PRODUCTION OF THE NUTRACEUTICAL CAROTENOID ASTAXANTHIN IN CAMELINA SEEDS

By

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I. Abstract

The light- harvesting, colorful pigments which are most widely produced by plants are known as carotenoids. These compounds are both high in number and diversity, creating a vast array of bright colors seen in fruits, vegetables, and algae. Carotenoids can only be synthesized *de novo* by photosynthetic organisms and microbes, but are present in humans through dietary intake. A handful of these compounds display various health benefits. Furthermore, dietary intake of this carotenoid greatly minimizes the risk of Vitamin A deficiency induced blindness.

Fortunately, bioengineering of plants provides an avenue to fortify food crops with elevated levels of Vitamin A, which has been a goal of researchers for decades. The Schmidt laboratory has successfully enhanced *Camelina sativa* seeds with enhanced β-carotene, Vitamin A's precursor compound. This current multi-year project involved the introduction and subsequent accumulation of a b-carotene derived keto-carotenoid, astaxanthin. This project involves the engineering of Camelina sativa seeds for the production of astaxanthin by extending the b-carotene carotenoid pathway and comparing two separate strategies, the introduction of crtS, a gene isolated from the red yeast *Xanthophyllomyces dendrorhous*, or the introduction of BKTChamy from the green algae *Haematococcus pluvialis*. The current project's objectives were to both confirm the presence and expression of the introduced transgenes and detect and quantify the introduced carotenoid astaxanthin in the seeds. This has been completed by genomic PCR using gene- specific primers to verify gene integration as well as RT-PCR to detect transgene transcription. PCR results proved incorporation of the genes, with BKTChamy being produced in higher quantities, evident by the much more noticeable orange seed color. Results of a phenotypic analysis revealed a reddish coloring of seeds with the β-carotene/ β-carotene ketolase cassettes, confirming elevated levels of astaxanthin not found in wild-type seeds.

II. Introduction

Carotenoids are naturally produced colorful pigments found in nature. These compounds exist in plants, animal structures, and microbes. Of over 750 identified carotenoids (Takaichi, 2011), several have been studied for their benefits to human health, primarily prevention of eye disease, which is considered to be a result of antioxidant activity (Johnson,

2002). One such carotenoid, β-carotene, confers the characteristic bright orange color seen in carrots as well as other fruits and vegetables. Notably, β-carotene is a precursor to Vitamin A and displays great potential in genetic engineering of food crops. A familiar example of Vitamin A biofortification is yellow colored Golden Rice, a genetically modified crop which was invented in the 1990s by German scientists Ingo Potrykus and Peter Beyer. Potrykus and Beyer utilized the phytoene synthase gene and lycopene cyclase under the control of an endosperm- specific glutelin promoter (Beyer, Et al., 2002). However, the Vitamin A precursor beta-carotene was not produced in significant quantities, with only 1.6 μg/g in uncooked rice. For reference, adults and teenagers require between 30-100 mg of the carotenoid per day for sufficient Vitamin A production (Mayo Clinic, 2023). Eventually, Syngenta redesigned Golden Rice, producing 23 times more beta-carotene, an amount that still does not satisfy daily human allotments (Enserink, 2008). Golden Rice 2 was created by isolating a new phytoene synthase gene, this time from maize, and combined with the *crtI* gene from *Erwinia uredovora* used in the original Golden Rice, leading to the improved quantities observed (Paine, Et al., 2005).

Numerous strategies have been employed to engineer a variety of other crops with unique carotenoids. This paper is a continuation of prior research on *Camelina sativa* conducted by Schmidt and Pendarvis in 2016 to enhance beta-carotene, completed by engineering the carotenoid pathway to overexpress the phytoene synthase gene.

Camelina sativa, a member of the Brassicaceae family of cruciferous, flowering mustard plants, has generally been neglected in terms of large-scale agricultural production. This oilseed crop is comparable to Canola, from which oils are extracted from seeds and sold as a commercially available food product. Additionally, Camelina offers healthful omega-3 fatty acids and may be a future source of low-cost biofuel (Lu and Kang, 2008). Camelina displays perhaps great potential as a novel substitute to Canola due to its ease of transformation (Lu and Kang, 2008). The carotenoid of interest, astaxanthin, is a lipid- soluble compound with a unique chemical structure reported to exhibit antioxidant and anti-inflammatory properties (Donoso, Et al., 2021). Astaxanthin is a biologically produced compound which is commonly used to supplement salmon feed and is responsible for the reddish coloring (Fakhri, Et al., 2018). A series of enzymatic reactions taking place in certain bacteria and algae allow the keto-carotenoid to be naturally produced from β-carotene.

