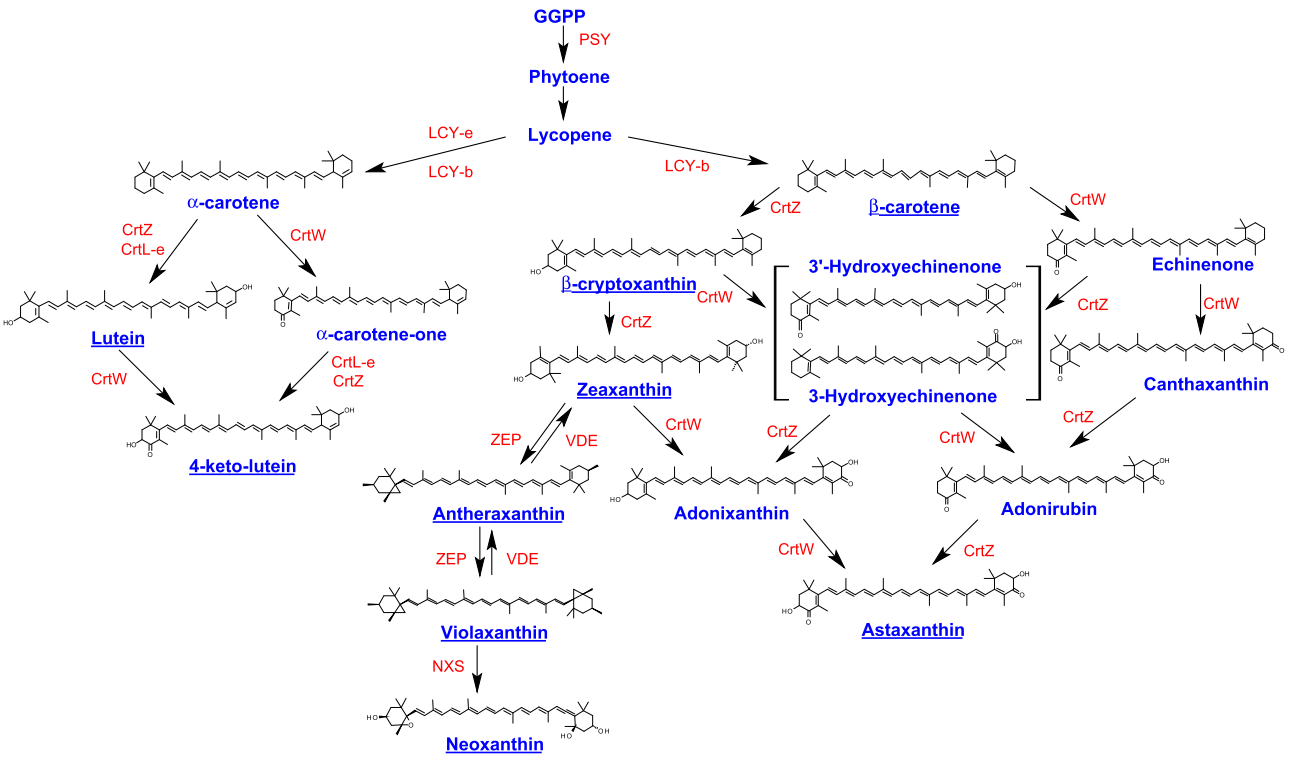


**Fig. 1.** Potential biosynthetic pathway to ketocarotenoid formation in transgenic potato expressing *Brevundimonas CrtZ* and *CrtW* genes. Carotenoids detected in transgenic potato lines by HPLC are underlined. Enzymes are indicated by the following annotation: CrtL-e, ε-ring hydroxylase; crtW, β-carotene ketolase; crtZ, β-carotene hydroxylase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; NXS, neoxanthin synthase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin de-epoxidase.

### 2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from powdered tuber samples from three biological replicates for each line as described in [41]. Quantitative RT-PCR was performed as described [42], with primer sequences listed in Supporting Information Table S1. Each experiment was repeated in triplicate using independent cDNAs. Relative expression levels were calculated using the Pfaffl method [43] using data obtained with the elongation factor-1 alpha specific primers as an internal reference control [44].

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.014.

### 2.6. Microarray analysis of gene expression

A custom Agilent microarray was designed to the predicted transcripts from assembly v.3.4 of the DM potato genome as described [45]. A single-channel replicate block microarray design was utilised, and the experimental design and complete datasets are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/; accession E-MTAB-XXXX). RNA labelling and downstream microarray processing were as recommended in the One-Color Microarray-Based Gene Expression Analysis protocol (v.6.5; Agilent) using the Low Input Quick Amp Labelling kit (Agilent). Following microarray scanning using an Agilent G2505B scanner, data were extracted from images using Feature Extraction (v.10.7.3.1) software and aligned with the appropriate array grid template file (033033_D_F_20110315). Intensity data and QC metrics were extracted using the FE protocol (GE1_107_Sep09). Entire FE datasets for each array were loaded into GeneSpring (v.7.3) software and data were normalised using default one-colour Agilent settings. Principal components analysis identified the three most similar replicates in each group which were selected for downstream analysis. Spot flags from FE (present or marginal) were used to remove probes with no consistent expression, leaving 25,291 probes. Statistical filtering was performed using volcano analysis ($P$-value <0.01, fold-change > 2×). Data were visualised using PageMan [46] and a gene tree heatmap in GeneSpring using default Pearson correlation.

## 3. Results

### 3.1. Effect of parental genotype on astaxanthin accumulation

In order to optimise the level of tuber astaxanthin accumulation in transgenic potato, it was reasoned that the parental genotype should have high total tuber carotenoid content but low lutein and high zeaxanthin content. Screening the tubers from the 106 offspring of a bi-parental diploid *S. tuberosum* Group Phureja (subsequently referred to as Phureja) population (01H15) enabled the identification of clone 57 producing tubers that fulfilled these criteria, with tubers containing ca. 54 μg/g DW total carotenoid, 81% of which was zeaxanthin and ca. 6% lutein in the saponified extracts (Tables 1A and 1B).

