Research Reports

Title: Engineering of tomato for the sustainable production of ketocarotenoids and its evaluation in aquaculture feed

Marilise Nogueira[1], Eugenia M. A. Enfissi[1], Maria Martinez[2], Guillaume N. Menard[3], Lothar Driller[2], Peter Eastmond[3], Wolfgang Schuch[2], Gerhard Sandmann[4], Paul D. Fraser[1]

[1]School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom
[2]Fraunhofer Chile Research, Avenida Mariano Sánchez Fontecilla 310, Las Condes, Santiago, 7550296, Chile
[3]Plant Biology & Crop Science, Rothamsted Research, West Common, Harpenden AL5 2JQ, United Kingdom
[4]Biosynthesis Group, Molecular Biosciences, Goethe University Frankfurt, Frankfurt, Germany

Corresponding author: Paul D. Fraser, School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, United Kingdom, Tel: +44 (0)1784 443894, Fax: +44 (0)1784 414224, P.Fraser@rhul.ac.uk.

Short running title: Ketocarotenoids for aquaculture feed

Classification: Biological Sciences - Applied Biological Sciences

Ketocarotenoids are high-value pigments used commercially across multiple industrial sectors as colorants and supplements. Chemical synthesis using petrochemical derived precursors remains the production method of choice. Aquaculture is an example where ketocarotenoid supplementation of feed is necessary to achieve product viability. The biosynthesis of ketocarotenoids such as canthaxanthin, phoenicoxanthin or astaxanthin in plants is rare. In the present study, complex engineering of the carotenoid pathway has been performed to produce high-value ketocarotenoids in tomato fruit (3.0 mg/g DW). The strategy adopted involved pathway extension beyond β-carotene through the expression of the β-carotene hydroxylase (*Crt*Z) and oxyxgenase (*Crt*W) from *Brevundimonas* sp. in tomato fruit, followed by β-carotene enhancement through the introgression of a novel lycopene β-cyclase (β-*Cyc*) allele from *S. galapagense* background. Detailed biochemical analysis, carried using chromatographic, UV/VIS and Mass Spectrometry approaches identified the predominant carotenoid as fatty acid (C14:0 and C16:0) esters of phoenicoxanthin, present in the *S* stereoisomer configuration. Under a field-like environment with low resource input, scalability was shown with the potential to deliver 23 kg of ketocarotenoid/hectare. To illustrate the potential of this GRAS (Generally Recognized as Safe) material with minimal, low-energy bioprocessing, two independent aquaculture trials were performed. The plant-based feeds developed were more efficient than the synthetic feed to color trout flesh (up to 2-fold increase in the retention of the main ketocarotenoids in the fish fillets). This achievement has the potential to create a new paradigm in the renewable production of economically competitive feed additives for the aquaculture industry and beyond.

Keywords: Carotenoids, genetic intervention, tomato, aquaculture.

Significance Statement

Ketocarotenoids are high-value pigments used in the food and feed industry to confer color. Aquaculture is a good example where the addition of carotenoids to the feed is essential for the coloration of trout or salmon flesh and thus product viability. In this article, complex engineering has been carried out to produce a new renewable source of ketocarotenoids for use as feed additives. Production in tomato fruit has enabled the testing of this novel GRAS (Generally Recognized as Safe) material with low energy minimal bioprocessing in aquaculture trials to demonstrate production, technical and economic feasibility of the system. This achievement represents a new potential paradigm in the bioproduction of specialty and bulk chemicals without our reliance on fossil fuel derived chemical processes.

Introduction

Carotenoids represent one of the largest classes of pigments found in nature (1), however only a small number are used commercially. Ketocarotenoids such as astaxanthin or canthaxanthin are among the highest value carotenoid pigments on the market (2). These carotenoids possess a characteristic chemical keto moiety on the 4 and/or 4´ position on the β-ionone ring and can also exhibit hydroxyl groups on the 3 and 3´ position (Fig. 1). The decoration of the β-ionone ring present in cyclic carotenoids can only be performed by a limited number of enzymes. These enzymes are promiscuous and thus a myriad of intermediates/products can arise. The best characterized ketocarotenoid forming enzymes are those from marine bacteria (3).

The predominant commercial use of ketocarotenoids are as feed supplements in aquaculture and poultry industry to convey aesthetic color and nutritional benefit. Without these supplements, adequate coloration of fish flesh cannot be achieved and an

economically viable product cannot be obtained (4). In addition, the pigments also confer beneficial animal husbandry aspects that enable intensification of the industry (5). It is estimated that 15 to 25% of the total feed costs associated with aquaculture production are due to the price of the carotenoid feed supplements required.

To date, chemical synthesis has been the production method of choice. Like many such processes, it is intrinsically linked to the chemical refining of fossil fuels, using by-products as precursors. The procedures are expensive, have detrimental environmental impact and lead to a final product that contains reaction contaminants and a mixture of stereoisomers of which the non-natural form typically predominates. The consumers demand for "non-artificial" colorants has driven the industry to identify and develop new sources of carotenoids to replace chemical synthesis (6). For example, algal platforms have been used but logistical problems linked to their slow growth has inhibited broad implementation (7). Other microbial sources, such as *Xanthophyllomyces dendrorhous* (formally *Phaffia rodozyma*) and *Paracoccus carotinifaciens* (Panaferd-AX), have been and are presently used. However, on a production cost-basis a plant-based source remains the most economically viable (8, 9). The only plant capable of ketocarotenoid (astaxanthin/phoenicoxanthin) formation is *Adonis aestivalis* which is not amenable to agricultural production and contains toxic alkaloids (10, 11). Thus, a genetic engineering approach of an agricultural crop offers a viable alternative.

