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Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production

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Abstract

Carotenoids have drawn much attention recently because of their potentially positive benefits to human health as well as their utility in both food and animal feed. Previous work in canola (*Brassica napus*) seed over-expressing the bacterial phytoene synthase gene (*crtB*) demonstrated a change in carotenoid content, such that the total levels of carotenoids, including phytoene and downstream metabolites like β-carotene, were elevated 50-fold, with the ratio of β- to α-carotene being 2 : 1. This result raised the possibility that the composition of metabolites in this pathway could be modified further in conjunction with the increased flux obtained with *crtB*. Here we report on the expression of additional bacterial genes for the enzymes geranylgeranyl diphosphate synthase (*crtE*), phytoene desaturase (*crtI*) and lycopene cyclase (*crtY* and the plant *B. napus* lycopene β-cyclase) engineered in conjunction with phytoene synthase (*crtB*) in transgenic canola seed. Analysis of the carotenoid levels by HPLC revealed a 90% decrease in phytoene levels for the double construct expressing *crtB* in conjunction with *crtI*. The transgenic seed from all the double constructs, including the one expressing the bacterial *crtB* and the plant lycopene β-cyclase showed an increase in the levels of total carotenoid similar to that previously observed by expressing *crtB* alone but minimal effects were observed with respect to the ratio of β- to α-carotene compared to the original construct. However, the β- to α-carotene ratio was increased from 2 : 1 to 3 : 1 when a triple construct consisting of the bacterial phytoene synthase, phytoene desaturase and lycopene cyclase genes were expressed together. This result suggests that the bacterial genes may form an aggregate complex that allows in vivo activity of all three proteins through substrate channeling. This finding should allow further manipulation of the carotenoid biosynthetic pathway for downstream products with enhanced agronomic, animal feed and human nutritional values.
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1. Introduction

Carotenoids are pigments essential for photosynthetic growth in higher plants and for protection against singlet oxygen and peroxy radicals in heterotrophic organisms (Demmig-Adams et al., 1996). These compounds are currently being studied for their substantial health benefits. In the human diet carotenoids have been shown to protect against cancer and other diseases (Fullmer and Shao, 2001; Giovannucci et al., 1995; Kohlmeier et al., 1997). Specifically, β-carotene, a precursor of vitamin A (retinol), can alleviate deficiencies leading to night blindness and other related nutritional insufficiencies (Olson, 1996). Carotenoids are also used to supplement animal feed and their dietary absorption is responsible for the pigmentation of fish, eggs and lobster (Johnson and Schroeder, 1996).

Carotenoids used for nutritional or feed purposes are commonly derived either by extraction from bacteria, fungi or algae, or by chemical synthesis from petroleum byproducts (Johnson and Schroeder, 1996; Nelis and De Leenheer, 1991). However, the carotenoid content in these organisms varies depending on the environmental conditions, and extracting large quantities of carotenoids is very expensive. Thus, the primary source of carotenoids found in health supplements remains chemical synthesis in spite of the perceived value of "natural" sources for these compounds. Biosynthetic carotenoids could be made more cost-effective by developing a crop that can synthesize a spectrum of

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different carotenoids at different levels, providing food and feed manufacturers with alternatives to the synthetic sources.

Carotenoids are isoprenoid compounds, formed by the condensation of two molecules of geranylgeranyl diphosphate (Cunnningham and Gantt, 1998, Fig. 1) which gives rise to the first carotenoid, phytoene. This reaction, catalyzed by phytoene synthase, is the initial step of the carotenoid pathway and leads to the accumulation of phytoene, a colorless C_{40} hydrocarbon. In higher plants, this step is generally followed by four sequential desaturations and isomerizations of the phytoene molecule. Two enzymes catalyze these reactions that result in the introduction of additional double bonds giving rise to phytofluene, ζ-carotene, neurosporene, and lycopene. Lycopene is the precursor of the cyclic carotenoids. The synthesis of α- or β-carotene requires additional enzymes: lycopene β-cyclase introduces a ring at both ends of the linear symmetrical lycopene to form the bicyclic β-carotene. A combination of enzymatic activities between lycopene β-cyclase and lycopene ε-cyclase are needed to convert lycopene to α-carotene. In contrast, the desaturation of phytoene to generate lycopene in bacteria can be catalyzed by one single enzyme (crtI) (Sandmann, 1994), and cyclization to β-carotene requires one enzyme (crtY) as well. There is no α-carotene produced in bacteria. The fact that plant ε-cyclases cannot catalyze the introduction of a second ε-ring, but plant β-cyclases can introduce two β-rings, might reveal a mechanism whereby the production of a β,β- and a β, ε-carotene is controlled in higher plants (Cunningham and Gantt, 2001; Cunningham et al., 1996). Further downstream hydroxylation of β- or α-carotene leads to the formation of the xanthophylls zeaxanthin and lutein, respectively.