Transgenic potato lines were generated in the Mayan Gold and 01H15.57 backgrounds using a construct containing the *Brevundimonas crtZ* and *crtW* genes in tandem, driven by the constitutive Cauliflower Mosaic Virus 35S promoter (described in Section 2). Initially independent transgenic lines (6–12 per construct in each parental genotype) were screened for total tuber carotenoid content and tubers from the lines with the highest carotenoid content were analysed in detail by HPLC. In the transgenic lines generated in the Mayan Gold background, the tuber carotenoid profile was similar to that observed on over-expression of the *bkt-1* gene, previously reported [36]. Whilst total tuber carotenoid levels were not significantly different to the wild type, approximately 70% of the tuber carotenoid was ketocarotenoid, 46% ketolutein and 27%

**Table 1A**
Tuber carotenoid content (μg/g DW) of non saponified 01H15.57 and Mayan Gold derived transgenic lines.

| Line | Neo | Vio | Keto lut | Asta | Ant | Lut | Zea | β-Crypto | Keto other | Car other | Keto ester | Car ester | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01H15.57 | 0.2 ± 0.1 | 0.8 ± 0.3 | n.d. | n.d. | 1.9 ± 0.3 | 2.9 ± 0.7 | 41.6 ± 0.6 | 1.0± 0.3 | n.d. | 2.2 ± 0.8 | n.d. | 3.3 ± 0.3 | 53.9 ± 2.0 |
| 01H15.57 Or | 0.4 ± 0.1 | 1.3 ± 0.3 | n.d. | n.d. | 6.3 ± 1.0** | 7.8 ± 1.6* | 67.6 ± 1.9*** | 0.3 ± 0.1* | n.d. | 3.1 ± 0.8 | n.d. | 1.6 ± 0.3* | 88.3 ± 3.2*** |
| 01H15.57 crtZW | n.d. | n.d. | n.d. | 28.0 ± 2.0 | n.d. | n.d. | 1.9 ± 0.5*** | 0.1 ± 0.0** | 2.0 ± 0.2 | 1.2 ± 0.1 | 18.8 ± 1.6 | 5.7 ± 0.3** | 57.7 ± 3.4 |
| 01H15.57 crtZW Or A | n.d. | n.d. | n.d. | 49.1 ± 3.6 | n.d. | n.d. | 5.3 ± 1.2*** | 0.2 ± 0.1* | 4.4 ± 0.1 | 3.9 ± 2.0 | 29.4 ± 5.2 | 6.1 ± 0.7* | 98.5 ± 9.9** |
| 01H15.57 crtZW Or B | n.d. | n.d. | n.d. | 43.6 ± 2.4 | n.d. | n.d. | 4.4 ± 1.1*** | 0.1 ± 0.0** | 4.2 ± 0.4 | 2.1 ± 0.2 | 29.8 ± 0.6 | 5.6 ± 0.6* | 89.7 ± 4.9** |
| 01H15.57 crtZW Or D | n.d. | n.d. | n.d. | 53.2 ± 1.2 | n.d. | n.d. | 6.5 ± 0.9*** | 0.1 ± 0.0** | 5.6 ± 0.6 | 2.1 ± 0.6 | 22.0 ± 2.5 | 4.1 ± 0.8 | 93.5 ± 3.5*** |
| Mayan Gold | 0.9 ± 0.1 | 1.3 ± 0.1 | n.d. | n.d. | 1.6 ± 0.2 | 8.9 ± 0.4 | 4.3 ± 0.5 | 0.1 ± 0.0 | n.d. | 1.7 ± 0.2 | n.d. | 4.3 ± 0.4 | 23.1 ± 0.8 |
| Mayan Gold Or | 1.4 ± 0.2 | 3.7 ± 0.2*** | n.d. | n.d. | 4.3 ± 0.6*** | 10.3 ± 0.3* | 4.0 ± 0.6 | n.d. | n.d. | 3.3 ± 0.2 | n.d. | 8.9 ± 0.4*** | 35.7 ± 1.5*** |
| Mayan Gold crtZW | n.d. | n.d. | 5.5 ± 0.4 | 4.0 ± 0.1 | n.d. | 0.2 ± 0.1*** | 0.3 ± 0.1*** | n.d. | 1.5 ± 0.2 | 1.2 ± 0.1 | 8.4 ± 0.7 | 2.1 ± 0.2** | 23.2 ± 1.2 |
| Mayan Gold crtZW Or | n.d. | n.d. | 9.2 ± 1.0 | 7.1 ± 0.9 | n.d. | 0.2 ± 0.1*** | 0.5 ± 0.1*** | n.d. | 2.4 ± 0.6 | 2.2 ± 0.3 | 16.3 ± 1.8 | 2.8 ± 0.2* | 40.7 ± 3.7** |

Values shown are for saponified mature stage extracts (μg/g DW). Values are the mean of three replicates ± standard error of mean. Statistical analysis of the differences in transgenic lines with respect to wild type was done using Students *t*-test and significance of differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: Wt, wild type; Neo, neoxanthin; Vio, violaxanthin; Keto lut, keto lutein; Asta, astaxanthin; Ant, antheraxanthin; β-crypto, β-cryptoxanthin; Lut, lutein; Zea, zeaxanthin; Car other, carotenoid other; β-car, β-carotene; Total, total tuber carotenoid content.

**Table 1B**
Tuber carotenoid content (μg/g DW) of saponified 01H15.57 and Mayan Gold derived transgenic lines.

| | Neo | Vio | Keto lut | Asta | Ant | Lut | Zea | β-crypto | Keto other | Car other | β-car | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01H15.57 | 0.1 ± 0.0 | 0.5 ± 0.2 | n.d. | n.d. | 3.5 ± 0.3 | 3.0 ± 0.4 | 43.6 ± 1.0 | n.d. | n.d. | 1.6 ± 0.2 | 1.4 ± 0.2 | 53.9 ± 2.0 |
| 01H15.57 Or | 0.4 ± 0.1* | 2.4 ± 0.2** | n.d. | n.d. | 5.5 ± 0.2 | 7.2 ± 1.5 | 66.7 ± 2.4*** | n.d. | n.d. | 3.6 ± 0.5 | 2.4 ± 0.2 | 88.3 ± 3.2*** |
| 01H15.57 crtZW | 0.2 ± 0.1 | 0.7 ± 0.1 | n.d. | 44.1 ± 1.7 | n.d. | 0.2 ± 0.1** | 2.4 ± 0.2*** | n.d. | 5.0 ± 0.8 | 3.3 ± 0.8 | 1.7 ± 0.3 | 57.4 ± 3.4 |
| 01H15.57 crtZW Or A | 1.5 ± 0.2** | 1.2 ± 0.2 | n.d. | 76.2 ± 9.4 | n.d. | 0.4 ± 0.1** | 2.6 ± 0.3*** | n.d. | 11.5 ± 0.6 | 2.8 ± 0.3* | 2.6 ± 0.6 | 98.5 ± 9.9** |
| 01H15.57 crtZW Or B | 0.3 ± 0.1 | 1.0 ± 0.1 | n.d. | 67.1 ± 2.4 | n.d. | 0.3 ± 0.1** | 4.7 ± 0.9*** | n.d. | 8.3 ± 1.8 | 5.0 ± 0.3** | 3.1 ± 0.3** | 89.7 ± 4.9** |
| 01H15.57 crtZW Or D | 0.2 ± 0.1 | 0.7 ± 0.1 | n.d. | 77.1 ± 4.0 | n.d. | 0.3 ± 0.1** | 3.6 ± 0.5*** | n.d. | 6.0 ± 0.6 | 3.3 ± 0.5 | 2.3 ± 0.2* | 93.5 ± 3.5*** |
| Mayan Gold | 0.9 ± 0.1 | 4.6 ± 0.3 | n.d. | n.d. | 3.5 ± 0.4 | 8.7 ± 0.4 | 4.0 ± 0.1 | n.d. | n.d. | 0.6 ± 0.1 | 0.7 ± 0.1 | 23.1 ± 0.8 |
| Mayan Gold Or | 2.5 ± 0.5* | 8.3 ± 1.5 | n.d. | n.d. | 6.3 ± 1.0 | 10.0 ± 1.4 | 5.5 ± 0.2 | n.d. | n.d. | 1.8 ± 0.1** | 1.3 ± 0.1** | 35.7 ± 4.1*** |
| Mayan Gold crtZW | 0.4 ± 0.1* | 1.7 ± 0.6* | 10.9 ± 1.0 | 6.3 ± 0.7 | n.d. | 0.2 ± 0.1*** | 0.4 ± 0.1 | n.d. | 2.6 ± 0.1 | 0.4 ± 0.1 | 0.8 ± 0.3 | 23.2 ± 1.2 |
| Mayan Gold crtZW Or | 0.8 ± 0.1 | 2.7 ± 0.5* | 18.6 ± 1.6 | 11.3 ± 1.4 | n.d. | 0.3 ± 0.1*** | 0.8 ± 0.2 | n.d. | 3.7 ± 0.5 | 0.7 ± 0.1 | 1.7 ± 0.3* | 40.7 ± 3.7** |