Previous attempts of astaxanthin bioengineering have proved successful in a variety of plants by different mechanisms. One such method took place in tobacco plants, using β-carotene ketolase from *Haematococcus pluvialis* algae and the *Pds* promoter from tomato (Mann, Et al., 2000). Researchers achieved a final dry weight concentration of 2000 μg/g (Mann, et al., 2000). A later experiment fortified tomato crops, with researchers utilizing co-expression of β-carotene ketolase isolated from algae species *Chlamydomonas reinhardtii* and *Haematococcus pluvialis* and complementation in *E. coli* (Huang, e al., 2013). Increased accumulations of astaxanthin were found in fruits (16.1mg/g) as a result of the experimentation. In terms of seed fortification, research has also been successful in astaxanthin engineering in soybean. More recently, a team utilized the *crtB* gene from *Pantoea anantis* in combination with the *bkt1* gene, producing astaxanthin from *Haematococcus pluvialis* to transform the soybean cultivar Jack, thereby accumulating other keto-carotenoids in the pathway including astaxanthin. Using a seed specific promoter, a final astaxanthin concentration of 2-7 μg/g was achieved (Pierce, et al., 2015).

This project utilizes an approach to quantify the levels of transgenically produced astaxanthin by two separate engineering strategies. Previous work involved the synthesis of two codon-optimized astaxanthin producing genes, crtS, from the red yeast *Xanthophyllomyces dendrorhous*, and BKTChamy from the green algae *Haematococcus pluvialis,* the same algae species typically used in previous experiments conducted by other authors. These genes were placed under the regulation of strong seed-specific promoters in expression cassettes also producing seed-specific crtB gene. Transgenic *Camelina sativa* plants were produced via *Agrobacterium*-transformation and subsequently grown to homozygosity. In this project, the lines were confirmed by expression analysis and carotenoid analysis that the introduced astaxanthin was successfully produced and accumulated in the transgenic seeds.

III. Materials and Methods

DNA Extraction/ PCR

DNA was isolated from a small quantity of leaf tissue and ground by hand then extracted using 600 ul of cetyl trimethyl ammonium bromide (CTAB buffer) (Doyle, 1990). This

extraction protocol was repeated for wildtype (nontransgenic control) samples, B control (crtB containing lines), and lines containing crtB transgene with either BKTchamy or crtS. Additional control plants consisted of transgenic lines containing the BKTchamy or the crtS transgenes without the crtB gene but expressing the visual marker green fluorescent protein (GFP) to aid in the identification of transgenic lines. In order to verify presence of the transgenes, several polymerase chain reactions (PCRs) were performed, allowing for visualization of bands to prove transgene incorporation. For each gel, a set of unique primers was used to isolate the amplicon of interest. These primers are a lectin promoter forward and crtB reverse to verify the B1 line, and a glycinin forward/ crtS or BKTchamy for the remaining transgenic lines. The primers were used at 10 μM concentration and a green master mix containing Taq polymerase (ThermoScientific)**.** The reaction was run using the following cycling parameters: initial 94 ˚C 5 minutes, then 35 cycles of 94 ˚C 30 seconds, 55 ˚C 30 seconds, 72 ˚C for 1 minute, and a final extension at 72 ˚C for 7 minutes. A 1% agarose gel containing ethidium bromide for visualization allowed for observation of the samples under ultraviolet light.

Table 1. Primer Sequences and Amplicon Sizes

Primer Name	Sequence	Amplicon Size
Lectin Promoter (5’) CrtB Reverse (3’)	5’ GTGCAATTTAGCTGAAGC 3’ 5’ CGGTTAGCATAAGGACTAGG 3’	300 bp
Glycinin Promoter (5’) CrtS Reverse (3’)	5’ CCTCATTCACCTTCCTCTCTT 3’ 5’ CGGTTAGCATAAGGACTAGG 3’	100 bp
Glycinin Promoter (5’) BKTChamy Reverse (3’)	5’ CCTCATTCACCTTCCTCTCTT 3’ 5’ GTTCCCAATGCTTTCTATGC 3’	300 bp

RNA Extraction/ RT-PCR

A 1mL Trizol solution was used as per the manufacturer's instructions for isolation of RNA from Camelina seeds. Approximately 50 seeds, 100 mg, were ground and homogenized with the Trizol reagent. The RNA samples were subjected to a DNase enzyme treatment for 1.5 hours to eliminate any residual genomic DNA. Following RNA isolation, the ThermoScientific First Strand cDNA kit was used per the manufacturer’s instructions to produce cDNA. Briefly, RNA was diluted to 2 ng/ μL for a 20 μL reaction.1 μL each of gene specific forward and reverse primers were designed for each line and added to the reaction mix. Control primers using

sequences from endogenous Camelina genes with large intronic sequences were designed and tested as well to ensure cDNA, rather than genomic DNA, was amplified for mRNA detection. The control gene used was a Camelina sativa LEAFY gene. Upon completion of cDNA synthesis, RT-PCR amplifications were performed for the control and transgenes following the standard PCR conditions listed above.