To date numerous proof of concept studies have been reported that have shown how complex pathway and cellular engineering can deliver dramatic changes in desirable compounds. However, very few reports exist that actually show the effectiveness of the approaches under "real-life" scenarios. In the present article, natural variation in combination with complex engineering has been performed to create a new plant-based

renewable source of ketocarotenoids. The production, technical and economic feasibility of the material has been demonstrated in comparison to existing products presently used in the aquaculture industry. The data generated has generic implications for the production of high-value specialty and bulk chemical production for renewable sources.

Results

Generation of a high ketocarotenoids tomato line (ZWRI). A stable ZWRI tomato line was generated from the genetic crossing of ZW and RI tomato. ZW lines overexpress the bacterial genes carotene hydroxylase (*Crt*Z) and carotene ketolase (*Crt*W), which are essential for the production of ketocarotenoids from endogenous plant carotenoids, primarily β-carotene (Fig. 1). Interestingly, ZW expressing lines do not produce high levels of ketocarotenoids (e.g. ZWRIØ ~70 µg/g DW, SI appendix, Table S1) due to the lack of the biosynthetic precursor β-carotene. RI are orange fruited recombinant inbred lines accumulating high levels of β-carotene. They derive from crossing the cultivated tomato *Solanum lycopersicum* with the wild *S. galapagense* accession (12, 13). Analysis of this collection identified concurrent high β-carotene fruit content with the presence of high comparative expression of the fruit ripening enhanced lycopene β cyclase (β-*Cyc*).

Two ZW events were crossed with two RI lines. The best combination in terms of ketocarotenoids levels were selected and kept as a hemizygous state for ZW genes, in order to prevent detrimental effects on plant vigor, and a homozygous state for the *S. galapagense* lycopene cyclase (β-*Cyc*) gene. The greater supply of immediate precursor (β-carotene) in ZWRI overcame biosynthetic limitations to ketocarotenoid formation

and high ketocarotenoid lines containing about 3 mg/g DW in the fruit material (40-fold increase compared to ZWRIØ) were generated (SI appendix, Table S1).

Biochemical characterization of ZWRI. In addition to the ZWRI line, the double azygous control (ZWØRIØ), which lost both the *Crt*Z and *Crt*W genes (ZW) plus the *S. galapagense* β-*Cyc* promoter (RI), and the azygous controls (ZWØRI and ZWRIØ) were studied. ZWRIØ deep red fruit were defined by a high level of lycopene (77% of total carotenoids) and a small level of ketocarotenoids (2%) (SI appendix, Table S1). ZWØRIØ were red tomatoes predominantly accumulating lycopene (68% of total carotenoids), ZWØRI tomatoes had an orange color representative of their β-carotene content (66%) and the ZWRI tomatoes had a deep red color reflecting the presence of the ketocarotenoids (87%) (SI appendix, Fig. S1 and Table S1). Chromatographic analysis of the ZWRI line revealed a complex ketocarotenoid profile (Fig. 2). The main ketocarotenoids found were phoenicoxanthin (in its free and esterified forms, ~45%) and canthaxanthin (~35%) (SI appendix, Table S1). The stereoisomer of phoenicoxanthin was determined as an *S* configuration (Fig. 2). High resolution MS/MS was used to identify phoenicoxanthin esters (C14:0 and C16:0). No statistically significant differences of total fatty acid content of the tomatoes was observed (SI appendix, Fig. S2). Astaxanthin, phoenicoxanthin and canthaxanthin will be described in this study as the coloring ketocarotenoids as they all harbour two ketone moieties, giving them the greatest spectra characteristic (λ_{max} > 470 nm) of all the ketocarotenoids and therefore the most intense red hue.

Scalability of the production platform. Following robust glasshouse production of ketocarotenoids from the ZWRI lines, production scalability was assessed. Cultivation of over 200 plants under rudimentary polytunnel containment devoid of supplementary

lighting and heating was performed over one growing cycle in the UK with an early and late season as defined by commercial growers. The composition of the ketocarotenoid profiles of the ZWRI tomatoes grown in different conditions did not alter (SI appendix, Table S1). There was a noticeable decrease of total ketocarotenoid content under polytunnel cultivation compared to greenhouse conditions. However, the greatest differences were observed between the early and late seasons of the crop. Despite this change arising from environmental effectors, the levels of ketocarotenoids in the ripe fruit still reached levels of 2.0 mg/g DW total ketocarotenoids in the late season crop (SI appendix, Table S1). Over the ZWRI tomatoes growth cycle in the polytunnel, an average yield of 12 tons per hectare could be extrapolated, which represents 23 kg of coloring ketocarotenoids (astaxanthin, phoenicoxanthin (free and esterified) and canthaxanthin) per hectare.

