

RESEARCH ARTICLE

Functional Analysis of the *Brassica napus* L. Phytoene Synthase (PSY) Gene Family

Ada López-Emparán^{1,2}, Daniela Quezada-Martinez¹, Matías Zúñiga-Bustos², Víctor Cifuentes³, Federico Iñiguez-Luy¹, María Laura Federico^{1*}

1. Genomics and Bioinformatics Unit, Agriaquaculture Nutritional Genomic Center, CGNA, Temuco, La Araucanía, Chile, 2. Instituto de Biología Vegetal y Biotecnología, Universidad de Talca, Talca, Maule, Chile, 3. Depto. Cs. Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Región Metropolitana, Chile

*maria.federico@cgna.cl



OPEN ACCESS

Citation: López-Emparán A, Quezada-Martinez D, Zúñiga-Bustos M, Cifuentes V, Iñiguez-Luy F, et al. (2014) Functional Analysis of the *Brassica napus* L. Phytoene Synthase (PSY) Gene Family. PLoS ONE 9(12): e114878. doi:10.1371/journal.pone.0114878

Editor: Enamul Huq, University of Texas at Austin, United States of America

Received: August 19, 2014

Accepted: November 14, 2014

Published: December 15, 2014

Copyright: © 2014 López-Emparán et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1090726), Proyecto Fortalecimiento R13F1001, Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) Regional Program and the Araucanía Regional Government /CGNA/R10C1001. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Phytoene synthase (PSY) has been shown to catalyze the first committed and rate-limiting step of carotenogenesis in several crop species, including *Brassica napus* L. Due to its pivotal role, PSY has been a prime target for breeding and metabolic engineering the carotenoid content of seeds, tubers, fruits and flowers. In *Arabidopsis thaliana*, PSY is encoded by a single copy gene but small PSY gene families have been described in monocot and dicotyledonous species. We have recently shown that PSY genes have been retained in a triplicated state in the A- and C-*Brassica* genomes, with each paralogue mapping to syntenic locations in each of the three “*Arabidopsis*-like” subgenomes. Most importantly, we have shown that in *B. napus* all six members are expressed, exhibiting overlapping redundancy and signs of subfunctionalization among photosynthetic and non photosynthetic tissues. The question of whether this large PSY family actually encodes six functional enzymes remained to be answered. Therefore, the objectives of this study were to: (i) isolate, characterize and compare the complete protein coding sequences (CDS) of the six *B. napus* PSY genes; (ii) model their predicted tridimensional enzyme structures; (iii) test their phytoene synthase activity in a heterologous complementation system and (iv) evaluate their individual expression patterns during seed development. This study further confirmed that the six *B. napus* PSY genes encode proteins with high sequence identity, which have evolved under functional constraint. Structural modeling demonstrated that they share similar tridimensional protein structures with a putative PSY active site. Significantly, all six *B. napus* PSY enzymes were found to be functional. Taking into account the specific patterns of expression exhibited by these PSY genes during seed development and recent knowledge of PSY suborganellar localization, the selection of transgene candidates for metabolic engineering the carotenoid content of oilseeds is discussed.

Introduction

The first committed step of the carotenoid biosynthetic pathway, the formation of phytoene from two molecules of geranylgeranyl pyrophosphate (GGPP), is catalyzed by the enzyme phytoene synthase (PSY). Since GGPP also serves as a precursor for the synthesis of tocopherols, chlorophylls, plastoquinones and gibberellins, *PSY* gene expression is highly regulated and represents an important checkpoint for controlling the flux (of carbon) into the carotenoid biosynthetic pathway [1–6].

PSY is encoded by a single copy gene (*AtPSY*, At5g17230) in *Arabidopsis thaliana*, therefore, controlling the flux and responses of the pathway is limited to regulating this unique enzyme [5]. Most plant species, however, contain small *PSY* gene families composed of two or three members [7–12]. In these plant species, subfunctionalization of *PSY* gene expression has been described mainly between photosynthetic and non photosynthetic tissues [7, 8, 13, 14]. This subfunctionalization has allowed for *PSY* overexpression in flowers, fruits, seeds or tubers without the detrimental effects that excessive carotenoid accumulation throughout the plant would have caused on photosynthesis [9] since carotenoids and chlorophylls are required to accumulate in a defined stoichiometric ratio in chloroplasts. In addition, it has been shown that specific *PSY* paralogues can mediate the stress-induced production of abscisic acid (ABA) in roots [11, 12, 15].

Brassica napus L. (AACC; $n=19$) is an allotetraploid species originated from the relatively recent hybridization of two base diploid genomes [16]. These diploid parental species, *Brassica rapa* L. (AA; $n=10$) and *Brassica oleracea* L. (CC; $n=9$), are considered ancient polyploids, each one of them composed of three paralogous “*Arabidopsis*-like” subgenomes [17–20]. These paralogous subgenomes are by no means equal, exhibiting variable gene content with interspersed gene losses and insertions [21–25]. We have recently reported that *PSY* genes have been retained in a triplicated state in both A- and C- *Brassica* diploid genomes and that *B. napus* bears the largest plant *PSY* gene family described to date [14]. All *Brassica* *PSY* gene family members are expressed, exhibiting overlapping redundancy and signs of subfunctionalization. In *B. napus*, expression of homeologues *BnaC.PSY.a* and *BnaA.PSY.b* were detected in all tissues, but homeologous gene pairs *BnaA.PSY.d/BnaC.PSY.f* and *BnaA.PSY.c/BnaC.PSY.e* exhibited preferential expression in chloroplast- and chromoplast-rich tissues, respectively [14].

PSY genes have been retained in *Brassica* species in spite of the well characterized fractionation of the subgenomes [25]; a process by which a genome tends to return to its original gene complement after a genome duplication event [26]. Remarkably, only 10% of *A. thaliana* predicted gene models have been found to be retained as syntenic orthologues in the three subgenomes of the sequenced *B. rapa* and *B. oleracea* genomes [19, 20]. Undoubtedly, the retention of six *PSY* genes in *B. napus* underpins the importance of this enzyme and could be at least partially explained by the selective advantage provided by increased levels of gene product in floral organs [14].

PSY activity has been shown to be a rate-limiting factor for carotenoid biosynthesis in non-photosynthetic tissues [27]. In *B. napus*, this limitation was demonstrated through pioneer work on metabolic engineering when the sole over-expression of a bacterial phytoene synthase gene (*CrtB*) in seeds raised β -carotene levels 50-fold [28]. The use of different PSY transgene sources, however, can enhance carotenoid content to various degrees, as observed in transgenic rice callus and grains [29], highlighting the importance of PSY transgene efficacy. In this context, it was important to investigate whether all six *B. napus* PSY genes were functional and determine which ones could represent better transgene alternatives to metabolically engineer the carotenoid content of oilseed crops. Therefore, the objectives of the present work were to: isolate, characterize and compare the complete protein coding sequences (CDS) of the *B. napus* PSY genes; to model their predicted tridimensional enzyme structures; to determine whether they all encode functional phytoene synthases using a heterologous complementation system and to evaluate their specific expression patterns during seed development.

Materials and Methods

Plant materials and nucleic acid isolation

Brassica napus cv. Westar plants were grown in a controlled greenhouse under 16-h-day/8-h-night cycle. For genomic DNA (gDNA) extraction, flower buds were harvested and lyophilized. DNA isolation was conducted following the CTAB procedure described by Kidwell and Osborn [30]. For total RNA extraction, cotyledons; seedlings with 3–4 true leaves; young leaves from mature plants; seeds and petals were harvested, immediately frozen in liquid nitrogen and stored at -80°C .

Rapid amplification of cDNA ends (RACE) of *B. napus* PSY genes

Total RNA was extracted using RNA-Solv Reagent (Omega Bio-Tek, GA, USA) following the manufacturer's instructions. Total RNA (2 μg) from all tissues were treated with RQ1 DNase (Promega, WI, USA) and first strand cDNA synthesis was carried out using an oligo(dT) primer and M-MuLV Reverse Transcriptase (New England BioLabs, MA, USA) as previously described [14]. Several *BnaX.PSY*-specific primers (S1 Table in [S1 File](#)) were designed in order to clone the 5' and 3' cDNA ends of six *B. napus* PSY genes using the GeneRacer kit (Invitrogen, CA, USA). Total RNAs from leaf, petal and seedling tissues were used to synthesize RACE-ready cDNA as described in Federico et al [31] with minor modifications. The resulting PCR fragments were gel-purified (E.Z.N.A. Gel Extraction Kit, Omega Bio-Tek, GA, USA) and cloned into the StrataClone Blunt vector (Agilent Technologies, CA, USA). After *E. coli* transformation, several colonies per clone were sequenced.