Table 2. Primer Sequences and Amplicon Sizes

Primer Name	Sequence	Amplicon Size
Camelina sativa LEAFY gene (Control)	Forward: 5' CAGAGGGAGCATCCGTTTATC 3' Reverse: 3' AGGAATCGAAAGATACCTTGGTG 5'	Genomic DNA: 490 cDNA: 175
crtB	Forward: 5' ACATGAGCTGCGAGATGATATAC 3' 3' TCGATCCACTCATGCCTCTA 5'	cDNA: 146 bp
crtS	Forward: 5' CGACTATCCGAAACCTGGTATG 3' Reverse: 3' GATCCTTCGATGTCGCTTATGA 5'	cDNA: 103 bp
BKTChamy	Forward: 5' TGTCTTCGCTGCCCTTG 3' Reverse: 3' TACACACCTCAAGCCATCAC 5'	cDNA: 184 bp

Seed Phenotypic Screening

To visualize phenotypic differences in seed color among wildtype and transgenic lines, seeds were placed under white light to capture high resolution images.

IV. Results

Transformed Camelina seeds

The astaxanthin-produced transgenes, BKTChamy and crtS, integration was confirmed in Camelina lines by genomic PCR. Gene-specific combinations of forward and reverse primers were used to ensure stable transformation of crtB or the astaxanthin producing genes.