Trout feeding trials. The potential of the ZWRI derived tomato material as feed supplements for the coloring of rainbow trout (*Oncorhynchus mykiss*) fillets was investigated in two geographical locations (Germany and Chile), where different conditions (fresh and brackish water, respectively) were used to assess the robustness of the platform. Four to five feed treatments were tested (basic, control tomato, ZWRI tomato, ZWRI extract and commercial feeds). Their compositions are described in SI appendix, Fig. S3a and Tables S2 and S3. A standard tomato variety devoid of ketocarotenoids but containing a similar total carotenoid content compared to ZWRI tomatoes (2-3 mg/g DW) were used as control tomatoes (SI appendix, Table S1). The tomato feeds were made using freeze dried tomato powder (SI appendix, Fig. S3b). The ZWRI extract feed was based on an oily carotenoid extract of the ZWRI tomatoes (SI appendix, Fig. S3c). The synthetic BioMar supplement and carophyll pink® pigments were utilized for the commercial feed in the fresh and brackish experiment,

185 respectively. Levels of total ketocarotenoids in the ZWRI and commercial feeds were
186 targeted at 75-80 ppm and clarified after feed processing (SI appendix, Table S4). Trout
187 with a starting weight of 100 g and 40 g were fed with the different treatments for 50
188 days and up to 80 days under fresh and brackish conditions, respectively.
189 **ZWRI tomatoes color trout fillets.** Following the feeding trials, a pink stripe along
190 the lateral line of the fish (from gills to tail) was observed on the fish fed with the ZWRI
191 tomato, ZWRI extract and commercial treatments. These same fish harbored colored
192 fillets with an orange to pink hue whereas the fish fed with the basic and control tomato
193 feeds had white fillets (Fig. 3a). Fillet color estimation using the DSM SalmoFan^{TM}
194 lineal showed that ZWRI feeds provided comparable fillet color compared to the
195 commercial feeds (SI appendix, Fig. S4) despite the commercial feed for the fresh water
196 trial having a greater initial ketocarotenoid content (SI appendix, Table S4). The
197 ketocarotenoid composition in the feed and in the fillet remained the same for the
198 commercial treatments and was predominantly astaxanthin. However, for the ZWRI
199 treatments, the main change was the loss of the ketocarotenoid esters in the fillet
200 compared to the feed (Fig. 3b). The ketocarotenoids found in the ZWRI fillets were
201 phoenicoxanthin, canthaxanthin and some astaxanthin. Most of the endogenous tomato
202 carotenoids such as lycopene and β-carotene were also not found in the trout fillet (SI
203 appendix, Table S5). The retention of (keto) carotenoid indicates quantitatively how
204 these compounds were retained in the fillet, compared to their initial amount in the feed
205 and is represented as a percentage. For the trout trial in fresh water, the retention of the
206 coloring ketocarotenoids (phoenicoxanthin, canthaxanthin and astaxanthin) in the fillet
207 was more than 2-fold greater in the ZWRI treatment compared to the commercial
208 supplement (Table 1). In particular, astaxanthin and phoenicoxanthin had exceptionally
209 high retention while in the case of the brackish water experiment, the retention of the

coloring ketocarotenoids was similar when comparing the ZWRI tomato, ZWRI extract and the commercial treatments (Table 1). Carotenoids were also quantified in the feces of the fresh water trout. Levels of coloring ketocarotenoids were 7 times greater in the feces of trout fed with the commercial treatment compared to the ZWRI tomato one and the retention was 1.2-fold higher for the commercial treatment (SI appendix, Table S6).

Chemical and physiological analysis of the trout showed substantial equivalents. No significant difference was observed when comparing the weight of the fish across the experiments (SI appendix, Fig. S5). Levels of carotenoids deposited in the eyes and livers of the trout obtained in the fresh water trial were minimal (~10 µg/g DW) and actually lower in ZWRI compared to the commercial treatment (SI appendix, Table S6). No significant difference was detected in cholesterol contents in the fillets and livers of the fresh water trout from the various feed conditions (SI appendix, Fig. S6a and S6b). The retinoid, retinyl acetate and apocarotenal, β-apo-14´-carotenal were found at similar levels in the trout livers from the basic and control tomato conditions. However, their levels were both increased in livers of trout fed with the ZWRI tomato and commercial feeds (2.4 to 4-fold and 3.3-fold, respectively). No significant difference in retinyl acetate and β-apo-14´-carotenal contents was noticed between the two latter treatments (SI appendix, Fig. S7). Fatty acids were also quantified in the different feeds and fillets from the fresh water trial. No significant difference in total fatty acid content was observed between the different feeds (SI appendix, Fig. S8a). The main fatty acids in the feeds were C18:1, C18:2 and C16:0. In the fillets, these fatty acids were still predominant but C22:6 increased considerably in the fillets compared to the feeds (~3.3-fold on average). The fatty acid composition of the fillets reflected that of the feeds used (SI appendix, Fig. S3a and S8b and Table S3b). Moreover, a global analysis

of the non-polar metabolites present in the fillets, derived from the different feed treatments tested, displayed no discernable clustering/separation following Principal Component Analysis (PCA) on the basis of the feed supplement used (SI appendix, Fig. S9).