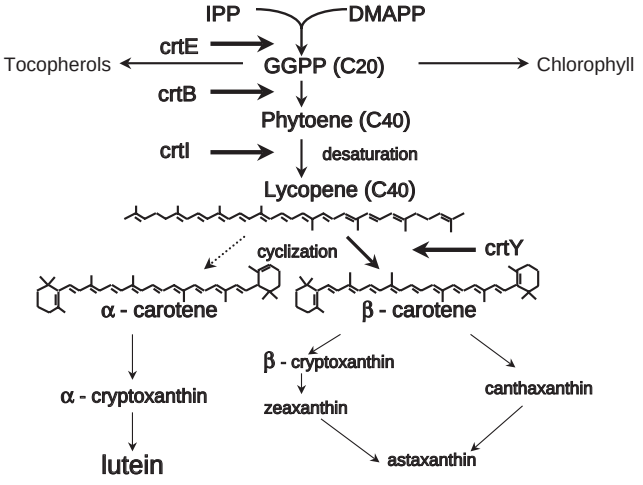

Fig. 1. A simplified diagram of the carotenoid pathway illustrating intermediates and enzymes involved in β- and α-carotene synthesis. Dotted arrows represent multiple enzymatic steps. The genes corresponding to the enzymes expressed in transgenic plants used in this study are highlighted in bold.

In plants, carotenoid biosynthesis which takes place in the plastid (Britton, 1990; Lichtenthaler et al., 1997), is not an independent pathway, but instead there are numerous other plastid localized prenyl-lipid-forming activities competing for intermediates (Chappell, 1995a, b). Reactions leading to the formation of chlorophylls, tocopherols, quinones and gibberellins all compete for the geranylgeranyl diphosphate substrate (Fig. 1). Many of these prenyl-lipid products are extremely important for the plant, and the metabolic flux through these pathways must be reliably regulated to achieve a coordinated supply of end products. The carotenoid biosynthetic enzymes are proposed to be structurally organized in a multi-enzyme complex within the plastid thylakoid membrane. The enzymatic interactions between these proteins have been characterized in some detail in daffodil and pea chloroplasts (Bonk et al., 1997; Beyer et al., 1985). At the periphery of this multi-enzyme complex reside at least two enzymes: geranylgeranyl diphosphate synthase (GGDS) and phytoene synthase. GGDS appears to be membrane bound upon import into pea chloroplasts but it may be bound to other enzymes that rely on the coordinated supply of geranygeranyl diphosphate such as chlorophyll synthase and phytoene synthase. Phytoene synthase has been shown to be tightly membrane bound in its active form (Camara, 1993). Import studies into pea chloroplasts demonstrated its binding to the thylakoid membrane. However, given that chloroplast envelopes contain carotenoids and other active enzymes, it is assumed that a portion of the active enzyme is bound to this membrane as well. The phytoene desaturation process is membrane associated and mediated by phytoene desaturase and ζ-carotene desaturase. These two enzymes are thought to interact closely. The intermediate ζ-carotene accumulates to very low amounts in vivo, providing evidence of channeled substrate flow (Bonk et al., 1997; Beyer et al., 1985; Camara, 1993). The formation of lycopene and its cyclization are tightly interlinked as well.

Previously, the phytoene synthase gene *crtB* from the bacterium *Erwinia uredovora* was over-expressed in a seed specific fashion using the napin promoter in canola (*Brassica napus*) seed (Shewmaker et al., 1999). High levels of carotenoids (approximately 50-fold over wild type) accumulated in the seed. The predominant carotenoids produced in these plants were β- and α-carotene in a ratio of 2:1, and phytoene. Other metabolite levels such as the cytoplasmically synthesized sterols remained essentially similar to those found in wild type canola, but the plastidially synthesized tocopherol levels decreased significantly compared to non-transgenic controls. These results suggest the possibility that the ratio of β- to α-carotene could be further manipulated, to either increase the levels of β-carotene harnessed for nutritional purposes, or

alternatively, impact the concentrations of downstream metabolites like the xanthophylls. These possible manipulations could significantly impact many commercial applications. To this end, additional carotenoid genes from the bacteria as well as a plant gene were introduced in canola seed.