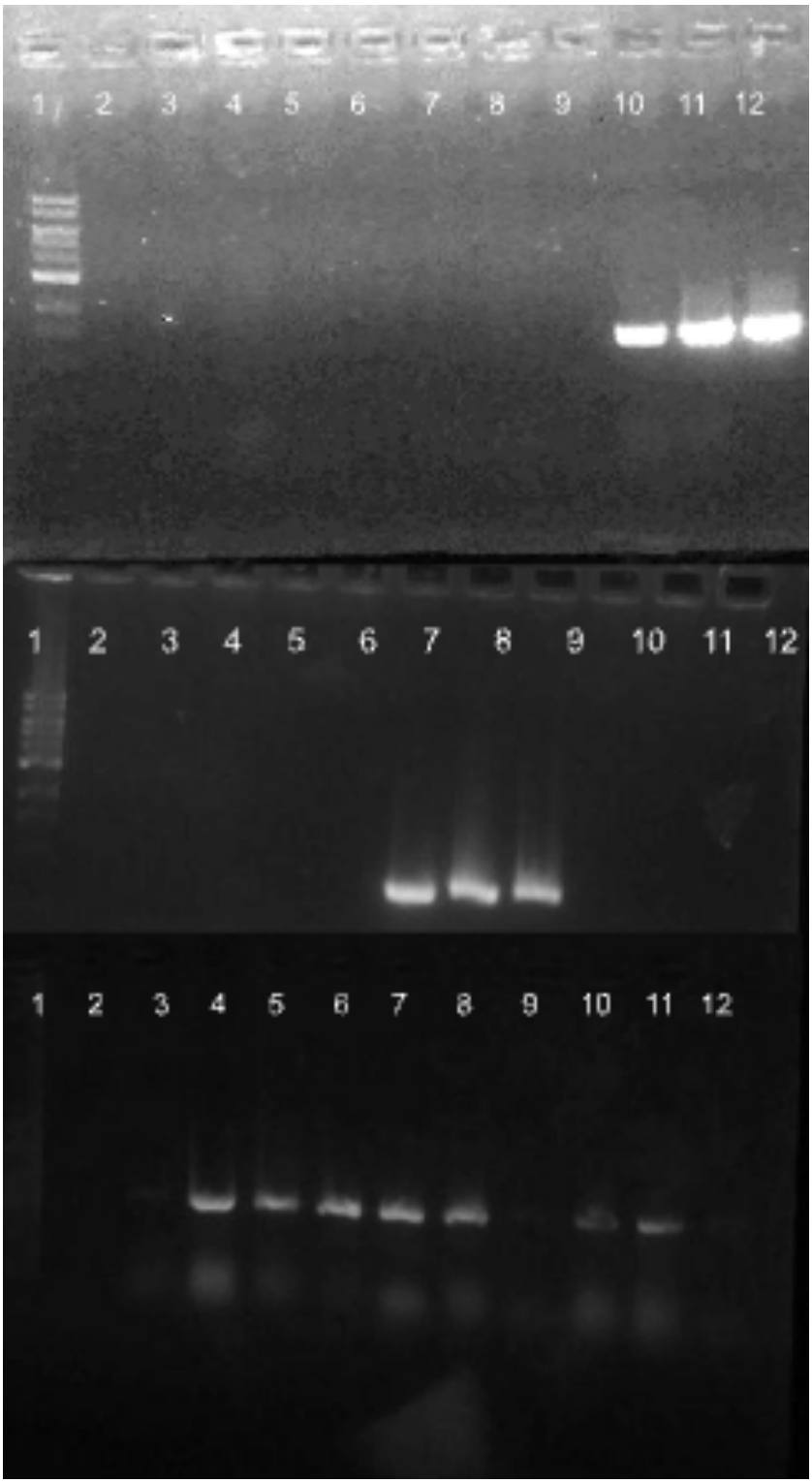

Figure 1. Agarose gels displaying the results of a PCR with transformed Camelina DNA to verify successful incorporation of transgenes.

Top gel: For verification of the presence of crtS (Gly forward primer, S reverse primer). Lane 1: DNA ladder, Lane 2: H2O control, Lane 3: wildtype, Lane 4: B1, Lane 5: null Lane 6: null Lane 7: BCH1, Lane 8: BCH3, Lane 9: GFPCh2, Lane 10: BS1 Lane 11: BS2 Lane 12: GFPS1. A positive amplification in lanes 10-12 indicates successful incorporation of the transgene into the genome.

Middle gel: For verification of the presence of BKTChamy (Gly forward primer, Ch reverse primer). Lane 1: DNA ladder, Lane 2: H2O control, Lane 3: wildtype, Lane 4: B1, Lane 5: null Lane 6: null Lane 7: BCH1, Lane 8: BCH3, Lane 9: GFPCh2, Lane 10: BS1 Lane 11: BS2 Lane 12: GFPS1. A positive amplification in lanes 7-9 indicates successful incorporation of the transgene into the genome.

Bottom gel: For verification of the presence of crtB (Le forward primer, B reverse primer). Lane 1: DNA ladder, Lane 2: H2O control, Lane 3: wildtype, Lane 4: B1, Lane 5: null Lane 6: null Lane 7: BCH1, Lane 8: BCH3, Lane 9: GFPCh2, Lane 10: BS1 Lane 11: BS2 Lane 12: GFPS1. A positive amplification in lanes 4 and 7-8, 10-11 indicates successful incorporation of the transgene into the genome.