Values shown are for saponified mature stage extracts (μg/g DW). Values are the mean of three replicates ± standard error of mean. Statistical analysis of the differences in transgenic lines with respect to wild type was done using Students *t*-test and significance of differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: Wt, wild type; Neo, neoxanthin; Vio, violaxanthin; Keto lut, keto lutein; Asta, astaxanthin; Ant, antheraxanthin; β-crypto, β-cryptoxanthin; Lut, lutein; Zea, zeaxanthin; Car other, carotenoid other; β-car, β-carotene; Total, total tuber carotenoid content.

astaxanthin. In the 01H15.57 *crtZW* line, again total tuber carotenoid levels were not significantly elevated and over 80% of the carotenoid was present as ketocarotenoid. In this case however ca. 77% of the carotenoid was present as astaxanthin (Tables 1A and 1B).

### 3.2. Effect of the Or gene on tuber carotenoid accumulation when expressed in the Phureja backgrounds

Previous studies have demonstrated that expression of the *Or* transgene in potato tubers leads to increased carotenoid levels [38–40]. These studies have been carried out in the low tuber carotenoid cultivar Desiree, where transgenic expression of *Or* results in up to 6-fold higher tuber carotenoid levels in fresh tubers with a further increase (up to 100 $\mu$g/g DW) after a period of cold storage [40]. In the current study, the effect of *Or* expression in the higher tuber carotenoid genotypes (Mayan Gold and 01H15.57) was investigated. Independent transgenic lines were generated using the same construct as in the Desiree study [39], in which transgene expression is driven by the tuber-specific granule bound starch synthase (GBSS) promoter. Independent transgenic lines (6–12 per parental genotype) were screened for total tuber carotenoid content and tubers from the lines with the highest carotenoid content were analysed in detail by HPLC (Tables 1A and 1B). Significantly elevated levels of tuber carotenoid were produced in the *Or* transgenic lines produced from both genotypes (63% increase in the 01H15.57 transgenics, 55% for the Mayan Gold). The tuber carotenoid profile of the *Or* transgenics however was substantially different to that published for the Desiree *Or* transgenics [39], with most of the enhanced carotenoid content due to increases in zeaxanthin in the 01H15.57 lines and violaxanthin, antheraxanthin and lutein in the Mayan Gold transgenics with only small changes in $\beta$-carotene content measured in both backgrounds.

### 3.3. Enhanced astaxanthin accumulation in transgenics co-expressing Or and crtZW

The *Or* gene is clearly a useful tool to enhance tuber carotenoids and a further aim of the current study was to investigate the effects of stacking the *Or* and *crtZW* genes in transgenic potato. The selected *Or* lines in the Mayan Gold and 01H15 backgrounds (the *Or* lines with the highest level of tuber carotenoid) were re-transformed with the *crtZW* construct, primary transgenics were screened for tuber carotenoid content and those with the highest levels (88.3 $\mu$g/g DW, analysed by HPLC (Tables 1A and 1B). Three independent *Or/crtZW* transgenic lines in the 01H15.57 background exhibited very similar carotenoid profiles. Compared with tubers from *Or* lines the *Or/crtZW* lines, had a small increase in total carotenoid content (98.5 compared with 88.3 $\mu$g/g DW) although not statistically significant. Most notably in the 01H15.57 background, the *Or/crtZW* transgenics accumulated relatively high levels of astaxanthin (up to 77 $\mu$g/g DW, 82% of the total carotenoid in the saponified extracts). Co-expression of all three transgenes in tubers of the transgenic lines was confirmed by qRT-PCR analysis (Fig. 2).

### 3.4. Accumulation of ketocarotenoids in leaves and stems

The carotenoid levels in leaves and stems were investigated in the parental 01H15.57 clone, Mayan Gold and the transgenic lines derived from them (Tables 2 and 3). For both genotypes, total carotenoid content of leaves and stems remained unchanged on transformation compared with the wild type. As the *Or* transgene expression was driven by the tuber-specific GBSS promoter, no change in the *Or* lines was to be expected. In the 01H15.57-derived transgenic lines that expressed the *crtZW* transgene (driven by the