Cloning of *B. napus* PSY protein coding sequences

Based on a previous report [14], oligonucleotide primers (Bna.PSY.15F and Bna.PSY.8R, S1 Table in [S1 File](#)) were used to amplify *BnaC.PSY.a* (GenBank JF920037) and *BnaA.PSY.b* (GenBank JF920038) complete coding sequences (CDS) with KOD Hot Start DNA Polymerase (Novagen, WI, USA) from leaf, petal and seed *B. napus* cDNAs. Specific PCR products (1290 bp) were cloned into the pGEM-T vector (Promega, WI, USA) and several colonies per clone were sequenced to confirm identity. Specific oligonucleotide primers (S1 Table in [S1 File](#)) were designed and used to amplify the remaining four CDS (*BnaX.PSY.c-f*) using Go Taq DNA polymerase (Promega) from petal and cotyledon *B. napus* cDNAs. Specific PCR fragments were cloned in pCR2.1-TOPO vector with the TOPO TA cloning kit (Invitrogen) and several colonies per clone were sequenced to confirm identity.

B. napus PSY protein coding sequence analysis

Sequence reads from each *BnaX.PSY* clone including cDNA, RACE and genomic DNA amplifications were assembled using the contig tool of DNA Baser Sequence Assembler v3.x (Heracle BioSoft SRL Romania, <http://www.DnaBaser.com>) and compared to *BnaX.PSY.a-f* GenBank accessions (JF920037-JF920042). Nucleotide and protein sequence alignments of complete CDS were performed using ClustalW2 [32]. The Arabidopsis PSY gene (At5g17230) was included as a reference. The presence of chloroplast transit peptides was predicted using the ChloroP 1.1 server [33]. Protein conserved domains were identified using the NCBI CDD tool [34]. Nucleotide replacement (Ka) and synonymous (Ks) substitutions were estimated using DNASP5 [35]. The evolutionary history was inferred using the Neighbor-Joining method [36] with a 500-bootstrap replication support [37] using MEGA4 software [38]. Tree branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the Poisson correction method [39]. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 321 positions in the final dataset. Genbank accessions of PSY enzymes used in the analysis are as follows: Arabidopsis (AtPSY, AAA32836), cassava (MePSY1, ACY42666; MePSY2, ACY42670), maize (ZmPSY1, P49085; ZmPSY2, AAQ91837; ZmPSY3, ABC75827), pepper (CaPSY1, ACE78189.1), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, ABC75828), sorghum (SbPSY1, AAW28996; SbPSY2, XP002442578; SbPSY3, AAW28997) and tomato (SlPSY1, P08196.2; SlPSY2, ABV68559.1; SlPSY3, XP_004228928.1). The cassava MePSY3 protein sequence was obtained from Phytozome (<http://www.phytozome.net/cassava.php>) as described in Arango *et al* [12].

Molecular Modeling of *B. napus* PSY proteins

Protein structure predictions were made by a combination of *ab initio* folding and threading the amino acid sequence of each *BnaX.PSY* gene without its chloroplast transit peptide onto multiple squalene synthase and carotenoid dehydrosqualene

synthase templates using the I-TASSER online server [40]. The threaded structures were evaluated on the ProSA server [41, 42]. To reduce the steric strains present in the structures, energy minimization and molecular dynamics simulations of 2 ns were performed for each modeled structure using the NAMD software [43]. During molecular dynamics simulations, each system was solvated into a TIP3P water box and neutralized with NaCl to mimic biological conditions.

PSY functional complementation test in *E. coli*

To test whether each of the six *B. napus* PSY proteins have phytoene synthase activity, a heterologous complementation assay was carried out. Briefly, two *Escherichia coli* BL21-Gold strains were used in the complementation assay (S1A Figure in [S1 File](#)). One β -carotene producer strain (DS1B) transformed with plasmid pDS1B, a pBAD33 vector carrying *Erwinia uredovora* carotenogenic genes *crtE*, *crtB*, *crtI*, *crtY* and *CrtX* and a non-producer strain (DS1B- Δ *crtB*) transformed with plasmid pDS1B- Δ *crtB* which has a deletion of the *Eu crtB* gene [44]. Strain DS1B accumulates β -carotene and served as a positive control. Strain pDS1B- Δ *crtB* transformed with an empty pETblue1 expression vector (Novagen, USA) served as negative control. Each *B. napus* PSY coding sequence without its corresponding signal peptide (S2 Figure in [S1 File](#)) and encoding an ATG start codon was amplified using KOD Hot Start DNA Polymerase (Novagen, USA) from corresponding minipreps using specific primers (S1 Table in [S1 File](#)). PCR products were gel-purified (E.Z.N.A. Gel Extraction Kit, Omega Bio-Tek, GA, USA) and cloned into the *EcoRV* site of pETblue-1 (S1B Figure in [S1 File](#)). Each resulting *B. napus* PSY pETblue-1 expression vector was transformed into strain pDS1B- Δ *crtB* using an ECM600 electroporation system (BTX, MA, USA) to test for enzyme activity. Transformants were grown in 15 ml of Luria-Bertani (LB) medium containing appropriate antibiotics (100 μ g ml⁻¹ ampicillin, 34 μ g ml⁻¹ chloramphenicol) overnight at 37°C. Cultures (1 ml) were used to inoculate 100 ml of supplemented LB and grown at 37°C until they reached an optical density of 0.5–0.8 at 600 nm. Then, they were grown at 30°C for 64 h in the dark to maximize carotenoid production. At that time, 15 ml were used to calculate the dry weight of the cultures. A total of 80 ml were used to extract carotenoids. Cultures were prepared in triplicates. Extraction proceeded with centrifugation at 3000 g for 20 min, pellets were washed with water and carotenoids extracted three times with acetone and once with petroleum ether. Extracts were concentrated to dryness under a stream of nitrogen, resuspended in acetone, filtered and subjected to HPLC analysis as described previously [45]. Beta-carotene was identified by comparing retention times and absorption pattern spectra with that of a natural standard (DHI, Denmark). Differences in β -carotene content were established using Tukey's Honestly Significant Difference (HSD) test at ($p < 0.05$) after analysis of variance using JMP Genomics 6.1.

RT-PCR analysis during *B. napus* seed development

For reverse transcriptase polymerase chain reaction (RT-PCR) analysis, total RNA from developing seeds collected at 20, 30, 45 and 60 days post anthesis (dpa) and leaves were extracted using the Absolutely RNA RT-PCR Miniprep Kit (Agilent Technologies, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Ca, USA) from 2 µg of total RNA in a 30 µl reaction. cDNA synthesis was tested by PCR amplification of the *B. napus* 18S gene (EX119428) using the Bna.18S primer pair (S1 Table in [S1 File](#)). Absence of gDNA contamination was tested by PCR amplification of the *B. napus* Actin gene (AF111812) with the BnActin primer pair (S1 Table in [S1 File](#)). This primer pair generates a predicted 725-bp fragment for cDNA and a 900-bp fragment for gDNA due to presence of an intron. RT-PCRs were performed using GoTaq Flexi DNA Polymerase (Promega, WI, USA). Amplification started with a 95°C denaturation step (5 min), followed by 40 cycles of 30 sec at 95°C, 30 sec at 55–57°C and 1 min at 72°C, with a final 72°C extension of 5 min. RT-PCR products were run and visualized on ethidium bromide-stained 1% agarose gels. Primer specificity was checked by testing primer pairs using plasmids containing each of the six *B. napus* PSY genes as templates and running the resulting PCR products along with RT-PCR products on single stranded conformation polymorphism (SSCP) gels (S3 Figure in [S1 File](#)). For SSCP gels, 20 µL of PCR products were gel-purified (E.Z.N.A. Gel Extraction Kit, Omega Bio-Tek, GA, USA) and eluted in 20 µL. Purified products (6 µL) were then mixed with 12 µL of SSCP loading buffer (95% formamide, 10 mM NaOH, 0,25% (w/v) xylene cyanol, 0,25% (w/v) bromophenol blue). For plasmid controls, 3 µL were mixed with 15 µL of loading buffer. SSCP analysis was essentially as described in [\[14\]](#).