Seed Phenotype Screening

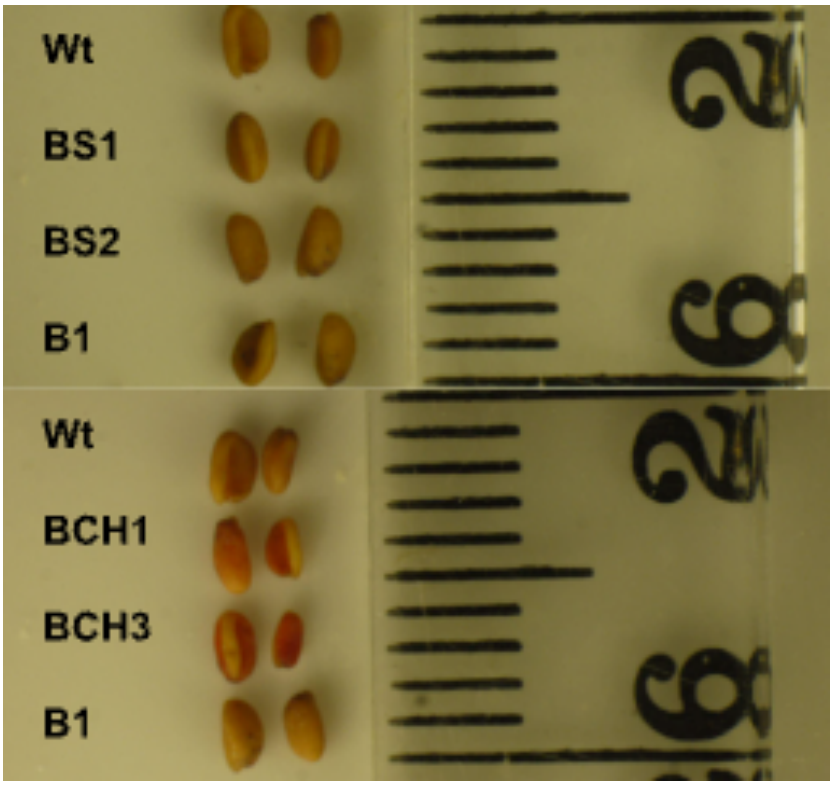

Figure 2. Seeds from the carotenoid producing constructs compared to wildtype (Wt) *Camelina sativa* seeds. The top seed set of crtS producing seeds displays subtle color differences as opposed to the noticeably brighter orange BKTChamy producing seeds.

V. Discussion

The aim of this project was to develop on prior research conducted on *Camelina sativa* seeds containing elevated levels of β-carotene by using expression genes crtS and BKTChamy to convert the carotenoid into astaxanthin. Previous work involved the successful assembly of the constructs necessary to achieve the production of astaxanthin, transformation of Camelina and the initial screening of positive transgenic lines by both visualization of orange seed colors and genomic PCR of the selectable marker kanamycin. This year's work expanded upon this, using gene specific primers to verify lines contained stable transformation of the desired astaxanthin-producing gene combinations. Another aim of current experimentation was to quantify the levels of gene expression of the astaxanthin producing lines, but this step remains incomplete due to technical issues with the reverse-transcriptase PCR from extracted RNA. Likely the quality of thec DNA produced from the isolated RNA extracted from developing seeds is the reason for the RT-PCR not being successful. However, the phenotypic color differences among lines indicates a promising level of success. Follow-up research will involve chemical isolation and quantification of astaxanthin in these transgenic seeds.

Previous results were undetectable levels of β-carotene in non-transgenic lines as compared to an average of 274 ± 4.69 μg β-carotene/g in crtB transgenic seeds (Schmidt and Pendarvis, 2017). For strategies employed to produce astaxanthin from precursor carotenoids, a 2013 experiment fortified tomato crops and observed increased accumulations of astaxanthin in fruits (16.1mg/g) (Huang, et al., 2013). In terms of astaxanthin specifically in seeds, a team of researchers was able to achieve final concentrations of 2-7 μg/ g dry weight using the *bkt1* gene from *Haematococcus pluvialis* to transform cultivar Jack of soybean (*Glycine max*) (Pierce, et al., 2015).

The eventual quantification of astaxanthin concentration as a result of our work would hope to not only rival these numbers, but also provide a more economic and sustainable source of the keto-carotenoid. The reported recommended minimum daily intake of astaxanthin is 2-4 mg/ day (Ambati, et al. 2014), with levels of up to 12 mg/ day acceptable as a therapeutic dose.

The main source of astaxanthin on the market currently aside from supplements would be salmon, due to the fortification of fish feed with the carotenoid (Fakhri, Et al., 2018). However, to obtain the reported beneficial daily level of 3.6 mg, consumption of 165 grams of wild caught salmon per day is required (Ambati, et al. 2014), or roughly a 6-ounce serving. In theory, this is possible but in practice unrealistic.

To improve bioavailability of the fat- soluble compound astaxanthin, absorption is increased when consumed with oils (Ambati, et al. 2014). This is where *Camelina sativa* proves advantageous over the tomato fruit or soybean used in other experimentation. Camelina oil is easily extracted from seeds using hexane, providing a yield of about 35.9% (Mondor, Hernández-Álvarez, 2021). Additionally, Camelina oil rivals its Canola oil counterpart in other nutritional attributes, including fatty acids and phenolic compounds (Mondor, Hernández-Álvarez, 2021). Camelina is highly desirable and lucrative as a crop due to its short life cycle of 85-100 days and potential for high yields of 1500–3000 kg/ hectare/year (Stamenković, Et al., 2021), as well as its ability to withstand diverse stress conditions.

The nutritional, economical, and agricultural implications of *Camelina sativa* cultivation evidence its newly recognized potential as not only an alternative oilseed crop, but a vessel for the biofortification with carotenoids. Following the completed steps, the project will continue on to quantify the levels of astaxanthin produced by RT-PCR and certified laboratory analysis. Of the two genes of interest, crtS and BKTChamy, the higher producer will be selected for continued trials and observation.

VI. References

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