Discussion

Over the last decades biotechnology has successfully delivered agronomical input traits. (14). The consumers demand for improved quality, global food security issues and the dwindling reserves of fossil fuels, has provided the economic and social impetus to switch from chemical refining to a bioeconomy based structure. To achieve this goal the development of output traits represent a major agricultural objective. “Golden Rice” and high oleic acid soya are two examples of output traits with the potential to make a difference. In addition to these two examples, numerous proof of concept studies have been reported that confer enhanced output traits associated with quality. However, only a few had the opportunity to show technical and production feasibility (15).

In the present study, a new plant-based source of ketocarotenoids has been achieved and scalability demonstrated in a contained manner under a field-like environment with low resource input (SI appendix, Table S1). The effectiveness of the tomato based material or its derived ketocarotenoid extract to act as an aquaculture feed supplement, responsible for coloring salmonid flesh, has been demonstrated and bench marked against two existing chemically synthesized products on the market. Over two trials in different geographical locations, in both brackish and fresh water conditions, the addition of the tomato material as an admix out performed existing industry products in terms of ketocarotenoid retention in the trout fillets (Table 1). No adverse effects on animal husbandry or yield parameters were observed and chemical substantial

equivalence was determined (SI appendix, Fig. S5, S6 and S9). The high ketocarotenoid tomato extracts also had the potential to color trout fillets (Fig. 3). However, the tomato matrix seemed to improve the retention of the ketocarotenoids in the fillets by nearly 2-fold (Table 1). Further work is required to ascertain the mechanism underlying this important phenomena, although we speculate that the lipid microenvironment within the material may enhance ketocarotenoid absorption into the gastrointestinal tract of the trout, as fat and oil are known to improve carotenoid solubilization into micelles and therefore their bioavailability (16). Furthermore, analysis of the carotenoid distribution in the specific organs/tissues of the trout (fillet, eye and liver) showed that distinct chemical classes of carotenoids were deposited in a differential manner (SI appendix, Tables S5 and S6). This phenomena could be due to the different lipid transport mechanisms that exist but also other factors such as carotenoid concentration and composition or the presence of other competing molecules. In the present study, carotenes were exclusively found in the liver while non-esterified xanthophylls were deposited in the fillet with the exception of echinenone and canthaxanthin that could also be found in the liver and eye. In a generic manner, these data corroborate previous reports describing carotenoid distribution in chicken tissues fed on carotenoid enhanced maize (17). The ketocarotenoids present in the tomato material and derived extracts are predominantly esterified but those ketocarotenoids present in the flesh of the trout are non-esterified. This observation supports previous findings where esterified ketocarotenoids have been shown to be cleaved in the intestine of the trout prior to deposition in the flesh (18). To date, the literature is inconclusive with regard to the potential of the esterified carotenoid forms being more bioavailable to fish (19, 20).

The rudimentary approach to formulation used in this study and the results achieved suggest that further optimization of the process will deliver an improved product

beyond the prototype used to date. The use of an admix also greatly improves the environmental impact of the process as no organic solvents are required in the down-stream processing or formulation process. In addition to its improved environmental credentials, the reduction in costs are significant. Although a full Life Cycle Analysis (LCA) is necessary, our estimates suggest that the keto tomato admix could provide an approximate 10-fold cost saving, as presently the production cost of the synthetic feed is in the range of US$1000 to 2000 per kg. Using the data generated in this study, the production costs for tomato material containing a kilogram of coloring ketocarotenoids are in the region of US$150. It is important to note that the reason such an admix can be effective is because of the levels reached in the selected tissues used. In this particular case, tomato fruit is the ideal sink tissue because it is intrinsically adapted to isoprenoid production. Although ketocarotenoids have been produced in lettuce (21), potato (22), maize (23), canola (24) and soybean seeds (25), the levels are over two orders of magnitude lower than those achieved in tomato. These low levels make the vast amounts of seed material required for incorporation into feed formulation impractical. Presumably, a major reason why these sources are limited is because they have evolved and been selected for starch and oil accumulation. It is interesting that the non-endogenous ketocarotenoids produced existed, where chemically possible, in an esterified form. Precisely how this phenomena arises and their potential to facilitate sequestration awaits further elucidation. Tomato fruit is also an established food, which is readily digestible and regarded as Generally Recognized as Safe (GRAS). Non-food sources such as tobacco do not have these credentials, no sink organs are readily amenable for production, this means that pleotropic effects are likely to occur in vegetative tissues when high levels (above 3% dry weight) are reached and substantial down-stream processes essential.