Here we report on the cloning and seed specific expression of the genes coding for other carotenoid enzymes expressed in conjunction with *crtB*. The tested constructs contained a combination of phytoene synthase (*crtB*), and either of the genes for geranylgeranyl diphosphate synthase (*crtE*), or phytoene desaturase (*crtI*), or a lycopene β-cyclase (bacterial *crtY* or plant β-cyclase). While the expected decrease in phytoene was seen when phytoene desaturase was combined with phytoene synthase, the alteration in the ratio of β/α carotene required more than one additional enzyme and raised the possibility of channeling in carotenoid biosynthesis in plants.

2. Results and discussion

2.1. Expression of bacterial carotenoid genes in Brassica napus

Expressing the bacterial gene encoding *crtB* using the napin promoter in canola seed, leads to a 50-fold increase in the level of carotenoids (Shewmaker, et al., 1999). In this seed, the percentage of phytoene varied anywhere between 11% and 29% of the total seed carotenoid and the ratio of β- to α-carotene averaged 2:1 (Table 1). The bacterial genes encoding geranylgeranyl diphosphate synthase (*crtE*), phytoene desaturase (*crtI*) and lycopene cyclase (*crtY*) were each cloned individually in conjunction with *crtB* behind the seed specific promoter, napin (Fig. 2). Additionally, two more gene constructs were tested, one coding for the *crtB* gene and the endogenous canola lycopene β-cyclase and one where the genes for *crtB*, *crtI* and *crtY* were cloned in tandem and driven by the napin promoter. For proper chloroplast import all bacterial genes were fused to the transit peptide from the pea ribulose bisphosphate carboxylase (Rubisco) small subunit (SSU). Transgenic canola plants were generated using each of the constructs and the seeds for each transgenic line were visually monitored throughout the development of the plant. At approximately 35–40 days post-anthesis (dpa), both green and orange embryos could easily be discerned, demonstrating segregation of the carotenoid trait. It was therefore also possible to determine the segregation ratio and consequently the number of loci present in these T2 (R1) plants, by determining the ratio of orange to green number of seeds (as indicated in Table 1) for each construct tested.

2.2. Plastid targeted expression of different bacterial carotenoid genes leads to accumulation of processed gene products

Developing seed ($\sim$35 dpa) from the transgenic plants was analyzed for the presence of each of the bacterial proteins by Western immunoblotting. For this analysis two to four transgenic lines with variable segregation ratios were selected from each transformation event. The antibodies used in each case were raised to each of the purified denatured mature proteins expressed in *E. coli*. This analysis demonstrated that the mobility of all proteins corresponds to the predicted size of the mature peptide. Based on the predicted molecular weight of the processed proteins, one would thus assume that the SSU leader sequence was correctly processed from the N-terminus of the protein (Fig. 3). Each tested line revealed the presence of the expected heterologous protein. In the Western blot done using the crtB antibody (Fig. 3A), two bands were observed. An upper band of the molecular weight of approximately 33 kDa corresponding to the predicted processed crtB protein, and a lower one of approximately 28 kDa. This smaller protein band had been observed previously (Shewmaker et al., 1999) and may be a degradation product of the protein. When probed with the polyclonal antibody raised to mature crtE protein, the three transgenic lines tested all demonstrated the presence of an approximately 32.5 kDa band corresponding to the predicted molecular weight of the processed crtE protein. Since an increase in total carotenoid levels has not been observed without expression of *crtB*, the transgenic lines in panels B. and C. are shown for the protein expressed in conjunction with phytoene synthase. As such, in the case of the crtB + crtI lines (Fig. 3B lanes 3–5), a $\sim$55.0 kDa protein is visible for the blot probed with an anti-crtI polyclonal antibody. Likewise, a protein product for crtY (Fig. 3B middle), and for the canola lycopene β-cyclase (lower panel) were detected using polyclonal antiserum specific for these proteins, confirming their respective expression *in planta*. Using the antibody to the endogenous lycopene β-cyclase protein a band corresponding to this protein expressed from the crtB-only transgenic line is detectable, albeit to slightly lower levels than for the three transgenic lines expressing the lycopene β-cyclase enzyme from the napin promoter. Since an equivalent amount of total protein was loaded in each lane, this could indicate an over-expression above the endogenous levels for this particular enzyme in this seed. Finally, four transgenic lines were tested for the triple construct transformed plants, expressing the crtB, crtI and crtY proteins concertedly. These transgenic seeds were tested for the presence of all enzymes using the specific polyclonal antibodies. Fig. 3C shows the data for crtI and crtY. As can be seen, proteins of the correct molecular weights are detected for both crtI