**Table 2**
Leaf carotenoid content ($\mu$g/g DW) of 01H15.57 and Mayan Gold derived transgenic lines.

| | Neo | Vio | keto lutein | Asta | Keto other | Ant | Lut | Zea | β-Car | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 01H15.57 | 131.0 ± 5.1 | 221.5 ± 12.7 | n.d. | n.d. | n.d. | 19.6 ± 4.1 | 845.4 ± 78.3 | 22.9 ± 4.8 | 746.2 ± 97.0 | 1986.7 ± 162.7 |
| 01H15.57 Or | 154.3 ± 23.8 | 188.8 ± 20.7 | n.d. | n.d. | n.d. | 30.8 ± 2.7 | 943.4 ± 123.3 | 24.5 ± 0.9 | 681.6 ± 36.9 | 2023.6 ± 175.5 |
| 01H15.57 crtZW | 57.8 ± 9.1** | 103.4 ± 14.5** | 324.3 ± 44.0 | 53.7 ± 8.8 | 167.5 ± 17.1 | 50.4 ± 13.7 | 660.1 ± 34.1 | 40.5 ± 6.2 | 335.6 ± 13.3* | 1793.5 ± 128.1 |
| 01H15.57 crtZW Or A | 72.1 ± 12.8* | 125.5 ± 20.7* | 342.9 ± 47.8 | 46.8 ± 2.4 | 178.4 ± 9.4 | 57.3 ± 11.9* | 822.3 ± 131.6 | 47.7 ± 8.0 | 404.7 ± 10.2* | 2097.7 ± 234.7 |
| 01H15.57 crtZW Or B | 58.6 ± 3.0*** | 71.8 ± 7.4*** | 347.6 ± 30.2 | 59.5 ± 9.2 | 190.7 ± 22.7 | 73.4 ± 21.0* | 721.6 ± 55.1 | 41.2 ± 10.1 | 320.2 ± 21.1* | 1884.7 ± 161.2 |
| 01H15.57 crtZW Or D | 69.8 ± 3.3*** | 117.8 ± 6.3** | 328.8 ± 11.8 | 36.6 ± 2.6 | 133.2 ± 6.2 | 44.7 ± 4.8 | 764.0 ± 87.0 | 64.1 ± 11.8* | 369.0 ± 53.1* | 1928.2 ± 126.5 |
| Mayan Gold | 103.9 ± 8.3 | 157.9 ± 26.9 | n.d. | n.d. | n.d. | 55.1 ± 3.0 | 770.4 ± 15.6 | 12.3 ± 2.8 | 618.5 ± 15.7 | 1718.1 ± 47.4 |
| Mayan Gold Or | 79.9 ± 7.7 | 136.2 ± 8.8 | n.d. | n.d. | n.d. | 72.2 ± 4.9* | 697.1 ± 72.3 | 13.2 ± 1.8 | 634.5 ± 55.8 | 1633.0 ± 109.1 |
| Mayan Gold crtZW | 76.9 ± 16.0 | 53.4 ± 3.1* | 126.3 ± 7.4 | 74.4 ± 9.7 | 60.4 ± 10.1 | 51.4 ± 3.7 | 746.2 ± 45.1 | 13.6 ± 2.0 | 609.8 ± 40.5 | 1818.3 ± 89.1 |
| Mayan Gold crtZW Or | 51.3 ± 2.6** | 50.3 ± 6.0* | 138.3 ± 11.5 | 86.7 ± 7.6 | 81.6 ± 6.9 | 90.2 ± 10.5* | 660.6 ± 16.1** | 12.1 ± 1.3 | 584.1 ± 31.3 | 1663.2 ± 61.8 |

Values shown are the mean of three replicates ± standard error of mean. Statistical analysis of the differences in transgenic lines with respect to wild type was done using Students *t*-test and significance of differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: Wt, wild type; Neo, neoxanthin; Vio, violaxanthin; Keto lut, keto lutein; Asta, astaxanthin; Ant, antheraxanthin; β-crypto, β-cryptoxanthin; Lut, lutein; Zea, zeaxanthin; Car other, carotenoid other; β-car, β-carotene; Total, total tuber carotenoid content.

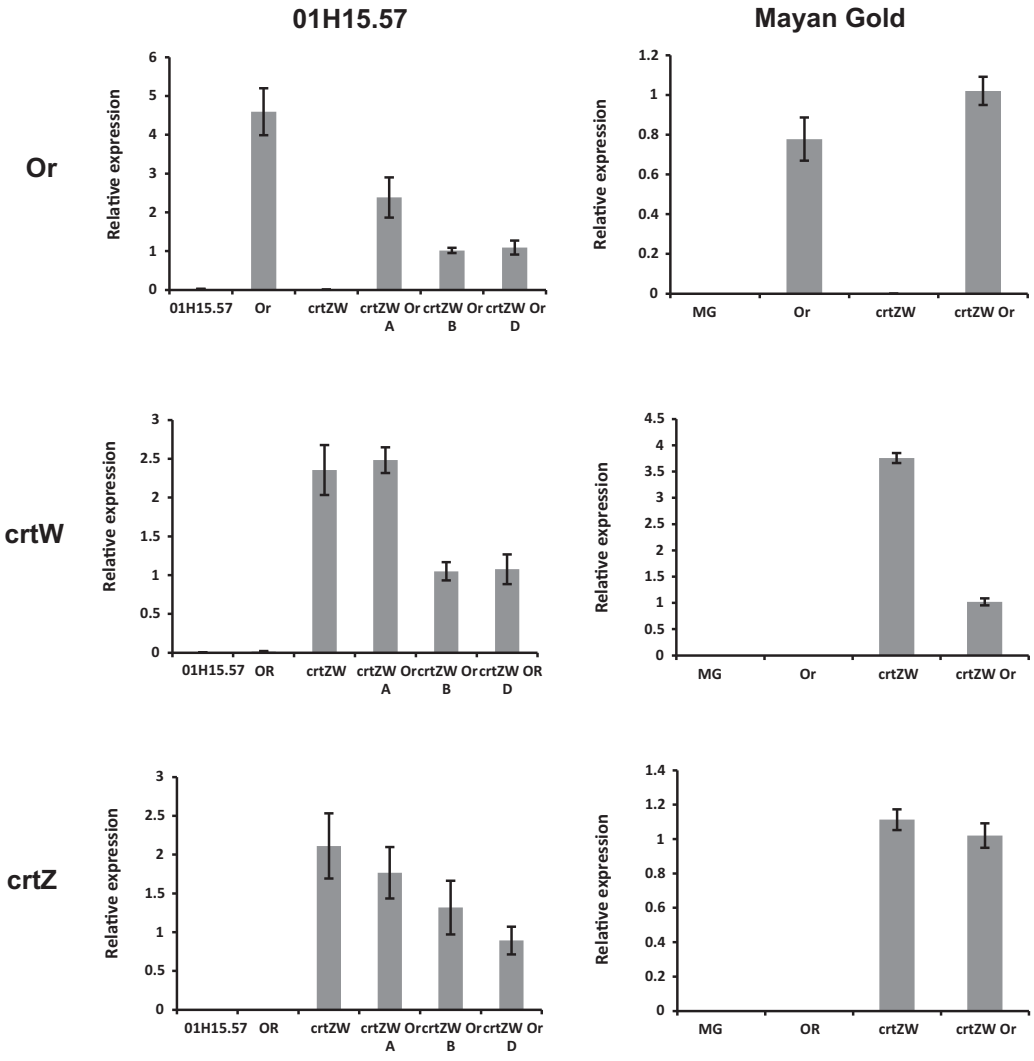


**Fig. 2.** *Or*, *crtZ* and *crtW* transgene expression levels in 01H15.57 and Mayan Gold derived transgenic lines. Comparison of gene expression levels of the *Or*, *crtW* and *crtZ* transgenes in the 01H15.57 and Mayan Gold derived transgenic lines as determined by q RT-PCR. Samples used were from summer grown mature stage tubers.

constitutive 35S CaMV promoter), ketocarotenoids accumulated to a similar extent in each of the four transgenic lines analysed. In leaves, ketolutein and astaxanthin were detected with levels reaching ca. 350 $\mu$g/g DW and 60 $\mu$g/g DW respectively. The combined ketocarotenoid content represented 32% of the total leaf carotenoid (Table 2). In the Mayan Gold background, the *crtZW* expressing lines also accumulated ketocarotenoid although levels were lower than in the 01H15.57 background, with a higher proportion of astaxanthin than ketolutein (Table 2).