The present study also shows that a mixture of ketocarotenoids can have the same coloring potential as astaxanthin solely, the main ketocarotenoid used in aquaculture feeds. Other natural colorants approved by the European Food Safety Authority, such as Panaferd-AX® (26) made from *Paracoccus carotinifaciens* a red carotenoid-rich soil bacterium, is also constituted of a mixture of carotenoids (27) (astaxanthin (2.2 %), phoenicoxanthin (1.3 %) and canthaxanthin (0.4 %) besides other carotenoids). The main ketocarotenoid in the ZWRI tomato preparation is phoenicoxanthin, although not abundant in nature, it can be found in mollusks, crustaceans (28), green alga (such as *Haematococcus pluvalis* (29) and *Chlorococcum* (30)) and Adonis flowers (31). The synthetic astaxanthin preparations used, contain unidentified reaction contaminants and a mixture of stereoisomers whereas the biosynthetically derived phoenicoxanthin used in the present study was exclusively present in its biologically active *S* configuration (Fig. 2), as found in *Adonis aestivalis* petals (32). One of the main concerns of novel foods is traceability. The salmonid fillets are the end-products for the food chain, which are effectively non-GM products with no foreign DNA. In effect, the approach is synonymous with the marketing of livestock products fed on GM feedstuffs such as soya and corn. One advantage of the present phoenicoxanthin product is that it offers an auditable biochemical marker. Previously, to achieve de-regulation of GM peppermint varieties unnatural stereoisomers had to be generated to create a traceable product within the market place (33).

The targeted chemical analysis of the experimental trout tissues has indicated no significant changes in steady state metabolite levels or composition between the trout consuming the present commercial product and the experimental tomato derived ketocarotenoid material. They both prove to enhance the level of one of the natural forms of vitamin A, retinyl acetate in the trout livers compared to the trout fed with

ketocarotenoid free feeds. This demonstrates that ketocarotenoids can also be used as vitamin A precursors in agreement with a previous *in vitro* study on rainbow trout intestine (34). Although further metabolomic analysis will help to confer the existence of substantial equivalents, based on the present chemical and physical data acquired the end-product would appear equivalent to similar products in the market in line with the US Food and Drug Administration terms. It could then be designated as "Generally Recognized as Safe" under the Federal Food, Drug, and Cosmetic Act and therefore avoid pre-market approval in the USA (35).

The approach described in this article demonstrates that using a combination of technologies presently available, new technology pipelines can be established to deliver renewable sources of high-value specialty chemicals. In this case, ketocarotenoids have been chosen and tomato fruit exploited as the production platform. Technical and production feasibility have been demonstrated. The pipeline developed is scalable, requires minimal down-stream processing, has improved environmental credentials and is economically competitive.

Materials and Methods

Plant material and cultivation. The Moneymaker variety of tomato *Solanum lycopersicum* had been previously transformed with the ZW construct, harboring the bacterial *Brevundimonas* sp strain SD212 genes carotene hydroxylase (*Crt*Z) and carotene ketolase (*Crt*W) both under the cauliflower mosaic virus 35S constitutive promoter (36), using the *Agrobacterium tumefaciens* strain LBA 4404 . The high β-carotene line used in this study (RI) derives from the crossing of the cultivated tomato *Solanum lycopersicum* (LA4024 in the TGRC database) with the wild *S. galapagense* accession (LA0483 in the TGRC database) (12, 13). Two ZW events (10-12 and 10-

17) were crossed with two RI lines (RI33 and RI1). The lines were cross pollinated. The best combination in terms of ketocarotenoids levels were selected (10-12 x RI33). The ZWRI plants were greenhouse grown (25°C day/15°C night), with supplementary lighting (16 h light/8 h dark) or under polytunnel containment devoid of supplementary lighting and heating, over one growing cycle in the UK with an early (June-July, ~ 24°C day) and late season (August-September, ~ 21°C day). Analyses were made on three pooled fruits from each studied plant in the greenhouse and on three samples from four individual batches of several kilos of tomatoes (~ 5 kg) from each season for the large scale study in the polytunnel.

Extraction and analysis of metabolites.

Carotenoids. Carotenoids were extracted from freeze dried tomatoes, freeze dried trout parts (fillets, livers and eyes), feces and feeds. Extractions and analyses were carried out following a protocol previously published (37). A detailed description is shown in SI appendix, SI text. **Fatty acids.** Fatty acids were extracted from 20 mg of freeze dried tomato powder or 50 mg of freeze dried trout fillet powder and feed and analyzed following the protocol from Menard et al (38). Details are described in SI appendix, SI text. **Retinoids.** A retinoid extraction method was adapted from Gesto, Castro, Reis-Henriques and Santos (39). A detailed description is given in SI appendix, SI text. **Non-polar compounds (including cholesterol).** Non polar compounds extraction from the trout fillets and livers was performed as described above for the carotenoids. The extracts were analyzed by gas chromatography-mass spectrometry analysis. A detailed protocol is given in SI appendix, SI text. **Phoenicoxanthin esters fatty acid determination.** The ketocarotenoid esters found in the tomato UPLC chromatogram profile were individually isolated for further characterization. First, the ketocarotenoid esters were saponified using the cholesterol esterase from *Pseudomonas* (Sigma, UK).