Accessions

B. napus PSY protein coding sequences described in this paper have been submitted to GenBank under the following accession numbers: *BnaC.PSY.a*, KF297333; *BnaA.PSY.b*, KF297330; *BnaA.PSY.c*, KF297331; *BnaA.PSY.d*, KF297332; *BnaC.PSY.e*, KF297334 and *BnaC.PSY.f*, KF297329.

Results

Sequence analysis of *B. napus* PSY genes

Gene cloning, DNA-SSCP and Southern blot analyses revealed the existence of at least six PSY homologues in *B. napus* [\[14\]](#). These PSY genes are expressed, exhibiting overlapping redundancy and signs of subfunctionalization among photosynthetic and non photosynthetic tissues [\[14\]](#). However, the question of whether this large PSY family actually encoded six functional enzymes remained to be answered. Therefore, we extended our previous work by cloning, characterizing and comparing the complete protein coding sequences (CDS) of

these six *B. napus* PSY genes. Using this complete CDS information, we were able to re-analyze their nucleotide and amino acid sequences (S2 Figure in [S1 File](#); [Fig. 1](#)). As illustrated in [Fig. 1](#), *B. napus* PSY genes encode proteins of similar length (414–424 aa) and contain characteristic motifs: a plastid transit peptide (TP), a conserved trans-isoprenyl diphosphate domain (trans-IPP) and a putative phytoene synthase active site (DXXXD) with 4 conserved aspartate residues [46]. This conserved trans-IPP domain is present in phytoene synthases, which catalyze the head to head (1'-1) condensation of two molecules of GGPP to produce phytoene. Protein sequence identity among the six PSY homologues ranged from 87.3 to 98.8 percent (S2 Table in [S1 File](#)) with most of the variability being found at the N-terminal regions, which encode TPs. When PSY proteins were evaluated without their TPs, sequence identity percentages among homologues increased, ranging from 91.6 to 99.4% (S2 Table in [S1 File](#)).

In order to investigate the evolutionary relationship between these *B. napus* PSY proteins and other plant PSYs whose functionality has been previously established [7, 8, 10–13, 15, 47], a phylogenetic analysis was conducted using 17 monocot and dicot PSYs. The phylogenetic tree showed that all *B. napus* PSY proteins cluster together with the Arabidopsis PSY (At5g17230) and that they are more closely related to dicot PSYs (MePSY1, MePSY2, SlPSY1, SlPSY2 and CaPSY1) than the extensively characterized monocot PSYs ([Fig. 2](#)). As expected, monocot PSYs formed distinct clades that exhibit different roles *in planta*, with stress-related PSYs (OsPSY3, ZmPSY3, SbPSY3) [11, 15] clustering together ([Fig. 2](#)).

Tridimensional enzyme structure prediction of *B. napus* PSY genes

Tridimensional (3D) structures of PSYs have not been determined experimentally and thus, modeling was performed using 3D information from enzymes that share conserved protein domains and catalyze similar enzymatic reactions (S4 Figure in [S1 File](#)). Like PSYs, squalene synthases (SQS) and carotenoid dehydrosqualene synthases also catalyze the head to head condensation of prenyl diphosphates to form linear terpenes [46, 48]. Our protein modeling predictions showed that the six *B. napus* PSY proteins are alpha-helix rich structures, with anti-parallel alpha-helices forming a large central cavity (S5 Figure in [S1 File](#)). As an example, the tridimensional structure prediction for the BnaC.PSY.a enzyme is shown in [Fig. 3](#). The two conserved aspartate-rich domains, DELVD and DVGED, localize to two alpha helices on opposite walls of the central cavity where the condensation of two molecules of GGPP is predicted take place to produce phytoene. Amino acid differences between the six *B. napus* PSY proteins do not alter or affect the structure of this putative active site region (S3 Figure in [S1 File](#)). Subtle differences in amino acid content found in this region, however, could have profound effects on enzyme activity as seen in other plant PSYs [50, 51].

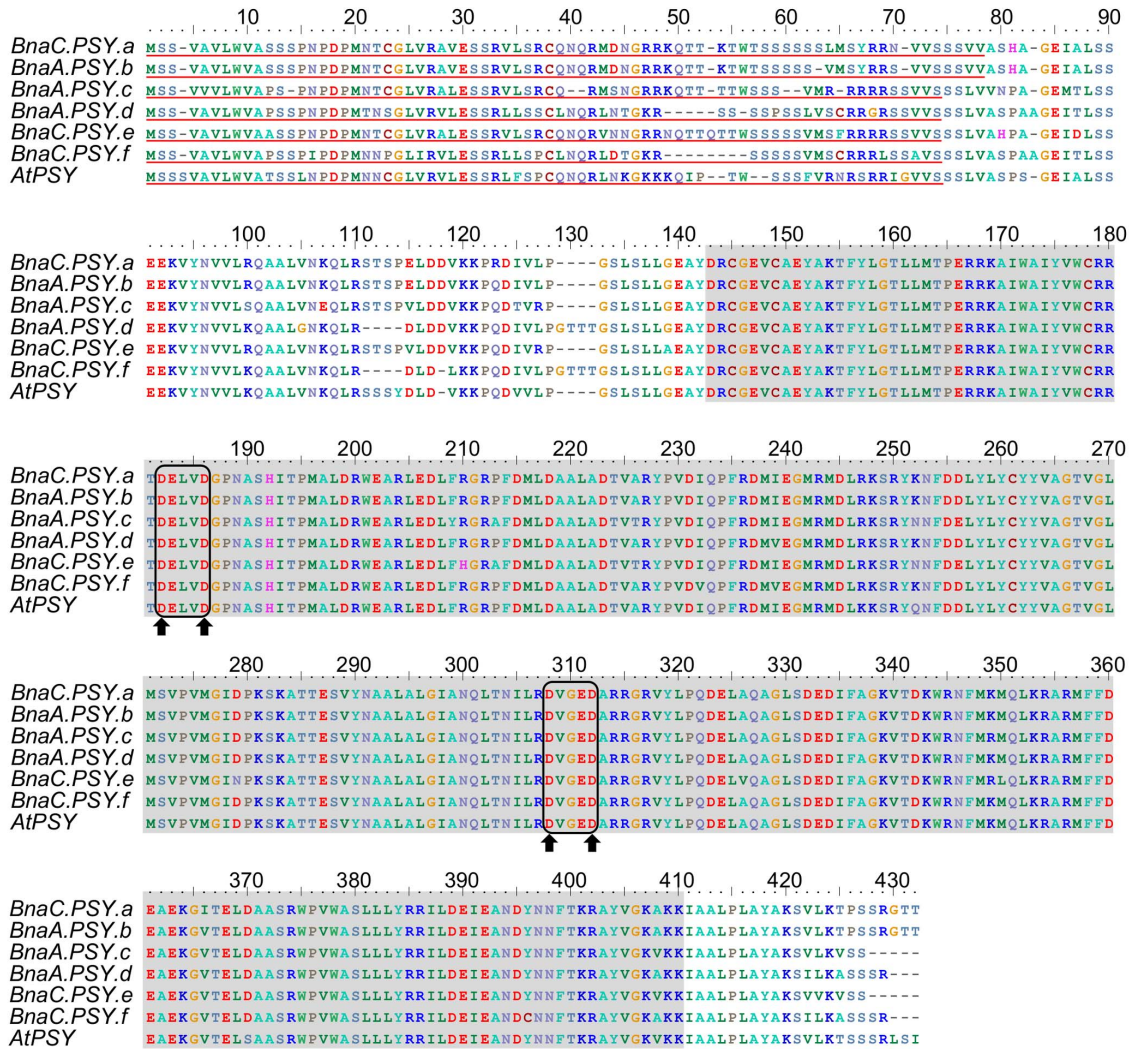


Fig. 1. Multiple alignment of *B. napus* PSY enzymes. All PSY enzymes contain a predicted chloroplast transient peptide at the N-terminal region (underlined in red) and a conserved trans-isoprenyl diphosphate synthase domain (highlighted in grey). The putative active site (DXXXD) is boxed in black. Black arrows indicate the presence of four conserved aspartate residues.