Table 1
Carotenoid concentrations (μg gFW^{-1}) of canola seed from the lines described in this report

Gene/line	Generation	Segregation ratio	Phytoene	Lycopene	α-Carotene	β-Carotene	Total	% Phytoene	β/α ratio
crtB-2	T2	3:1	192	6	372	721	1341	14.31	**1.9**
crtB-12	T2	15:1	171	10	400	739	1341	12.75	**1.8**
crtE + crtB4	T2	3:1	80	7	153	324	599	13.35	**2.1**
crtE + crtB-24	T2	15:1	168	7	277	439	866	19.39	**1.9**
crtE + crtB-25	T2	3:1	220	7	226	488	1023	21.5	**1.7**
crtB + crtI-1	T2	3:1	42	44	336	691	1157	3.6	**2**
crtB + crtI-16	T2	3:1	65	38	428	828	1409	4.6	**1.9**
crtB + crtI-42	T2	15:1	33	12	292	536	912	3.6	**1.8**
crtB + crtI-1	T3	homozygous	20	29	462	857	1412	1.4	**1.8**
crtB + crtY-13	T2	3:1	152	7	221	458	865	17.6	**2**
crtB + crtY-16	T2	15:1	207	9	235	459	935	22.1	**1.9**
crtB + crtY-24	T2	15:1	102	8	232	456	826	12.3	**1.9**
crtB + β-cyclase-17	T2	3:1	220	6	234	488	985	22.3	**2**
crtB + β-cyclase-24	T2	255:1	152	9	128	285	595	25.5	**2.2**
crtB + β-cyclase-23	T2	3:1	99	7	180	394	718	13.8	**2.1**
crtB + crtI + crtY-4	T2	63:1	18	9	225	714	1002	1.8	**3.1**
crtB + crtI + crtY-15	T2	255:1	21	8	210	704	983	2.1	**3.3**
crtB + crtI + crtY-16	T2	255:1	28	10	281	846	1229	2.3	**3**
crtB + crtI + crtY-24	T2	3:1	10	7	131	401	578	1.7	**3**

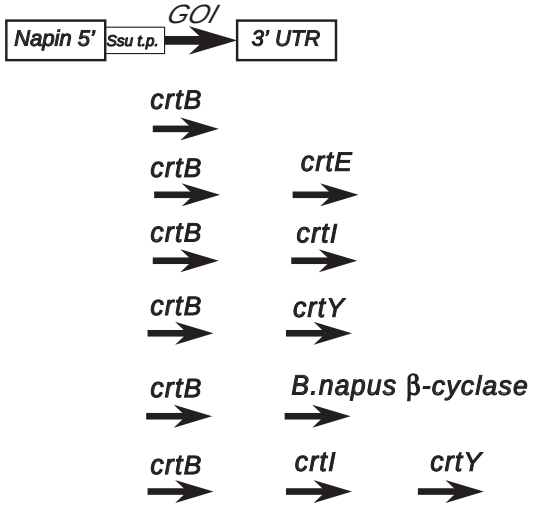

Fig. 2. Diagram illustrating the plasmid constructs used in this work. The top diagram represents the generic expression cassette containing the napin 5′ (including the promoter), the pea small subunit transit peptide, the gene of interest (GOI) followed by the untranslated 3′ region. Plasmids were engineered by subcloning relevant expression cassettes in a plant transformation vector. Plasmids contained one, two or three expression cassettes as shown.

and crtY proteins. Judging by the intensity of the signal, and based on the fact that equal quantity of total protein were loaded in each lane, there appears to be approximately equal amounts of protein (crtI and crtY) produced in these lines. Taken together, these results gave confidence that each of the transgenic expressed proteins was targeted to a subcellular organelle, the plastid, and the molecular mass of the separated polypeptides in immunoblots implied correct processing of the transit peptide in the plastids of canola seed.