Protocol was adapted from Jacobs, Leboeuf, Mccommas and Tauber (40) and Stalberg, Lindgren, Ek and Hoglund (41) and is described in SI appendix, SI text. The saponified ketocarotenoids were identified as pheonicoxanthin by comparison of spectral characteristic and retention time value of the authentic standard. To determine the fatty acids attached to the phoenicoxanthin esters, the compounds were analysed using mass spectrometry. Separations were performed by HPLC (Ultimate 3000, Dionex) prior to on-line MS using a RP C30 3 μm column (150×2.1 mm i.d., YMC) coupled to a 20×4.6 mm C30 guard column. The column temperature was maintained at 30°C. The mobile phase was comprised of (A) methanol containing 0.1% formic acid (by vol.) and (B) tert-butyl methyl ether containing 0.1% formic acid (by vol.). These solvents were used in a gradient mode starting at 100% (A) for 5 min, then stepped to 95% (A) for 4 min, followed by a linear gradient over 30 min to 25% (A). After this gradient (A) was a step down to 10% over 10 min. Initial conditions (100% A) were restored for 10 min after the gradient to re-equilibrate the system. The flow rate used was 0.2 ml/min. The HPLC system was coupled to maXis^{TM} quadrupole-time-of-flight (QTOF, Bruker, Germany). The ionisation mode employed was Atmospheric Pressure Chemical Ionisation (APCI) operating in positive mode. Capillary and APCI vaporisation temperatures were set at 250°C and 450°C respectively and the gas flow (nitrogen) at 4L/min. APCI source settings were as follows: nebuliser pressure 2.5 bar, corona current 4 μA and a capillary voltage of 4.5 kV. A full MS scan was performed from 300 to 1500 m/z and MS/MS spectra were recorded at the isolation width of 0.2 m/z. Identification of the fatty acids attached to the phoenicoxanthin was done by comparison with the expected mass in the MS and MS/MS profiles of the phoenicoxanthin esters. Instrument calibration was performed externally prior to each sequence with APPI/APCI calibrant solution (Agilent Technologies). Automated post-run internal calibration was performed by

injecting the same APPI/APCI calibrant solution at the end of each sample run via a six port divert valve equipped with a 20 µL loop. **Phoenicoxanthin optical isomerism analysis.** Fractions of phoenicoxanthin were collected and optical isomerism studied using a liquid chromatography method adapted from Wang, Armstrong and Chang (42), which is detailed in SI appendix, SI text.

Trout trials.

Feed preparation. A detailed description of the feed preparation is given in SI appendix, SI text. The composition of the feeds are described in SI appendix, Table S2 & S3. Figure S3a (SI appendix) gives an overview of the composition of the different feeds. **Feeding trout trial.** The experiments were conducted in compliance with the 3Rs (Replacement, Reduction, Refinement) principles and the 2010/63/EU directives. Ethical approval on animal experiments from the internal RHUL and the DISCO steering committees were obtained. **Fresh water experiment.** Rainbow trout (*Oncorhynchus mykiss*) about 100g were grown in 400 L tanks filled with 260 L of fresh water (flow rate:1.2 to 2 L/min, temperature: 11-13°C, oxygen content: 8-10 mg/L). They were fed for seven weeks with the different feed conditions (basic, control tomato, ZWRI tomato and commercial (BioMar Efico alpha Color 717 42/ 22) feeds), first with 2% of their weight and afterwards with 1.5%. Each feed was tested in three tanks containing 10 fish each. Feeds were delivered from the top of the tank at the surface of the water. Feeds were stored at 4°C in a dark room during the length of the experiment to prevent carotenoid degradation. Fish were sampled at the end of the experiment and the fillets, eyes, livers and feces of each trout were collected and kept at -80°C under N_2 atmosphere until analysis. **Brackish water experiment.** Rainbow trout (*Oncorhynchus mykiss*) about 30-40g were grown in 130 L tanks filled with brackish water (salt concentration: 30 PSU, flow rate: 8 to 9 L/min, temperature: 14-

15°C, oxygen content: 7.405 mg/L). Each tank contained 25 fish, twenty of whom were fed for 60 days and then sampled and the other five fish were fed for an extra 20 days, so in total 80 days. Each tank corresponded to one feed condition (basic, control tomato, ZWRI tomato, ZWRI extract and commercial (Carophyll pink®) feeds). Fillets of each fish were collected for analysis at the end of the experiments and kept at -80°C under N_2 atmosphere until the analyses were performed. **Statistical power of the study.** A Post-hoc power analysis was performed to assess the statistical power of the study (SI appendix, SI text). **Fillet color assessment.** Color of the fish fillets were assessed by three individuals in natural light, right after the slaughtering of the fish, using the DSM SalmoFan as a reference. The color indices of the fan associated with the different hues were used to estimate the color. The average of the indices from the three individuals were calculated and used as a representation of the fillet color of each trout.

Statistical analysis. For the study of plant material, three to five biological replicates with three technical replicates per biological replicates were analysed for every experiment. For the study of the trout material, five to fifteen biological replicates with three technical replicates per biological replicate were investigated for each experiment unless stated otherwise. IBM SPSS Statistics 21 software was utilized to determine significant differences between groups. A detailed explanation of the statistical tests performed is given in SI appendix, SI text. P-values were calculated and represented in figures as follow: $P < 0.05$, $P < 0.01$, and $P < 0.001$ were indicated by *, **, and ***, respectively, when appropriate. Table S7 (SI appendix) describes all the statistical tests performed in this paper and all the p-values obtained with the SPSS software. **Randomization.** Randomization technique was used whenever possible (SI appendix, SI text).