doi:10.1371/journal.pone.0114878.g001

Functional characterization of *B. napus* PSY genes

The existence of engineered strains of *E. coli* carrying different combinations of genes of the carotenoid biosynthetic pathway provide an extremely useful and practical system to assay for enzyme function [44, 52, 53]. Six *B. napus* PSY CDS, lacking their corresponding plastid TPs, were subcloned into the pETBlue1 expression vector (Novagen, USA) and transformed into *E. coli* harboring pDS1B- $\Delta crtB$ (S1 Figure in S1 File) to test for phytoene synthase activity [44]. *E. coli* cells produced the final end product of the transformed pathway, β -carotene, in the presence of the bacterial PSY gene *CrtB* (Fig. 4A) but not when the empty pETBlue1 vector was cotransformed with pDS1B- $\Delta crtB$ (Fig. 4B). These *E. coli* cells, DS1B and DS1B- $\Delta crtB$, served as positive and negative controls, respectively.

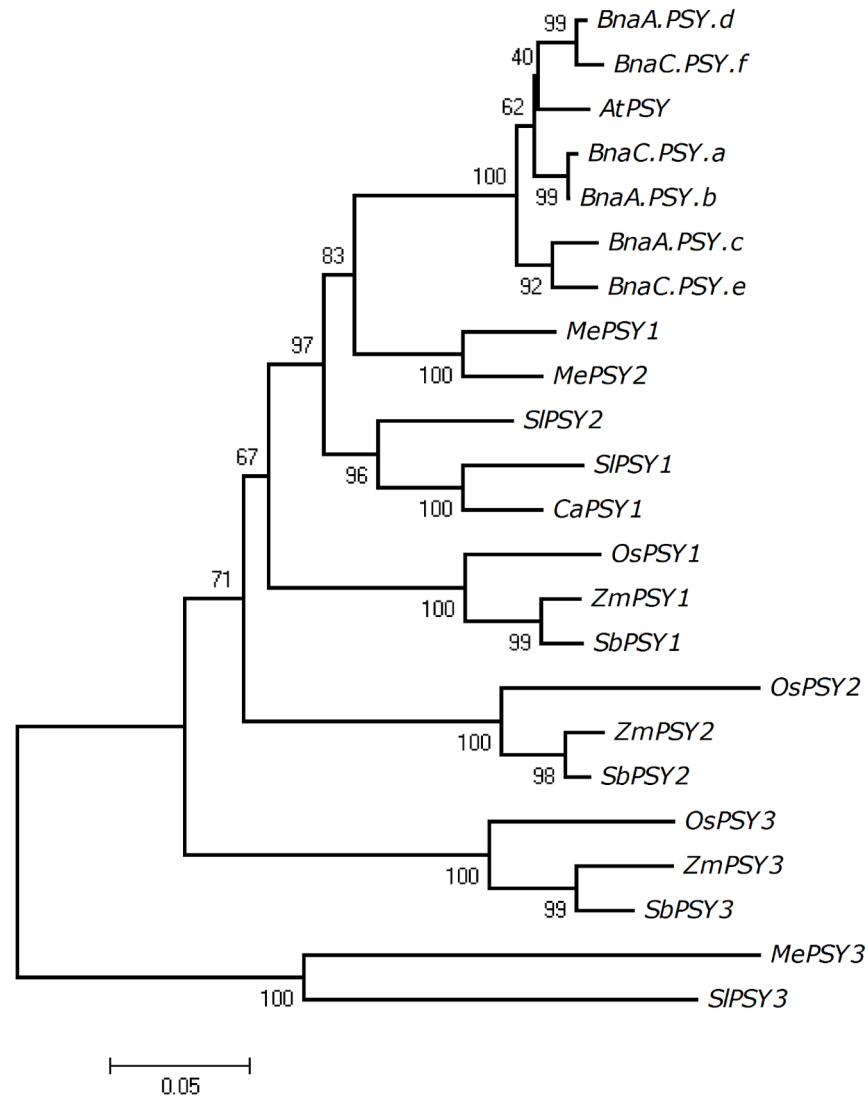


Fig. 2. Phylogenetic relationship of *B. napus* and selected monocot and dicot PSY enzymes. The evolutionary history was inferred using the neighbor-joining method using MEGA4 [38]. The percentage of replicate trees in which the associated taxa clustered together in the 500-bootstrap replication test is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units (number of amino acid substitutions per site) as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [39]. Arabidopsis (AtPSY, AAA32836), cassava (MePSY1, ACY42666; MePSY2; ACY42670), maize (ZmPSY1, P49085; ZmPSY2, AAQ91837; ZmPSY3, ABC75827), pepper (CaPSY1, ACE78189.1), rice (OsPSY1, AAS18307; OsPSY2, AAK07735; OsPSY3, ABC75828), sorghum (SbPSY1, AAW28996; SbPSY2, XP002442578; SbPSY3, AAW28997) and tomato (SIPSY1, P08196.2; SIPSY2, ABV68559.1; SIPSY3, XP_004228928.1). The cassava MePSY3 protein sequence was obtained from Phytozome (<http://www.phytozome.net/cassava.php>) as described in Arango *et al* [12].

doi:10.1371/journal.pone.0114878.g002

Significantly, *E. coli* cells harboring pDS1B- Δ crtB and cotransformed with each of the six *B. napus* PSY genes were capable of producing β -carotene (Fig. 4C-H). In all cases, HPLC analysis of carotenoid extracts from the *E. coli* cell cultures showed a β -carotene peak, which matched in retention time and spectrum with that

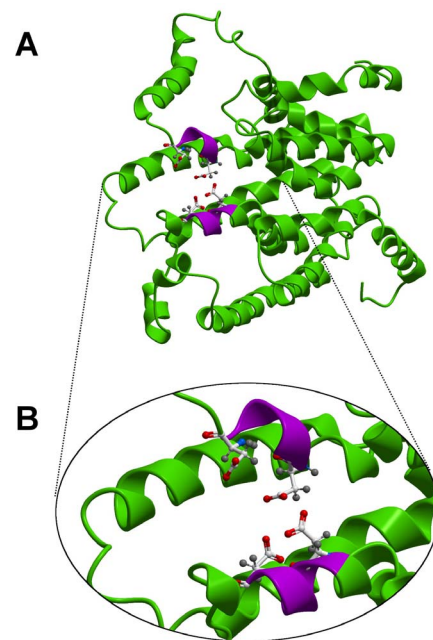


Fig. 3. *B. napus* PSY tridimensional enzyme structure prediction. A. Modeled structure of the BnaC.PSY.a protein, an alpha-helix rich structure. B. Putative active site. Alpha-helices are shown in green, the putative active site (DXXXD) in purple with the four conserved aspartate residues shown as licorice representations [49].

doi:10.1371/journal.pone.0114878.g003

observed for the positive control (Fig. 4A). Thus, we were able to confirm that this large PSY gene family encodes functional enzymes with *BnaA.PSY.d* producing the largest accumulation of β -carotene under this heterologous complementation system conditions (Fig. 4I).

***B. napus* PSY gene expression analysis during seed development**
Metabolic engineering of high carotenoid seed oil content could benefit from the overexpression of a PSY gene which naturally exhibits seed-specific expression, as seen with maize PSY1 [10, 13, 29]. Therefore, the expression of each *B. napus* PSY was assessed by RT-PCR using homologue-specific primers at four different stages of seed development. Transcripts of all six members of the PSY family were detected in developing seeds, primarily at early stages (Fig. 5). This observed expression pattern agreed with previous studies that indicated a critical stage of *B. napus* seed development in regards to cell proliferation, oil deposition and carotenoid accumulation starting at around 20 dpa [54-56]. Homologue pairs *BnaC.PSY.a/BnaA.PSY.b* and *BnaA.PSY.d/BnaC.PSY.f* could be detected at 20, 30, 45 and 60 dpa under tested conditions (Fig. 5). Interestingly, *BnaC.PSY.e*, one of the two homologies previously shown to be preferentially expressed in petals [14], was the PSY gene least expressed during seed development (Fig. 5).