2.3. Expression of crtI with crtB lowers phytoene levels, but only expression of crtI and crtY with crtB increases the ratio of β- to α-carotene

HPLC analyses were performed on the transgenic lines described above. These analyses were meant to provide the relative content of carotenoids present in the seed as well as reveal the composition of this class of compounds. The results are tabulated in Table 1. The baseline level of carotenoids and the composition of the *Brassica* seed are provided by the *crtB*-expressing construct, where the expression of crtB alone resulted in a 50-fold increase in total carotenoids, with phytoene content of approximately 11–29% and a level of β- to α-carotene at 2 : 1. By combining the expression of crtB with other enzymes in the pathway, we hypothesized that the amount of phytoene could be reduced and the ratio of β- to α-carotene could be elevated. Expressing crt E in conjunction with crt B had little or no obvious effect on the phytoene levels, the levels of total carotenoids, or on the ratio of β- to α-carotene relative to expression of crt B alone (Table 1). This may be due to the fact that geranylgeranyldiphosphate, the product

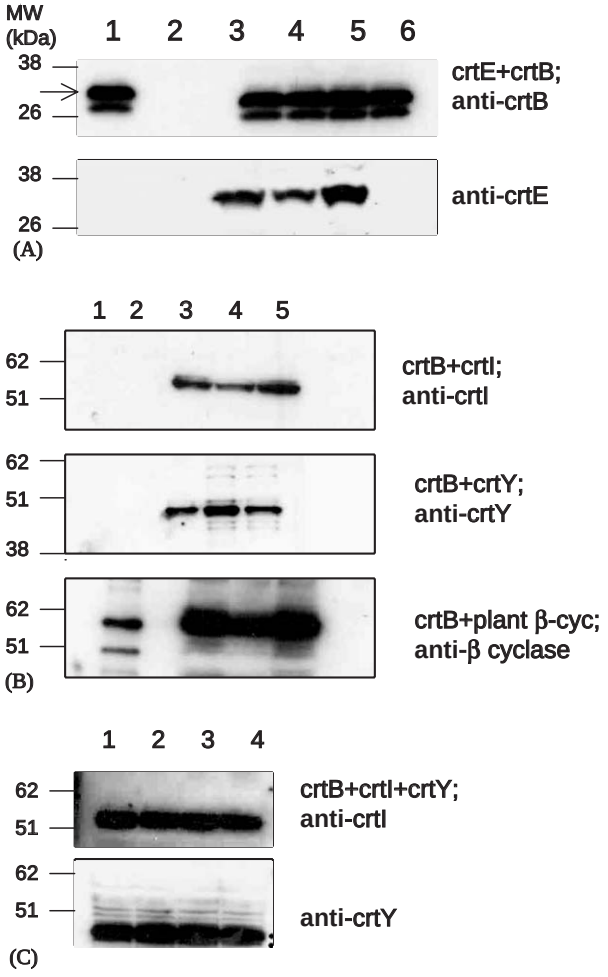

Fig. 3. Western analysis of the expressed protein products of the *E. uredovora* carotenoid biosynthetic genes in canola seed. Proteins were extracted from seed material of canola plants transformed with the constructs shown in Fig. 2. For each construct a minimum of three transgenic lines were tested. Approximately 10 μg of total protein was loaded in each lane. Panel A was tested using extracts from crtB and crtE + crtB transgenics. Lane 1 and lane 6 are control lanes using seed from lines crtB-2 and crtB-12, respectively. Lane 2 is a blank lane. Lanes 3–5 are proteins extracted from seed material of lines crtE + crtB-4, -24, and -25. Panel B shows Western blots tested with antiserum to crtI, crtY or lycopene β-cyclase protein. Lanes: 1, positive control line crtB-2; 2, blank; 3–5 are transgenic lines specific for the protein being expressed. For crtB + crtI the lines tested were (from left to right) -1, -16, and -42; for crtB + crtY the lines tested were -13, -16, and -24; for crtB + β-cyclase the lines tested were -17, -22, and -24. Panel C shows the results of the Westerns for the crtB + crtI + crtY triple construct tested with antiserum to the crtI and crtY proteins. Four lines were tested which correspond to lanes 1-4: line-4, -15, -16, -24. The location of the most relevant molecular weight markers is indicated to the left of each blot.