Acknowledgments. The authors are grateful to Dr Perez-Fons for MS assistance and Prof. Bramley for his continued support and advice. The authors thank Anne Beiermeister and Ulfert Focken from the Johann Heinrich von Thunen-Institut for their assistance with the trout trial in Germany. This work was supported through the European Union Framework Program DISCO (from DISCOvery to products: A next generation pipeline for the sustainable generation of high-value plant products, project No. 613513), BBSRC OPTICAR project-BB/P001742/1 and the CA COST Action EUROCAROTEN project (European network to advance carotenoid research and applications in agro-food and health, project No. CA15136).

REFERENCES

1. Fraser PD & Bramley PM (2004) The biosynthesis and nutritional uses of carotenoids. *Prog Lipid Res* 43(3):228-265.
2. Marz U (2015) The global market for carotenoids.
3. Fraser PD, Miura Y, & Misawa N (1997) In vitro characterization of astaxanthin biosynthetic enzymes. *Journal of Biological Chemistry* 272(10):6128-6135.
4. Alfnes F, Guttormsen AG, Steine G, & Kolstad K (2006) Consumers' willingness to pay for the color of salmon: A choice experiment with real economic incentives. *Am J Agr Econ* 88(4):1050-1061.
5. Steven DM (1948) Studies on animal carotenoids. I. Carotenoids of the brown trout (*Salmo trutta linn.*). *Journal of Experimental Biology* 25(4):369-387.
6. Olesen I, Alfnes F, Rora MB, & Kolstad K (2010) Eliciting consumers' willingness to pay for organic and welfare-labelled salmon in a non-hypothetical choice experiment. *Livest Sci* 127(2-3):218-226.
7. Lorenz RT & Cysewski GR (2000) Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends in Biotechnology* 18(4):160–167.
8. Ausich RL (1997) Commercial opportunities for carotenoid production by biotechnology. *Pure and Applied Chemistry* 69(10):2169–2174.
9. Gatlin DM, *et al.* (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac Res* 38(6):551-579.
10. Cunningham FX & Gantt E (2011) Elucidation of the Pathway to Astaxanthin in the Flowers of Adonis aestivalis. *Plant Cell* 23(8):3055-3069.
11. Maksyutova SS & Lazareva DN (1978) Pharmacological Properties of Adonis Sibir and Vernalis, Growing in Bashkiria. *Farmakol Toksikol* 41(2):223-226.
12. Goldman IL, Paran I, & Zamir D (1995) Quantitative Trait Locus Analysis of a Recombinant Inbred Line Population Derived from a Lycopersicon-Esculentum

X Lycopersicon-Cheesmanii Cross. *Theoretical and Applied Genetics* 90(7-8):925-932.
13. Paran I, Goldman I, Tanksley SD, & Zamir D (1995) Recombinant Inbred Lines for Genetic-Mapping in Tomato. *Theoretical and Applied Genetics* 90(3-4):542-548.
14. James C (2014) Global Status of Commercialized Biotech/GM Crops: 2014. *ISAAA* brief 49.
15. Newell-McGloughlin M (2008) Nutritionally improved agricultural crops. *Plant Physiol* 147(3):939-953.
16. Nagao A (2014) Bioavailability of Dietary Carotenoids: Intestinal Absorption and Metabolism. *Jarq-Jpn Agr Res Q* 48(4):385-391.
17. Moreno JA, *et al.* (2016) The distribution of carotenoids in hens fed on biofortified maize is influenced by feed composition, absorption, resource allocation and storage. *Sci Rep-Uk* 6.
18. White DA, Page GI, Swaile J, Moody AJ, & Davies SJ (2002) Effect of esterification on the absorption of astaxanthin in rainbow trout, Oncorhynchus mykiss (Walbaum). *Aquac Res* 33(5):343-350.
19. White DA, *et al.* (2003) The degree of carotenoid esterification influences the absorption of astaxanthin in rainbow trout, Oncorhynchus mykiss (Walbaum). *Aquaculture Nutrition* 9(4):247-251.
20. Bowen J, *et al.* (2002) Utilization of (3S,3 ' S)-astaxanthin acyl esters in pigmentation of rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition* 8(1):59-68.
21. Harada H, *et al.* (2014) Construction of transplastomic lettuce (Lactuca sativa) dominantly producing astaxanthin fatty acid esters and detailed chemical analysis of generated carotenoids. *Transgenic Res* 23(2):303-315.
22. Mortimer CL, *et al.* (2016) Product stability and sequestration mechanisms in Solanum tuberosum engineered to biosynthesize high value ketocarotenoids. *Plant Biotechnol J* 14(1):140-152.
23. Farre G, *et al.* (2016) Metabolic engineering of astaxanthin biosynthesis in maize endosperm and characterization of a prototype high oil hybrid. *Transgenic Res* 25(4):477-489.
24. Fujisawa M, *et al.* (2009) Pathway engineering of Brassica napus seeds using multiple key enzyme genes involved in ketocarotenoid formation. *Journal of Experimental Botany* 60(4):1319-1332.
25. Pierce EC, *et al.* (2015) Ketocarotenoid Production in Soybean Seeds through Metabolic Engineering. *PLoS One* 10(9):e0138196.
26. EFSA (2007) Safety and efficacy of Panaferd-AX (red carotenoid-rich bacterium Paracoccus carotinifaciens) as feed additive for salmon and trout. *The EFSA journal* 546:1-30.
27. Osterlie M & Lerfall J (2015) Pigments for Aquaculture of Salmonids. A Comparative Model Study of Carophyll Pink and Panaferd AX in Cod Liver Oil. *J Am Oil Chem Soc* 92(9):1321-1331.
28. Matsuno T (2001) Aquatic animal carotenoids. *Fisheries Sci* 67(5):771-783.
29. Yuan JP & Chen F (1998) Chromatographic separation and purification of trans-astaxanthin from the extracts of Haematococcus pluvialis. *J Agr Food Chem* 46(8):3371-3375.
30. Liu BH & Lee YK (2000) Secondary carotenoids formation by the green alga Chlorococcum sp. *J Appl Phycol* 12(3-5):301-307.