### 3.5. Effect of cold storage of tuber carotenoid levels

Previously a dramatic increase in tuber carotenoid content in *Or* transgenic lines on cold storage has been reported [40]. Over 5 months of storage at 5 °C, tuber carotenoid content increased ca. 10-fold, with most of the increase due to accumulation of $\beta$-carotene. The effect of cold storage on tuber carotenoid content was investigated in the genotypes used in this study. For the 01H15.57 genotype and transgenics derived from it, there was no significant change in total carotenoid content over six months of cold storage (Fig. 3A) and relatively little change in constituent carotenoids (Supporting Information Table S2). For Mayan Gold and transgenics derived from it, there were increases in the total carotenoid content ranging from ca. 50% to 100% over the six month storage period (Fig. 3B). Increases in lines expressing *crtZW* and parental Mayan Gold were of a similar magnitude to lines expressing the *Or* transgene. Interestingly, none of these genotypes accumulated appreciable amounts of $\beta$-carotene but for the *crtZW* expressing transgenic lines, most of increase in carotenoid content was accounted for by increases in ketocarotenoid (Supporting Information Table S2). In general for all *Or* expressing transgenic lines, *Or* transcript level decreased over the six month storage period by up to 4-fold compared with the levels at harvest, although in all cases the expression level was easily detectable (Fig. 4).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.014.

### 3.6. Effect of environment on tuber carotenoid content

Recently the effects of environment on tuber carotenoid accumulation have been reported [47] with significantly different levels of tuber carotenoid measured in the same cultivar grown under diverse environments. The 01H15.57 genotypes (wild type and the *Or* and *crtZW* transgenics) were grown under glasshouse conditions in summer and winter seasons as defined in Section 2. These genotypes were also grown under controlled conditions in growth cabinets. Whereas the glasshouse temperature was carefully

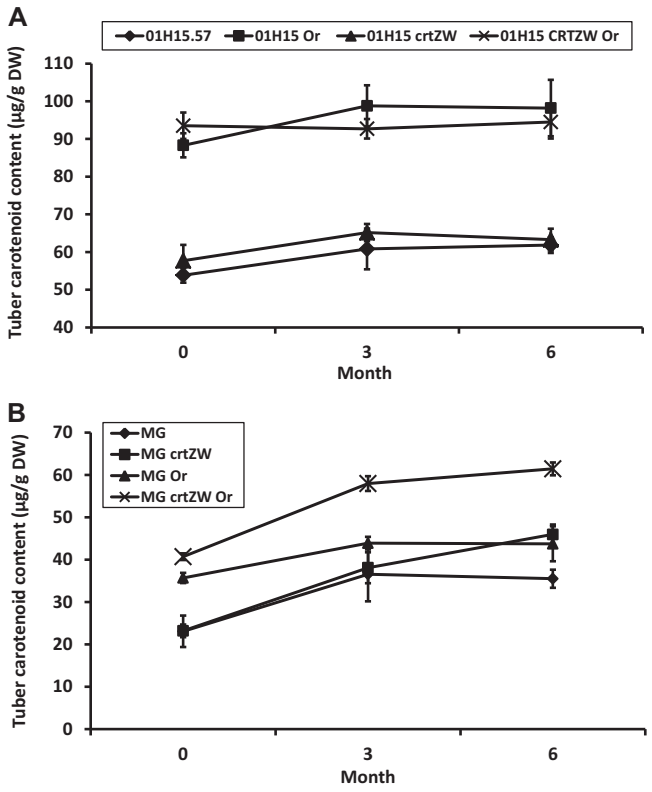


**Fig. 3.** Tuber carotenoid contents of Mayan Gold and 01H15.57 derived transgenic lines. Comparison of tuber carotenoid contents of (A) Mayan Gold and (B) 01H15.57 derived transgenic lines. Values shown are the means of three biological replicates. Error bars indicate the standard error.

regulated, day length and light quality were not tightly controlled. Comparing the winter and summer grown tubers, total tuber carotenoid content was not significantly different for any of the genotypes. However there were large changes in tuber carotenoid composition, with the 01H15.57 genotype showing a significant decrease in zeaxanthin content and an enhanced level of antheraxanthin and violaxanthin in winter (Table 4). These changes were also the most prominent effect on carotenoid profile in the 01H15.57 *Or* transgenic tubers. 01H15.57 *crtZW* lines showed an enhanced level of violaxanthin and antheraxanthin and reduced ketocarotenoid levels under the winter growth conditions. The conditions in the growth cabinet were designed to replicate the light intensity and day length conditions of the winter season. Tubers from plants grown under these conditions also contained reduced zeaxanthin in the 01H15 Wt and *Or* lines and enhanced antheraxanthin levels in the *Or* and *crtZW* transgenics compared with summer grown tubers (Table 4).

### 3.7. Changes in transcript levels associated with environmental and transgene induced changes in tuber carotenoids

In order to gain further insights into the transcriptional networks associated with the environmentally and transgenically perturbed tuber carotenoid levels observed in this study, microarray experiments were designed to identify genes that were differentially expressed in tubers expressing *Or*, *crtZW* and under different environmental conditions.

Between the 01H15.57 genotypes grown in summer and winter, 1278 genes were differentially expressed in tuber tissue using the statistical cut-off levels described in Section 2 (Supporting Information Table S3). For *Or* and *crtZW* expressing transgenic lines in the 01H15.57 background, 948 and 1573 genes were differentially expressed compared with the parental genotype respectively (Supporting Information Tables S4 and S5)