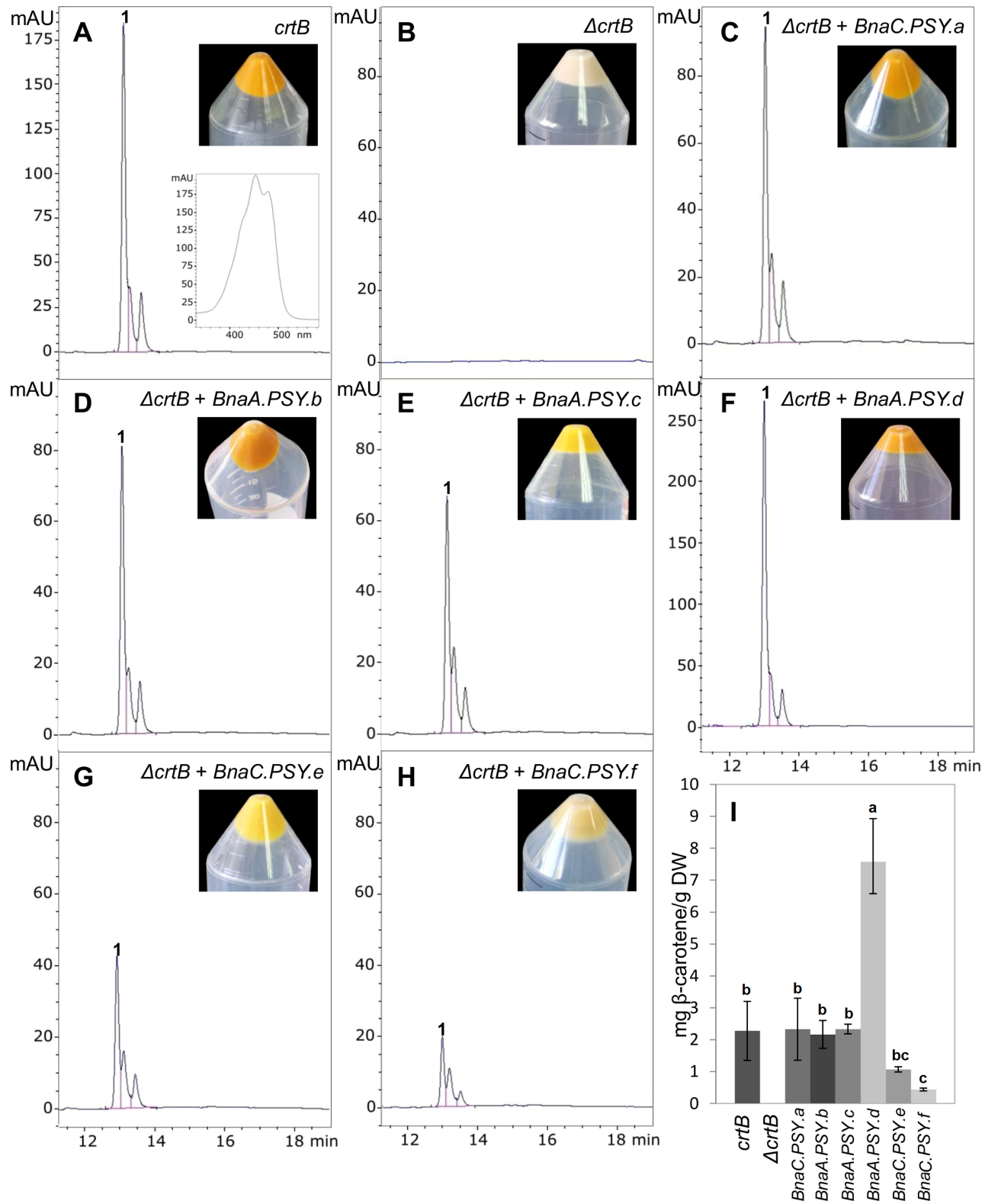


Fig. 4. Functional complementation of *B. napus* PSY genes. *E. coli* cells were transformed with: (A) pDS1B, a pBAD33 vector carrying *E. uredoovora* carotenogenic genes *crtE*, *crtB*, *crtl*, *crtY* and *CrtX*; (B) pDS1B- Δ *crtB* which has a deletion of the *Eu crtB* gene + pETBlue1 (empty vector); (C) pDS1B- Δ *crtB* + pETBnaC.PSY.a; (D) pDS1B- Δ *crtB* + pETBnaA.PSY.b; (E) pDS1B- Δ *crtB* + pETBnaA.PSY.c; (F) pDS1B- Δ *crtB* + pETBnaA.PSY.d; (G) pDS1B- Δ *crtB* + pETBnaC.PSY.e; and (H) pDS1B- Δ *crtB* + pETBnaC.PSY.f and HPLC chromatograms obtained at 450 nm are shown for each transformation. The spectral fine spectrum for beta carotene (peak 1) is shown as an example in the control panel (A). The amount (mg) of β -carotene produced by each complementation assay expressed as per gram of dry weight (DW) is shown in (I). Bars represent standard deviation calculated from three replications. For each vector combination, different letters indicate significant differences at $p < 0.05$ determined by Tukey's HSD test.

doi:10.1371/journal.pone.0114878.g004

Discussion

Carotenoids are isoprenoid compounds synthesized in plant cell plastids. They fulfill a variety of plant functions during photosynthesis, pollination, seed dispersal and stress [57–60]. Certain carotenoids also fulfill nutritional requirements in humans since they act as precursors of vitamin A (β -carotene) and help prevent age-related macular degeneration (lutein, zeaxanthin) and other diseases [61–63]. Classical breeding and metabolic engineering efforts have been mainly focused on increasing β -carotene content in edible plant tissues to alleviate vitamin A deficiency [64–68]. Successful stories of such efforts include the development of Golden Rice [29, 67]. However, predictable control of carotenoid biosynthesis has not been accomplished to date. Specific details about pathway bottlenecks, enzyme suborganellar localizations, metabolon assembly and activity are only partially known and could also be tissue and species specific [69].

Overexpression of bacterial and plant PSYs revealed that PSY activity could be rate-limiting for increasing carotenoid content in non-photosynthetic tissues [27–29], with certain PSY transgenes being more effective than others in promoting carotenoid accumulation in transgenic plants [29, 67]. Although the reason behind this different efficiency is not known [29, 69], it has been suggested that overexpressing a plant PSY gene in transgenic *B. napus* could result in higher levels of seed oil carotenoids than those obtained overexpressing *CrtB* [29]. Therefore, the importance of this study is two-fold. First, we have expanded our previous work demonstrating that all six members of the *B. napus* PSY gene family encode active phytoene synthases. Second, we provided sequence and functional information to help select transgene candidates for metabolic engineering the carotenoid content of oilseeds, including *B. napus*.