31. Cunningham FX & Gantt E (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of Adonis aestivalis. *Plant Journal* 41(3):478-492.
32. Maoka T, Etoh T, Kishimoto S, & Sakata S (2011) Carotenoids and Their Fatty Acid Esters in the Petals of Adonis aestivalis. *J Oleo Sci* 60(2):47-52.
33. Lange BM, *et al.* (2011) Improving peppermint essential oil yield and composition by metabolic engineering. *P Natl Acad Sci USA* 108(41):16944-16949.
34. White DA, Ornsrud R, & Davies SJ (2003) Determination of carotenoid and vitamin A concentrations in everted salmonid intestine following exposure to solutions of carotenoid in vitro. *Comp Biochem Phys A* 136(3):683-692.
35. Price WD & Underhill L (2013) Application of Laws, Policies, and Guidance from the United States and Canada to the Regulation of Food and Feed Derived from Genetically Modified Crops: Interpretation of Composition Data. *J Agr Food Chem* 61(35):8349-8355.
36. Mortimer CL, *et al.* (2017) The formation and sequestration of nonendogenous ketocarotenoids in transgenic *Nicotiana glauca*. *Plant Physiology* 173:1617–1635.
37. Nogueira M, Mora L, Enfissi EMA, Bramley PM, & Fraser PD (2013) Subchromoplast Sequestration of Carotenoids Affects Regulatory Mechanisms in Tomato Lines Expressing Different Carotenoid Gene Combinations. *Plant Cell* 25(11):4560-4579.
38. Menard GN, *et al.* (2017) Genome Wide Analysis of Fatty Acid Desaturation and Its Response to Temperature. *Plant Physiol* 173(3):1594-1605.
39. Gesto M, Castro LFC, Reis-Henriques MA, & Santos MM (2012) Tissue-specific distribution patterns of retinoids and didehydroretinoids in rainbow trout Oncorhynchus mykiss. *Comp Biochem Phys B* 161(1):69-78.
40. Jacobs PB, Leboeuf RD, Mccommas SA, & Tauber JD (1982) The Cleavage of Carotenoid Esters by Cholesterol Esterase. *Comp Biochem Phys B* 72(1):157-160.
41. Stalberg K, Lindgren O, Ek B, & Hoglund AS (2003) Synthesis of ketocarotenoids in the seed of Arabidopsis thaliana. *Plant Journal* 36(6):771-779.
42. Wang C, Armstrong DW, & Chang CD (2008) Rapid baseline separation of enantiomers and a mesoform of all-trans-astaxanthin, 13-cis-astaxanthin, adonirubin, and adonixanthin in standards and commercial supplements. *Journal of chromatography. A* 1194(2):172-177.

Figure legends

Fig. 1. Representative scheme of the ketocarotenoid pathway introduced in plant.

Enzyme names are as follow: CRTR-B1, plant carotene β-hydroxylase 1; CRTW,

bacterial carotene ketolase and CRTZ, bacterial carotene hydroxylase. The purple and

591 blue shadings depict the position of the newly added functional group (hydroxyl or
592 ketone, respectively).
593 **Fig. 2.** Chromatographic profiles of ZWRI tomato carotenoids and phoenicoxanthin
594 chirality. The chromatographic carotenoids profile was obtained by UPLC and
595 recorded at 470 nm. The insert shows that the chiral carbon of the ZWRI
596 phoenicoxanthin has an *S* configuration.
597 **Fig. 3.** ZWRI tomatoes color trout fillets. (a) Photographs of the trout fed with the basic,
598 commercial, control tomato, ZWRI tomato and ZWRI extract feeds, taken at the end of
599 the fresh and brackish water trials (50 and 80 days, respectively). (b) Chromatographic
600 profiles of carotenoids in the feed and fillet corresponding to the commercial and ZWRI
601 tomato treatments. 1, astaxanthin; 1#, unknown ketocarotenoid-1; 2, phoenicoxanthin;
602 3, canthaxanthin; 4, 3′-OH-echinenone; 5, 3-OH-echinenone; 6, echinenone; 7,
603 phoenicoxanthin-C14:0; 8, adonixanthin-C14:1; 9, phoenicoxanthin-C16:0; 10,
604 adonixanthin-C16:1; 11, β-carotene.
605
606