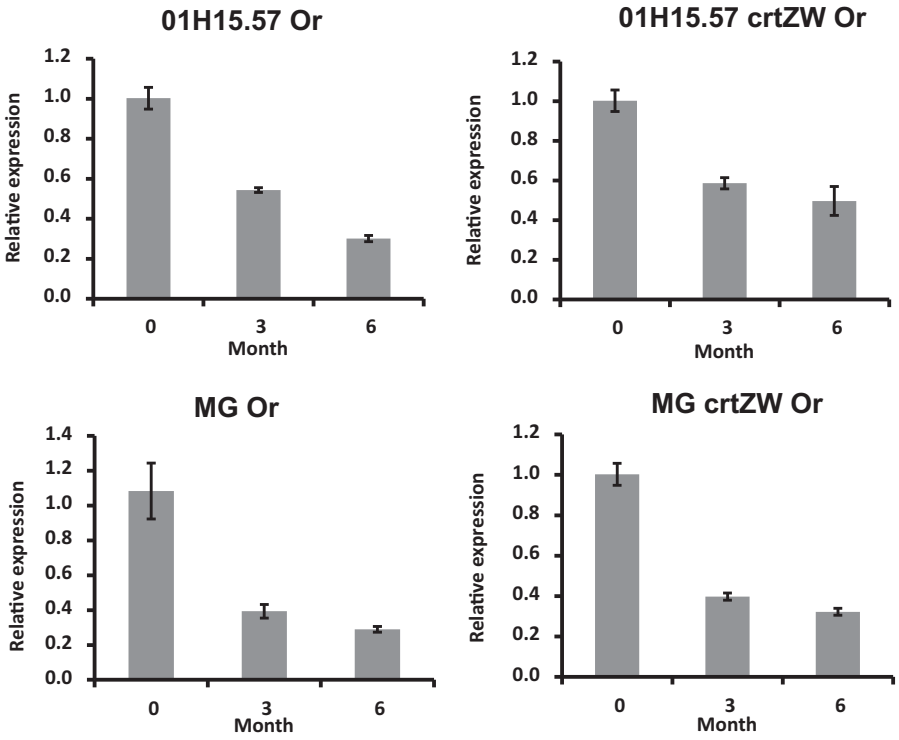


**Fig. 4.** *Or* gene expression in 01H15.57 and Mayan Gold derived transgenic lines during long term cold storage. Comparison of *Or* gene expression levels in 01H15.57 *Or*, 01H15.57 *crtZW Or*, Mayan Gold *Or* and Mayan Gold *crtZW Or* lines during long term cold storage as determined by qRT-PCR. Samples used were from summer grown mature stage tubers.

**Table 3**
Stem carotenoid content (μg/g DW) of 01H15.57 and Mayan Gold derived transgenic lines.

| | Neo | Vio | Keto lut | Asta | Keto other | Ant | Lut | Zea | β-car | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| 01H15.57 | 5.8 ± 0.3 | 6.4 ± 0.5 | n.d. | n.d. | n.d. | 10.8 ± 0.4 | 64.1 ± 2.7 | 50.0 ± 2.1 | 32.5 ± 1.1 | 169.5 ± 5.4 |
| 01H15.57 Or | 5.1 ± 0.4 | 6.4 ± 0.2 | n.d. | n.d. | n.d. | 10.5 ± 1.1 | 59.0 ± 2.4 | 59.9 ± 4.2 | 28.5 ± 1.9 | 169.3 ± 8.1 |
| 01H15.57 crtZW | 2.8 ± 0.2 | 4.7 ± 0.3 | 14.7 ± 0.7 | 26.0 ± 1.1 | 35.1 ± 1.9 | 5.9 ± 0.5 | 22.6 ± 0.2 | 7.4 ± 0.2 | 32.1 ± 2.5 | 151.5 ± 5.0 |
| 01H15.57 crtZW Or A | 2.7 ± 0.2 | 7.0 ± 0.1 | 17.3 ± 0.9 | 28.8 ± 0.1 | 40.5 ± 2.0 | 7.7 ± 0.5* | 24.4 ± 1.7*** | 9.5 ± 1.0*** | 32.8 ± 0.3 | 170.7 ± 4.9 |
| 01H15.57 crtZW Or B | 3.0 ± 0.2 | 5.7 ± 0.2 | 15.2 ± 0.1 | 29.2 ± 0.7 | 43.3 ± 2.0 | 7.3 ± 0.4* | 25.7 ± 0.6** | 9.3 ± 0.9*** | 35.8 ± 2.5 | 174.5 ± 4.6 |
| 01H15.57 crtZW Or D | 3.3 ± 0.3 | 4.0 ± 0.1 | 14.4 ± 1.2 | 27.9 ± 2.2 | 39.2 ± 1.9 | 7.0 ± 0.7* | 26.0 ± 2.4** | 11.1 ± 1.0*** | 31.8 ± 1.7 | 164.6 ± 8.9 |
| Mayan Gold | 2.3 ± 0.2 | 5.8 ± 1.5 | n.d. | n.d. | n.d. | 14.8 ± 0.8 | 84.9 ± 6.4 | 15.0 ± 2.3 | 46.6 ± 8.1 | 169.5 ± 9.4 |
| Mayan Gold Or | 2.9 ± 0.5 | 6.9 ± 1.1 | n.d. | n.d. | n.d. | 14.4 ± 0.5 | 79.9 ± 6.4 | 13.5 ± 2.4 | 47.2 ± 1.9 | 164.9 ± 6.6 |
| Mayan Gold crtZW | 1.0 ± 0.2 | 1.4 ± 0.2 | 18.1 ± 0.1 | 20.7 ± 2.6 | 19.2 ± 3.6 | 5.0 ± 0.6*** | 55.3 ± 1.7* | 8.2 ± 1.3 | 34.4 ± 0.7 | 163.4 ± 4.0 |
| Mayan Gold crtZW Or | 2.4 ± 0.2 | 2.8 ± 0.2 | 15.2 ± 4.6 | 22.5 ± 1.4 | 22.6 ± 5.7 | 7.1 ± 0.7** | 46.7 ± 3.9** | 10.4 ± 1.1 | 31.5 ± 1.1 | 161.2 ± 4.5 |

Values shown are the mean of three replicates ± standard error of mean. Statistical analysis of the differences in transgenic lines with respect to wild type was done using Students *t*-test and significance of differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: Wt, wild type; Neo, neoxanthin; Vio, violaxanthin; Keto lut, keto lutein; Asta, astaxanthin; Ant, antheraxanthin; β-crypto, β-cryptoxanthin; Lut, lutein; Zea, zeaxanthin; Car other, carotenoid other; β-car, β-carotene; Total, total tuber carotenoid content.

**Table 4**
Tuber carotenoid content (μg/g DW) of 01H15.57 and Mayan Gold Wt and derived transgenic lines grown under different environmental conditions.

