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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].