Carotenoid biosynthesis starts with the production of phytoene, a colorless carotenoid. This reaction is catalyzed by PSY, which is encoded by at least six different nuclear genes in *B. napus* (S2 Figure in S1 File). This gene family encodes enzymes with characteristic PSY motifs (Fig. 1) and a tridimensional structure which bears a putative PSY active site (Fig. 3, S5 Figure in S1 File). The highest level of sequence divergence among *B. napus* PSY proteins was found at the N-terminal TP region (Fig. 1) as previously observed in these Brassica and other PSY proteins [9, 10, 12, 14, 15]. In spite of this high sequence variability, TPs are recognized and bound by protein import complexes (translocons) in the outer (Toc) and inner (Tic) envelope membranes of plastids [70] successfully targeting nuclear-encoded proteins to plastids [71]. Different translocons (encoded by

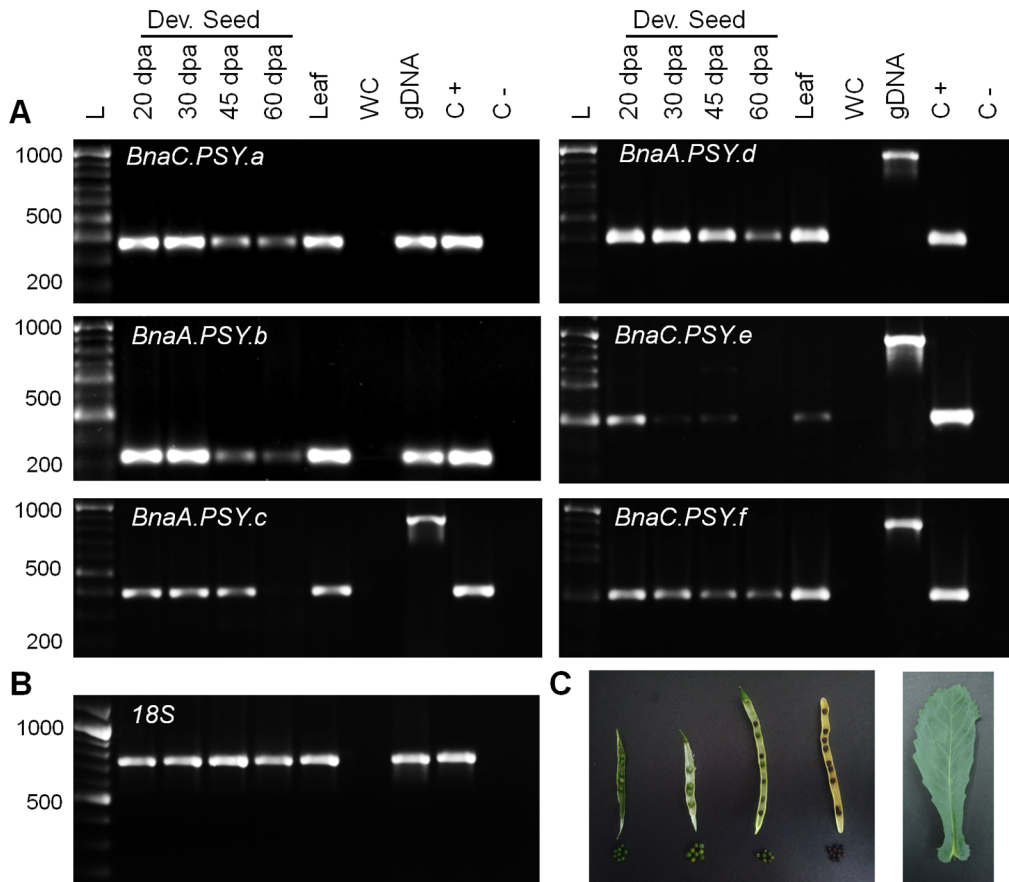


Fig. 5. *B. napus* PSY gene expression during seed development. A. *BnaX.PSY* gene expression was determined by RT-PCR using homologue-specific primers. B. *B. napus* *18S* gene expression (loading control). C. Sampling stages of *B. napus* seed development (from left to right: 20, 35, 40 and 60 days post anthesis) and leaf tissue. L: 100 bp ladder; WC: water control; gDNA: *B. napus* gDNA control; C+: PSY homologue-specific plasmid controls (positive); C-: PSY homologue plasmid controls (negative). RT-PCR (40 cycles) was performed in two biological replicates, only one is shown for simplicity.

doi:10.1371/journal.pone.0114878.g005

homologous genes) are assembled in plastids of different tissue types (e.g. photosynthetic vs. non-photosynthetic) and developmental stages [72]. No functional prediction, however, is currently possible solely based on TP sequence information.

The level of replacement and synonymous site nucleotide divergence ratio (K_a/K_s) suggest that all *B. napus* PSYs are likely undergoing purifying selection with K_a/K_s values lower than 0.28 for all gene pairs tested, which strongly indicates that these PSY proteins have evolved under functional constraint (S3 Table in S1 File). Even though *in silico* predictions (Fig. 3, S5 Figure in S1 File) indicated that these *B. napus* PSYs are likely active enzymes, only their functional characterization could confirm the ability of each of these PSYs to act as phytoene synthase. In fact, the use of a heterologous complementation system confirmed that all PSY genes could complement phytoene synthase deficient *E. coli* strains and produce β -carotene (Fig. 4). The amount of β -carotene produced by individual *B. napus* PSY genes varied, with *BnaA.PSY.d* producing the largest accumulation of β -carotene

(Fig. 4I). Albeit this differential accumulation of β -carotene in *E. coli* clones transformed with individual *B. napus* PSY genes could be the result of a variety of factors (e.g. protein stability, folding and/or solubility) related to the heterologous system itself, discrete differences in amino acid sequence found between the different PSY homologues could also be the cause. In cassava PSY2, for example, a single nucleotide polymorphism (SNP, D₁₉₁) leading to a single amino acid substitution (alanine to aspartic acid at position 191) was associated to the higher carotenoid content of yellow cassava roots [50]. Similarly, a mutation causing a proline to leucine substitution (P192L) in tomato PSY1 is linked to delayed carotenoid accumulation in the fruit, possibly due to reduced PSY enzymatic activity [73].

Arabidopsis and *B. napus* PSYs together with evaluated dicotyledonous PSY1 and PSY2 enzymes were found to be more related to cereal PSY1s which are preferentially expressed in seeds [10, 13]. Cassava and tomato PSY3s (MePSY3, SIPSY3), which were both discovered by homology searches due to their virtually undetectable gene expression levels, formed a separate branch [12, 74]. Interestingly, all six *B. napus* PSY genes were found to be expressed in seeds with homologue pairs *BnaC.PSY.a/BnaA.PSY.b* and *BnaA.PSY.d/BnaC.PSY.f* being detected at all developmental stages (Fig. 5). Taken together, the functional characterization of this *B. napus* PSY family (Fig. 4I, Fig. 5) indicates that *BnaA.PSY.d* could be the first transgene candidate of choice to enhance carotenoid content in oilseeds, followed by *BnaC.PSY.a*, *BnaA.PSY.b*, *BnaA.PSY.c* and *BnaC.PSY.f*.

Recently, the study of PSY suborganellar localization in maize cells revealed that different PSY1 allelic variants localize to distinct plastid compartments, underpinning the importance of enzyme and metabolome localization [51, 69]. Together with maize PSY2 and PSY3, rice and Arabidopsis PSYs, these six *B. napus* PSY enzymes possess serine (S) and proline (P) residues at positions equivalent to asparagine (N₁₆₈) and threonine (T₂₅₇) in maize PSY1, suggesting that they most likely localize to plastoglobuli. In addition, they also carry alanine (A) at an equivalent position to that of cassava PSY2 white allele, which could be exchanged for the aspartic acid (D) present in the yellow allele [50]. This A-to-D approach resulted in a 3-fold increase of AtPSY specific activity *in vitro* [50]. In this complex scenario, it will be worth engineering a series of *BnaA.PSY.d* mutagenized enzymes for seed-specific overexpression in transgenic *B. napus* and other related oilseed crops like *Camelina sativa*. Undoubtedly, a more detailed characterization of PSY and other carotenoid biosynthetic gene families will help to better design metabolic engineering strategies.