| | Neo | Vio | Asta | Ant | Lut | Zea | β-crypto | Keto other | Car other | Keto ester | Car ester | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 01H15.57 Summer | 0.2 ± 0.1 | 0.8 ± 0.3 | n.d. | 1.9 ± 0.3 | 2.9 ± 0.7 | 41.6 ± 0.6 | 1.0± 0.3 | n.d. | 2.2 ± 0.8 | n.d. | 3.3 ± 0.3 | 53.9 ± 2.0 |
| 01H15.57 Winter | 0.2 ± 0.1 | 4.4 ± 0.5* | n.d. | 16.1 ± 0.9*** | 3.1 ± 0.4 | 24.4 ± 1.6*** | 0.5 ± 0.3 | n.d. | 0.6 ± 0.2 | n.d. | 6.3 ± 1.7** | 54.4 ± 2.7 |
| 01H15.57 Cabinet | n.d. | 0.5 ± 0.1 | n.d. | 7.6 ± 0.9*** | 1.1 ± 0.1* | 27.9 ± 1.1*** | n.d. | n.d. | 0.7 ± 0.1 | n.d. | 4.4 ± 0.2* | 42.2 ± 2.2** |
| 01H15.57 Or Summer | 0.4 ± 0.1 | 1.3 ± 0.3 | n.d. | 6.3 ± 0.8 | 7.8 ± 1.6 | 67.6 ± 0.7 | n.d. | n.d. | 3.1 ± 0.8 | n.d. | 1.6 ± 0.2 | 88.3 ± 3.2 |
| 01H15.57 Or Winter | 0.2 ± 0.1 | 2.6 ± 0.3* | n.d. | 22.6 ± 1.2*** | 8.5 ± 0.5 | 51.1 ± 2.6** | n.d. | n.d. | 1.6 ± 0.3 | n.d. | 4.7 ± 0.9* | 91.2 ± 2.2 |
| 01H15.57 Or Cabinet | 0.1 ± 0.1 | 2.6 ± 0.3* | n.d. | 19.1 ± 0.9*** | 5.3 ± 0.4 | 53.3 ± 2.4** | n.d. | n.d. | 1.9 ± 0.3 | n.d. | 4.9 ± 0.5** | 87.2 ± 3.5 |
| 01H15.57 crtZW Or Summer | n.d. | n.d. | 53.2 ± 1.2 | n.d. | n.d. | 6.5 ± 0.9 | 0.1 ± 0.0 | 5.6 ± 0.6 | 2.1 ± 0.6 | 22.0 ± 2.5 | 4.1 ± 0.8 | 93.5 ± 3.5 |
| 01H15.57 crtZW Or Winter | 1.0 ± 0.1 | 9.6 ± 0.9 | 29.2 ± 0.5*** | 6.0 ± 0.6 | n.d. | 10.0 ± 0.7* | n.d. | 10.5 ± 0.8** | 1.2 ± 0.1 | 12.8 ± 1.0* | 5.4 ± 0.8 | 85.8 ± 3.2 |
| 01H15.57 crtZW Or Cabinet | 1.0 ± 0.1 | 6.3 ± 0.1 | 30.0 ± 2.9** | 5.9 ± 0.3 | n.d. | 7.4 ± 1.0 | n.d. | 6.9 ± 0.2 | 1.0 ± 0.1 | 15.0 ± 0.9 | 4.9 ± 0.4 | 78.4 ± 3.1* |

Values shown are in μg/g DW and are the mean of three replicates ± standard error of mean. Statistical analysis of the differences in transgenic lines with respect to wild type was done using Students *t*-test and significance of differences are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: Wt, wild type; Neo, neoxanthin; Vio, violaxanthin; Keto lut, keto lutein; Asta, astaxanthin; Ant, antheraxanthin; β-crypto, β-cryptoxanthin; Lut, lutein; Zea, zeaxanthin; Car other, carotenoid other; β-car, β-carotene; Total, total tuber carotenoid content.

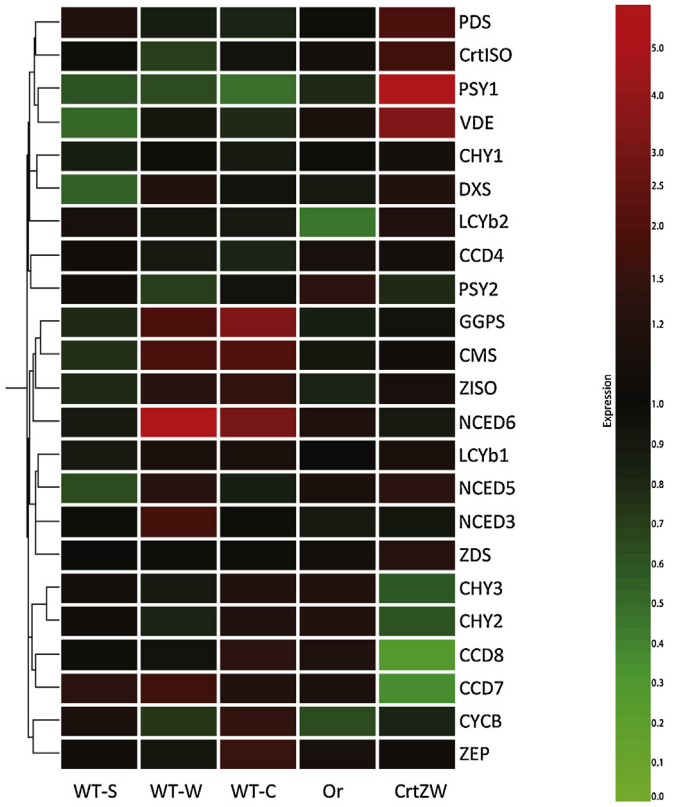


**Fig. 5.** Carotenoid gene tree heat map. Comparison of carotenoid biosynthetic pathway gene expression profiles between 01H15 wild type (WT) and *crtZW* (summer) and *Or* (summer) transgenic tubers. Heat map was generated using GeneSpring GX software as described in Section 2. S, summer; W, winter; C, cabinet.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.014.

As the focus of this study is on carotenoid metabolism a set of probes representing carotenoid biosynthetic genes was identified based on the annotation of the potato genome ([48], Supporting Information Table S6). Using data from these probes, the expression patterns of the carotenoid associated genes in the different transgenic lines and parental genotype is shown in Fig. 5. More detailed analysis of the expression levels of each gene including statistical significance data is shown in Supporting Information Fig. S1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.014.

Comparing tuber transcript profiles from winter and summer grown plants, a major re-structuring of the carotenoid biosynthetic pathway associated transcriptome is evident. In particular, several genes encoding carotenoid cleavage dioxygenases and violaxanthin de-epoxidase are expressed at higher levels in winter grown material than in summer. Interestingly in growth cabinet produced samples, the expression levels of these genes are generally intermediate between the winter and summer samples, as were the tuber carotenoid contents described in Table 4. For *Or* expressing transgenic tubers compared with the 01H15.57 parental material grown under the same environment (summer) there were significantly lower levels of lycopene $\beta$-cyclase-specific transcript, whereas violaxanthin de-epoxidase transcript levels were significantly higher than in the parental genotype. In tubers from *crtZW* expressing transgenics, significantly higher levels of phytoene synthase 1 and violaxanthin de-epoxidase transcript levels were measured whereas the levels of several other carotenoid associated transcripts were significantly lower (for example $\beta$-carotene hydroxylase).

The three gene lists for transcripts differentially expressed between summer and winter, *Or* associated transcriptional changes and those associated with *crtZW* expression were further analysed to investigate functional categories of transcript that changed in the various environmental conditions or transgenic lines (Supplementary Information Figs. S2–S4). PageMan analysis using the Wilcoxon test parameter revealed that the transcriptional changes observed were complex and likely to impact across wide areas of metabolism and development. For example, comparison of summer and winter grown 01H15.57 wild type revealed genes involved in cell wall, lipid, amino acid and hormone metabolism were down-regulated whereas genes involved in abiotic stress, redox processes, regulation of transcription and signalling were up-regulated (Supplementary Information Fig. S3). Interestingly, there is a major up-regulation in the isoprenoids branch of the secondary metabolism BINS and subBINs in the *crtZW* associated gene list whereas no up-regulation was observed for the *Or* associated differentially expressed genes compared with the summer grown wild type control (Supplementary Information Figs. S4 and S5). Input data used to generate the PageMan figures is provided in Supporting Information Tables S3–S5.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.01.014.