of geranylgeranyldiphosphate synthase, is a precursor for a number of other isoprenoids, such as tocopherols and chlorophylls. However, a significant reduction in phytoene was observed when the desaturase enzyme, crtI, was expressed in conjunction with crtB. The average percentage of phytoene in these seeds is about 3.3% compared to ~13% in the crtB-only transgenic seed. In addition, seeds from the crtB + crtI lines had on average higher lycopene levels and higher proportions of α- and β- carotene (Table 1). These seeds also have lower levels of γ- and total tocopherols, similar to what was observed for expression of crtB alone (Shewmaker et al., 1999). The increase in the percentage of α- and β-carotene suggests that the endogenous lycopene cyclases (ε- and β-cyclases) are naturally substrate-limited, and flux through cyclization reactions can increase when lycopene levels are elevated through transgenic manipulation. Chlorophyll levels were also lower compared to wild type seed indicating that expressing the combination of crtB and crtI draws GGPP for carotenoid biosynthesis and limits GGPP supply for chlorophyll production. Cumulatively the results from this construct indicated that expression of two enzymes in the pathway could improve the carbon flow towards colored carotenoid production.

Expression of *crtB* with either the gene encoding the bacterial β-cyclase (*crtY*), or the gene encoding the plant lycopene β-cyclase revealed that the total level of carotenoids and the percentage phytoene in the seed remained high, but no change in the amounts of β-carotene relative to α-carotene were observed. This indicated that the co-ordinate expression of *crtB* with a cyclase was not sufficient to alter carotenoid levels above that obtained with *crtB* alone. This result also suggests that the phytoene desaturation function is partially rate limiting, a consideration supported by the low levels of lycopene observed in these two transgenic lines (Table 1). Interestingly, when three bacterial genes, *crtB*, *crtI*, and *crtY* were expressed together, a 50% increase in the β- to α-carotene ratio from about 2 : 1 to about 3 : 1 was observed. In this seed, the levels of phytoene were decreased dramatically, even more so than in the crtB + crtI transgenic seed, and the levels of lycopene were reduced significantly. This indicated that the pool of lycopene was being further converted to cyclic carotenoids. The chlorophyll and tocopherol content of the triple construct was reduced (data not shown), indicating that carbon flux was effectively channeled towards the carotene portion of the pathway and away from other derivatives of GGPP.

We envision the three bacterial proteins forming a complex in or around the chloroplast thylakoid membrane and this structure facilitating the channeling of carbon units for efficient formation of β-carotene. For this flux to function properly, all bacterial enzymes in the pathway need to be coordinately expressed. This hypothesis is supported by the observation that when the enzyme crtI is absent some phytoene is not further utilized and the ratio of β-/α-carotene is not altered. The results presented in Table 1 are more visually followed in Fig. 4, where the HPLC traces of representative