Supporting Information

S1 File. Contains the following files: **S1 Figure.** PSY Heterologous Complementation System. A. Two *E. coli* BL21-Gold strains were used, a β -carotene producer strain (DS1B) transformed with plasmid pDS1B, a pBAD33

vector carrying *Erwinia uredovora* carotenogenic genes *crtE*, *crtB*, *crtI*, *crtY* and *CrtX* and a non-producer strain (DS1B- Δ *crtB*) transformed with plasmid pDS1B- Δ *crtB* which has a deletion of the *Eu crtB* gene. B. Six pETBlue1-BnaX.PSY vectors were used, each carrying a *B. napus* PSY homologue, without its corresponding signal peptide, cloned into the EcoRV site. **S2 Figure.** Multiple nucleotide *BnaX.PSY* sequence alignment. **S3 Figure.** Homologue-specific PCR primer control reactions. A. Primer specificity was tested by PCR using plasmids containing each of the six *B. napus* PSY genes. Primers BnaC.PSY.a and BnaA.PSY.b could not be tested against BnaA.PSY.d and BnaC.PSY.f (clones did not include 5'UTRs). **B.** SSCP analysis of *BnaC.PSY.a* RT-PCR reactions show that only two strands exhibiting the same exact pattern as the *BnaC.PSY.a* plasmid control are present, confirming primer specificity. **C.** SSCP analysis of *BnaA.PSY.b* RT-PCR reactions show that only two strands exhibiting the same exact pattern as the *BnaA.PSY.b* plasmid control are present, confirming primer specificity. L: 100 bp ladder; BnaX.PSY.a-f: plasmid DNA controls; gDNA: *B. napus* genomic DNA control; WC: water control; ND. Not determined; 20 dpa: seed cDNA 20 days post anthesis; Leaf: leaf cDNA. **S4 Figure.** Alignment of *B. napus* PSY proteins with squalene synthase and carotenoid dehydrosqualene synthase templates. 2zcs: *Staphylococcus aureus* Dehydrosqualene synthase complexed with BPH-700; 3acx: *Staphylococcus aureus* Dehydrosqualene synthase complexed with BPH-673; 4e9u: *Staphylococcus aureus* Dehydrosqualene synthase complexed with thiocyanate inhibitor; 2zco: *Staphylococcus aureus* Dehydrosqualene synthase; 4hdl: PpcA F15L mutant from *Geobacter sulfurreducens*; 3vj8: *Homo sapiens* Squalene synthase. **S5 Figure.** Tridimensional enzyme structure prediction of *B. napus* PSY homologous pairs. Alpha-helices are shown in green and the putative active site (DXXXD) in purple with the four conserved aspartate residues shown as licorice representations [49]. **S1 Table.** Oligonucleotide primers used in this study. **S2 Table.** BnaX.PSY protein sequence identity (%). **S3 Table.** Ka/Ks. [doi:10.1371/journal.pone.0114878.s001](https://doi.org/10.1371/journal.pone.0114878.s001) (DOCX)

Acknowledgments

The authors would like to thank Mariela Mora, Jimena Carril, Humberto Gajardo, Fernando Westermeyer and Orlando Acevedo for their technical assistance; Dr. León Bravo, Dr. Wendy González and Dr. Claudia Stange for providing academic support to ALE and MZ. CGNA acknowledges INIA and UFRO for their support providing equipment and infrastructure.

Funding

This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1090726), Proyecto Fortalecimiento R13F1001, Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) Regional Program and the Araucania Regional Government/CGNA/R10C1001.

Author Contributions

Conceived and designed the experiments: ALE MLF. Performed the experiments: ALE DQ MZ. Analyzed the data: ALE DQ MZ VC FIL MLF. Contributed reagents/materials/analysis tools: VC FIL MLF. Wrote the paper: MLF.

References

1. von Lintig J, Welsch R, Bonk M, Giuliano G, Batschauer A, et al. (1997) Light-dependent regulation of carotenoid biosynthesis occurs at the level of phytoene synthase expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis* seedlings. *Plant J* 12: 625–634.
2. Welsch R, Beyer P, Hugueney P, Kleinig H, von Lintig J (2000) Regulation and activation of phytoene synthase, a key enzyme in carotenoid biosynthesis, during photomorphogenesis. *Planta* 211: 846–854.
3. Welsch R, Medina J, Giuliano G, Beyer P, von Lintig J (2003) Structural and functional characterization of the phytoene synthase promoter from *Arabidopsis thaliana*. *Planta* 216: 523–534.
4. Rodríguez-Villalón A, Gas E, Rodríguez-Concepción M (2009) Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown *Arabidopsis* seedling. *Plant J* 60: 424–435.
5. Cazzonelli Ci, Pogson BJ (2010) Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci* 15: 266–274.
6. Toledo-Ortiz G, Huq E, Rodríguez-Concepción M (2010) Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. *Proc Natl Acad Sci U S A* 107: 11626–11631.
7. Bartley GB, Viitanen PV, Bacot KO, Scolnik PA (1992) A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. *J Biol Chem* 267: 5036–5039.
8. Bartley GE, Scolnik PA (1993) cDNA cloning, expression during development, and genome mapping of PSY2, a second tomato gene encoding phytoene synthase. *J Biol Chem* 268: 25718–25721.
9. Busch M, Seuter A, Hain R (2002) Functional analysis of the early steps of carotenoid biosynthesis in tobacco. *Plant Physiol* 128: 439–453.
10. Gallagher CE, Matthews PD, Li F, Wurtzel ET (2004) Gene duplication in the carotenoid biosynthetic pathway preceded evolution of the grasses. *Plant Physiol* 115: 1776–1783.
11. Li F, Vallabhaneni R, Wurtzel ET (2008) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress induced root carotenogenesis. *Plant Physiol* 146: 1333–1345.
12. Arango J, Wüst F, Beyer P, Welsch R (2010) Characterization of phytoene synthase from cassava and their involvement in abiotic stress-mediated responses. *Planta* 232: 1251–1262.
13. Li F, Vallabhaneni R, Yu J, Rocheford T, Wurtzel ET (2008) The maize phytoene synthase gene family: overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. *Plant Physiol* 147: 1334–1346.
14. Cárdenas PD, Gajardo HA, Huebert T, Parkin IA, Iniguez-Luy FL, et al. (2012) Retention of triplicated phytoene synthase (PSY) genes in *Brassica napus* L. and its diploid progenitors during the evolution of the Brassiceae. *Theor Appl Genet* 124: 1215–1228.
15. Welsch R, Wüst F, Bär C, Al-Babili S, Beyer P (2008) A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. *Plant Physiol* 147: 367–380.
16. Nagaharu U (1935) Genome analysis in Brassica with special reference to the experimental formation of *B. napus* and its peculiar mode of fertilization. *Jpn J Bot* 7: 389–452.
17. Lysak MA, Koch MA, Pecinka A, Schubert I (2005). Chromosome triplication found across the tribe Brassiceae. *Genome Res* 15: 516–525.
18. Parkin IA, Gulden SM, Sharpe AG, Lukens L, Trick M, et al. (2005). Segmental structure of the *B. napus* genome based on comparative analysis with *Arabidopsis thaliana*. *Genetics* 171: 765–781.

19. **The *Brassica rapa* Genome Sequencing Project Consortium** (2011) The genome of the mesopolyploid crop species *Brassica rapa*. *Nat Genet* 43: 1035–1039.
20. **Liu S, Liu Y, Yang X, Tong C, Edwards D, et al.** (2014). The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploidy genomes. *Nat Commun* 5: 3930, doi:10.1038/ncomms4930
21. **O'Neill CM, Bancroft I** (2000) Comparative physical mapping of segments of the genome of *Brassica oleracea* var. *alboglabra* that are homoeologous to sequenced regions of chromosomes 4 and 5 of *Arabidopsis thaliana*. *Plant J* 23: 233–243.
22. **Rana D, Van den Boogaart T, O'Neill CM, Hynes L, Bent E, et al.** (2004) Conservation of the microstructure of genome segments in *Brassica napus* and its diploid relatives. *Plant J* 40: 725–733.
23. **Park JY, Koo DH, Hong CP, Lee SJ, Jeon JW, et al.** (2005) Physical mapping and microsynteny of *Brassica rapa* ssp. *pekinensis* genome corresponding to a 222 kbp gene-rich region of *Arabidopsis* chromosome 4 and partially duplicated on chromosome 5. *Mol Genet Genomics* 274: 579–588.
24. **Town CD, Cheung F, Maiti R, Crabtree J, Haas BJ, et al.** (2006) Comparative genomics of *Brassica oleracea* and *Arabidopsis thaliana* reveal gene loss, fragmentation, and dispersal after polyploidy. *Plant Cell* 18: 1348–1359.
25. **Cheng F, Wu J, Fang L, Sun S, Liu B** (2012) Biased Gene Fractionation and Dominant Gene Expression among the Subgenomes of *Brassica rapa*. *PLoS One* 7(5): e36442. doi:10.1371/journal.pone.0036442
26. **Sankoff D, Chunfang Z, Qian Z** (2010) The collapse of gene complement following whole genome duplication. *BMC Genomics* 11: 313 doi:10.1186/1471-2164-11-313
27. **Maass D, Arango J, Wüst F, Beyer P, Welsch R** (2009) Carotenoid crystal formation in *Arabidopsis* and carrot roots caused by increased phytoene synthase protein levels. *PLoS One* 4: e6373.
28. **Shewmaker CK, Sheehy JA, Daley M, Colburn S, Ke DY** (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J* 20: 401–412.
29. **Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, et al.** (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotechnol* 23: 482–487.
30. **Kidwell KK, Osborn TC** (1992) Simple plant DNA isolation procedure. In: Beckman J, Osborn TC, editors. *Plant genomes: methods for genetic and physical mapping*. Kluwer, The Netherlands, pp 1–13.
31. **Federico ML, Kaeppler HF, Skadsen RW** (2005) The complex developmental expression of a novel stress-responsive barley *Ltp* gene is determined by a shortened promoter sequence. *Plant Mol Biol* 57: 35–51.
32. **Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al.** (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31: 3497–3500.
33. **Emanuelsson O, Nielsen H, von Heijne G** (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8: 978–984.
34. **Marchler-Bauer A, Zheng C, Chitsaz F, Derbyshire MK, Geer LY, et al.** (2013) CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res* 41: 384–352.
35. **Librado P, Rozas J** (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451–1452.
36. **Saitou N, Nei M** (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425.
37. **Felsenstein J** (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783–791.
38. **Tamura K, Dudley J, Nei M, Kumar S** (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596–1599.
39. **Zuckerkandl E, Pauling L** (1965) Evolutionary divergence and convergence in proteins. In: V Bryson and H.J Vogel, editors. *Evolving Genes and Proteins*. Academic Press, New York. pp.97–166.
40. **Roy A, Kucukural A, Zhang Y** (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5: 725–738.
41. **Wiederstein M, Sippl MJ** (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res* 35: 407–410.