## 4. Discussion

### 4.1. Co-expression of crtW and crtZ from Brevundimonas sp. SD212 in Phureja genotypes

Recently gene combinations have been tested in *E. coli* in order to determine efficient approaches for converting $\beta$-carotene to astaxanthin [26,49]. The co-expression of *crtW* and *crtZ* from *Brevundimonas* sp. *SD212* was more effective than expression of various *crtW* (or *crtO*) and *crtZ* (or *CYP175A*) genes from *Paracoccus* sp. *N81106* and *Synechocystis* sp. *PCC*6803. Subsequently, transplastomic co-expression of the *Brevundimonas* sp. *SD212 crtW* and *crtZ* genes in tobacco, resulted in the accumulation of extremely high levels of astaxanthin in leaves (greater than 0.5% dry weight [18]). In this study we aimed to investigate whether the *Brevundimonas crtZW* construct gave an increased level of astaxanthin when expressed in potato tubers. Previously we have produced transgenic lines expressing the *bkt1* gene from *H. pluvialis* in Mayan Gold [36]. The levels of ketocarotenoid in these *bkt1* expressing tubers were similar to the Mayan Gold line with the highest ketocarotenoid content that we generated in this study using the *Brevundimonas* genes, implying that in the Mayan Gold background other factors may limit ketocarotenoid accumulation.

### 4.2. Expression of the cauliflower Or gene in the Phureja background

Previously, the effects of expressing the cauliflower *Or* gene in potato have been described in detail [39,40]. Using the low tuber carotenoid cultivar Desiree as the parental genotype, expression of *Or* corresponded with an increase in total carotenoid content from ca. 5.5 $\mu$g/g DW to up to 30 $\mu$g/g DW. The increase was due to enhanced levels of several carotenoids, most prominently $\beta$-carotene. In the current study an increase in total carotenoid was also observed on *Or* expression, however in this case the increase was much more modest. In both genotypes a maximal total tuber carotenoid content increase of ca. 60% was observed. Furthermore the carotenoid profile of the *Or* transgenics was quite distinct in

the Phureja genotypes used in this study compared with that previously seen in Desiree, with increases in zeaxanthin, antheraxanthin, violaxanthin and lutein accounting for most of the increase. The effects of low temperature storage were also markedly different for the Phureja-based transgenic lines compared with Desiree. No significant increase in tuber carotenoid content was observed in any of the *Or* expressing transgenic lines in the 01H15.57 background. For Mayan Gold, carotenoid increases on cold storage were observed, however the extent of these increases were similar for wild type and all transgenic lines investigated and were maximally 2-fold higher after storage. The different effects of the *Or* gene in the higher tuber carotenoid backgrounds reported here may reflect different plastid structures in the different genotypes together with different expression levels of carotenoid biosynthetic pathway genes. The contrasting results reflect that there are still significant gaps in our understanding of how carotenoid pathways are regulated despite recent advances [50]. The higher expression levels of $\beta$-carotene hydroxylase and lower expression of *CCD4* in Phureja genotypes compared with Tuberosum [41] may well be important in this respect, with genes underlying further QTLs for tuber carotenoid content also likely to have an impact [42].

The effects of *Or* co-expression with other carotenoid pathway genes was also tested in this study. In both the Mayan Gold and 01H15 genotypes co-expression of *Or* with the *Brevundimonas* sp. *SD212 crtZW* genes had only a minor effect on total tuber carotenoid content compared with the expression of *crtZW* alone. Interestingly for both Mayan Gold and 01H15.57 co-expression of *Or* and *crtZW* resulted in higher levels of tuber ketocarotenoid, so that in the *crtZW/Or* transgenic lines astaxanthin levels reached 77 $\mu$g g DW. Thus although *Or* expression in the Phureja background did not result in such dramatic increases as observed in Desiree, these data demonstrate that *Or* co-expression with other carotenoid pathway genes is a useful tool for manipulating levels of specific carotenoids.

### 4.3. Effects of environment

Major environmental effects on tuber carotenoid accumulation were observed for the 01H15.57 genotype and the transgenic lines derived from it. For all genotypes, there was an increase in antheraxanthin content, a decrease in zeaxanthin but little overall change in total carotenoid content in winter grown plants compared with summer. Interestingly changes in tuber carotenoid composition but not overall carotenoid content dependent on environment have been observed [47]. In the present study, the increase in violaxanthin de-epoxidase transcript level observed in winter and cabinet grown tubers could be envisaged to impact directly on the levels of antheraxanthin and zeaxanthin. The decreased level of zeaxanthin in the winter grown tubers may account for the decreased astaxanthin levels seen in tubers from winter grown material and further implicates zeaxanthin as the precursor for astaxanthin biosynthesis.

The impact of season, *Or* and *CrtZW* expression on the tuber transcriptome was not confined to isoprenoid-associated transcripts. Each factor (season or transgene) had different effects on both the isoprenoid pathway transcripts and more widely. These data highlight the complexity of isoprenoid transcript networks and may be of use in future attempts to fully understand the connections between isoprenoid networks and global plant metabolism.

### 4.4. Significance of astaxanthin levels produced in transgenic potato

Previously, metabolic engineering of potato has achieved tuber levels of 13 $\mu$g/g DW astaxanthin and so the values obtained in this study (maximally 77 $\mu$g/g DW) represent a considerable improvement. The selection of appropriate parental germplasm and co-expression of the cauliflower Or gene have contributed to this improvement. However the levels of astaxanthin are still less than those achieved in carrot (90 $\mu$g/g FW [34]) and transplastomic tobacco (5440 $\mu$g/g DW [18]). Recently transgenic tomato fruit that accumulate 16 mg/g DW astaxanthin have been reported although it should be noted that these levels were only reported for primary transgenics [29]. Nevertheless, as potatoes are a staple part of the diet, the levels obtained in this study could be nutritionally significant. In fact a recommended dose of 5 mg astaxanthin per day has been proposed [51] and up to 6 mg of astaxanthin per day as algal supplements over 8 weeks was considered safe for human consumption [52]. Consumption of 55 g DW equivalent of potato (ca. 220 g FW) thus has the potential to provide the recommended 5 mg daily intake of astaxanthin.

The levels of ketocarotenoid produced in potato foliage in the transgenic lines reported in this study were also less than transplastomic tobacco. A transplastomic approach in potato may boost the levels achieved here and as potato foliage is generally destroyed by desiccation or flailing prior to tuber harvest, it may be possible to develop ketocarotenoid production in potato foliage as a co-product.

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