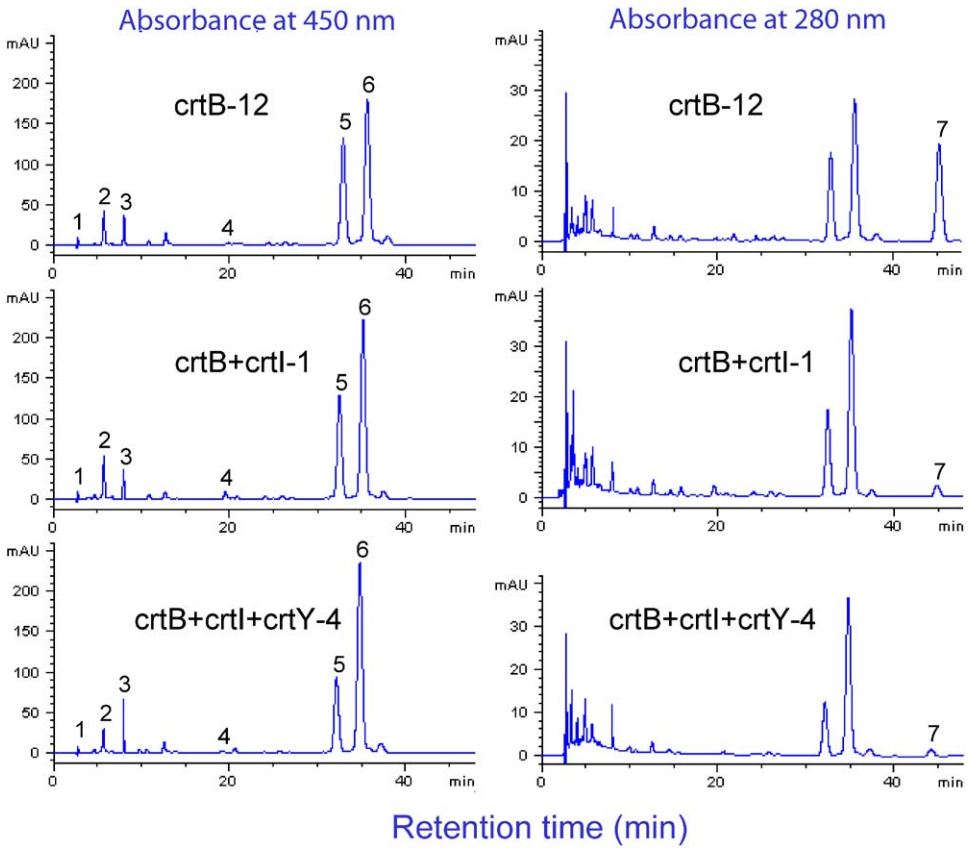

Fig. 4. HPLC analysis of mature control (crtB only), crtB + crtI or the triple construct crtB + crtI + crtY transgenic lines. The peaks correspond to the following: 1, solvent front; 2, lutein; 3, internal standard; 4, lycopene; 5, α-carotene; 6, β-carotene; 7, phytoene. Left panels show absorbance at 450 nm; right panels at 280 nm. Extracts were obtained from similar tissue weights.

crtB-only, crtB + crtI and crtB + crtI + crtY transgenic seed are illustrated. The left portion of this figure shows the absorption profiles at 450 nm, with peaks 5 and 6 corresponding to α- and β-carotene, respectively. On the right portion of Fig. 4, the absorbance at 280 nm measures the amount of the colorless phytoene present in the transgenic seed. The area under the labeled peaks corresponds to the amount of the particular compound present in the tested lines. For example, peak 5 (left panel) is reduced in the crtB + crtI + crtY transgenic seed (lowest graph) compared to its levels in the crtB-only and crtB + crtI seed (upper and middle graph), providing the difference in the β- to α-carotene ratio. Peak 7, corresponding to phytoene, appeared prominently in the crtB-only transgenic seed (Fig. 4, right panel, upper graph), but is reduced dramatically in the crtB + crtI transgenic seed (middle graph), when the desaturase enzyme is expressed concomitantly with phytoene synthase.

Overall, based on the biochemical specificities of the enzymes used in this study, we expected to observe an increase in phytoene, or an overall increase relative to the expression of *crtB* alone, when *crtB* and *crtE* were expressed together. However, no significant change in phytoene levels was observed for this double construct. Conversely, a decrease in phytoene and concomitant increase in β-carotene, similar to what has been reported previously (Ye et al., 2000), was expected when *crtB* and *crtI* were expressed together. However, while the phytoene levels did appear to decrease, there was no observed increase in β- to α-carotene ratio in these transgenic plants. In the case of the *crtB* expressed together with either *crtY* or the plant lycopene β-cyclase, an increase in the ratio of β- to α-carotene was expected. In these cases however, while total carotenoid levels in transgenic seed were increased, only one construct provided a significant change in the ratio of β- to α-carotene. This was the scenario when three bacterial genes, *crtB*, *crtI* and *crtY* were expressed coordinately from the napin promoter. We propose that these three bacterial gene products form a multi-enzyme complex in which all proteins are required for proper substrate channeling and function in plants. This principle, of all subunits from one particular species being required for correct activity of a specific end product in plants is unexpected. However, it may be a general phenomenon

not only restricted to this particular pathway in order for the enzymes involved in substrate channeling to perform the most efficient function.

Taken together, the data presented here indicates that expression of only two genes in the carotenoid pathway is not the most effective way for metabolic engineering of β-carotene in canola seed over-expressing *crtB*. However, supplying three enzymes from the same species seems to have worked the best in this case. Further research would need to be done to determine if enzymes from different bacterial species would function as effectively together or if all the enzymes need to be from the same species. Research of others has shown that supplying all enzymes of the carotenoid biosynthetic pathway in *trans* be it of bacterial or plant origin enables the biosynthesis of provitamin A in the endosperm of rice at very low levels (Ye et al., 2000; Burkhardt et al., 1997), demonstrating that the endosperm of a monocot plant and the germ of a dicot plant have different induction potentials or inherent capabilities for carotenoid biosynthesis. In other research yet, expression of only the bacterial phytoene desaturase gene *crtI* in tomato, was sufficient for a measurable increase in β-carotene levels (Romer et al., 2000) arguing for complex interactions between bacterial and plant proteins. Further manipulation of the genes described here with their plant orthologues in *Brassica* species, leading to combined expression of bacterial and plant proteins may also allow to modulate the function of the multi-protein complex and even modify the composition of the different carotenes more towards α- or β-carotene. One could envision, for example, a strategy whereby certain transcripts are down-regulated through antisense, sense suppression or RNA interference, while other genes are over-expressed leading to an even higher increase in relative β-carotene levels. Other alternatives could entail further manipulation of just the bacterial genes described here, by mutagenesis and/or other molecular approaches in the coding regions to increase the substrate binding ability and therefore improve flux through the pathway. Similarly, a breeding strategy could be employed whereby the transgenic lines presented here could be crossed to other transgenic lines to achieve different compositions of the final products. These products could lead to improved applications in human or animal nutrition.