42. **Sippl MJ** (1993) Recognition of Errors in Three-Dimensional Structures of Proteins. *Proteins* 17: 355–362.
43. **Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, et al.** (2005) Scalable molecular dynamics with NAMD. *J Comput Chem* 26: 1781–1802.
44. **Niklitschek M, Alcaíno J, Barahona S, Sepúlveda D, Lozano C, et al.** (2008) Genomic organization of the structural genes controlling the astaxanthin biosynthesis pathway of *Xanthophyllomyces dendrorhous*. *Biol Res* 41: 93–108.
45. **Sáez P, Bravo L, Latsague M, Toneatti M, Sánchez-Olate D** (2013) Light energy management in micropropagated plants of *Castanea sativa*, effects of photoinhibition. *Plant Sci.* 201–202: 12–24.
46. **Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, et al.** (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* 39: 225–229.
47. **Huh JH, Kang BC, Nahm SH, Kim S, Ha KS, et al.** (2001) A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theor Appl Genet* 102: 524–530.
48. **Pandit J, Danley DE, Schulte GK, Mazzalupo S, Pauly TA, et al.** (2000) Crystal structure of human squalene synthase. A key enzyme in cholesterol biosynthesis. *J Biol Chem* 275: 30610–30617.
49. **Humphrey W, Dalke A, Schulten K** (1996) VMD: Visual Molecular Dynamics. *J Mol Graph* 14: 33–38.
50. **Welsch R, Arango J, Bar C, Salazar B, Al-Babili S, et al.** (2010) Provitamin A accumulation in cassava (*Manihot esculenta*) roots driven by a single nucleotide polymorphism in a phytoene synthase gene. *Plant Cell* 22: 3348–3356.
51. **Shumskaya M, Bradbury LMT, Monaco RR, Wurtzel ET** (2012) Plastid Localization of the Key Carotenoid Enzyme Phytoene Synthase Is Altered by Isozyme, Allelic Variation, and Activity. *The Plant Cell* 24: 3725–3741.
52. **Gallagher CE, Cervantes-Cervantes M, Wurtzel ET** (2003) Surrogate biochemistry: use of *Escherichia coli* to identify plant cDNAs that impact metabolic engineering of carotenoid accumulation. *Appl Microbiol Biotechnol* 60: 713–719.
53. **Cunningham FX Jr, Gantt E** (2007) A portfolio of plasmids for identification and analysis of carotenoid pathway enzymes: *Adonis aestivalis* as a case study. *Photosynth Res* 92: 245–259.
54. **O'Hara P, Slabas A, Fawcett T** (2002) Fatty acid and lipid biosynthetic genes are expressed at constant molar ratios but different absolute levels during embryogenesis. *Plant Physiol* 129: 310–320.
55. **Dong J, Keller W, Yan W, Georges F** (2004) Gene expression at early stages of *Brassica napus* seed development as revealed by transcript profiling of seed abundant cDNAs. *Planta* 218: 483–491.
56. **Niu Y, Wu GZ, Ye R, Lin WH, Shi QM, et al.** (2009) Global analysis of gene expression profiles in *Brassica napus* developing seeds reveals a conserved lipid metabolism regulation with *Arabidopsis thaliana*. *Mol Plant* 2: 1107–1122.
57. **Demmig-Adams B, Gilmore AM, Adams WW 3rd** (1996) Carotenoids 3: in vivo function of carotenoids in higher plants. *FASEB J* 10: 403–412.
58. **Maluf MP, Saab IN, Wurtzel ET, Sachs MM** (1997) The *viviparous12* maize mutant is deficient in abscisic acid, carotenoids, and chlorophyll synthesis. *J Exp Bot* 48: 1259–1268.
59. **Hirschberg J** (2001) Carotenoid biosynthesis in flowering plants. *Curr Opin Plant Biol* 4: 210–218.
60. **Demmig-Adams B, Adams WW 3rd** (2002) Antioxidants in photosynthesis and human nutrition. *Science* 298: 2149–2153.
61. **Bollag W** (1996) The retinoid revolution. Overview. *FASEB J* 10: 938–939.
62. **Hammond BR Jr, Wooten BR, Snodderly DM** (1997) Density of the Human Crystalline Lens is Related to the Macular Pigment Carotenoids, Lutein and Zeaxanthin. *Optometry and Vision Sci* 74(7) : 499–504.
63. **Landrum JT, Bone RA, Joa H, Kilburn MD, Moore LL, et al.** (1997) A one year study of the macular pigment: the effect of 140 days of a lutein supplement. *Exp Eye Res* 65(1): 57–62.
64. **Farré G, Bai C, Twyman RM, Capell T, Christou P, et al.** (2011) Nutritious crops producing multiple carotenoids—a metabolic balancing act. *Trends Plant Sci* 16(10): 532–40.

65. **Ortiz-Monasterio JI, Palacios-Rojas N, Meng E, Pixley K, Trethowan R, et al.** (2007) Enhancing the mineral and vitamin content of wheat and maize through plant breeding. *J Cereal Sci* 46: 293–307.
66. **Wurtzel ET, Cuttriss A, Vallabhaneni R** (2012) Maize provitamin A carotenoids, current resources, and future metabolic engineering challenge. *Front Plant Sci* 10.3389/fpls.2012.00029
67. **Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, et al.** (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 14; 287(5451): 303–5.
68. **Ducreux LJ, Morris WL, Hedley PE, Shepherd T, Davies HV, et al.** (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. *J Exp Bot* 56: 81–89.
69. **Shumskaya M, Wurtzel ET** (2013) The carotenoid biosynthetic pathway: thinking in all dimensions. *Plant Sci* 208: 58–63.
70. **Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, et al.** (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* 403: 203–207.
71. **Li HM, Chiu CC** (2010) Protein transport into chloroplasts. *Annu Rev Plant Biol* 61: 157–180.
72. **Yan J, Campbell JH, Glick BR, Smith MD, Liang Y** (2014) Molecular characterization and expression analysis of chloroplast protein import components in tomato (*Solanum lycopersicum*). *PLoS ONE* 9(4): e95088. doi:10.1371/journal.pone.0095088
73. **Gady AL, Vriezen WH, Van de Wal MH, Huang P, Bovy AG, et al.** (2012) Induced point mutations in the phytoene synthase 1 gene cause differences in carotenoid content during tomato fruit ripening. *Mol Breed* 29(3): 801–812.
74. **Sato S, Tabata S, Hirakawa H, Asamizu E, Shirasawa K, et al.** Tomato Genome Consortium (2012) The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485: 635–641.