2.4. Experimental protocol

2.4.1. Binary vector construction

The *E. uredovora* bacterial carotenoid genes used in this study were obtained from N. Misawa (Kirin Brewery, Tokyo, Japan). The pea ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (SSU)-transit peptide (tp) was used to target the bacterial genes to the plastid of the cell by creating a fusion with the genes of interest. In the case of crtB, the fusion of the tp surrounding the cleavage site has been previously described (Shewmaker et al., 1999). In the case of crtE, crtI or crtY, the fusions had the following structure surrounding the cleavage site. For crtE: Val−Lys−Cys−Met−Thr−Val−Cys (the first four amino acids of the mature crtE protein are underlined); for *crtI*: Val−Lys−Cys−Met−Lys−Pro−Thr (previously described in Misawa et al., 1993); for *crtY*: Val−Lys−Cys−Met−Gln−Pro−His. The bacterial genes + tp, *crtE*, *crtB*, *crtY* and the plant lycopene β-cyclase (with its own transit peptide) were excised with appropriate restriction enzymes. Each gene with the exception of *crtI* (see below) was subcloned in the BlgII site of vector pCGN3323 (Kridl et al., 1991; Voelker et al., 1992) resulting in the gene being located between napin 5′ and napin 3′ regions. The *crtI* gene was cloned between a napin 5′ and 3′ nopaline synthase cassette. Each cassette was then excised using appropriate restriction enzymes (NotI or HindIII) and subcloned into the plant binary vector pCGN9003 that contains the SSU-tp-*crtB* fusion flanked by the napin promoter and 3′ untranslated regions. The resulting plasmid vectors used for transformation also contained the 35S-nptII gene for selection. All recombinant DNA techniques were performed using standard molecular biology technique (Sambrook et al., 1989).

2.4.2. Plant transformation and materials

Agrobacterium mediated transformation of hypocotyls explants of *Brassica napus* L. Cultivar Quantum (Q) were carried out as described previously (Radke et al., 1992). The generation notation is as follows: T1 (R0) refers to the original plants regenerated off the hypocotyls. T2 seed refers to the seed from the T1 plants. To determine the number of loci, ten 35 DPA siliques from T1 transgenic plants (T2 seeds) were collected and the number of orange and green seeds counted. Homozygote plants were obtained from T2 plant events with one locus (3 : 1 segregation) where the T3 seed scored at approximately 35 DPA contained all orange seed.

2.4.3. Antibody generation to crtE, crtI, crtY and lycopene β-cyclases

The genes for *crtE*, *crtI*, *crtY* and plant β-cyclase (without transit peptide sequence) were PCR amplified and cloned individually in the BamHI-SmaI sites of plasmid vector pQE30 (Qiagen Inc, Carlsbad, CA) and the resulting ligated plasmid product was transformed in bacterial cell line M15pREP4 used for induction of heterologous proteins. Individual clones were selected and tested for presence of the plasmid of interest. Three positive colonies were selected in each case and grown at 37°C to an $OD_{450} = 0.4$. Expression of the respective proteins was induced by addition of 1 mM (final

concentration) IPTG. The induced proteins were purified over Ni-NTA (Qiagen) as specified in the manufacturer's catalog. One milligram of each of the purified proteins was sent for antibody production (Babco/Covence). Antibody production was according to standard procedure in NZW female rabbits.

2.4.4. Western blot analysis

Protein extracts were prepared from control and transgenic *Brassica* seeds at various days post-anthesis as described previously (Bruneau et al., 1991). Approximately 25–30 frozen seeds per line to be analyzed were ground with a mortar and pestle in the presence of liquid nitrogen. The ground pulp was placed in a Falcon tube containing 2 ml of boiling DB + buffer (2% SDS, 40 mM Tris-HCl pH 8.0, 20% glycerol, 1% β-mercaptoethanol) and boiled in water for 10 min. The samples were then centrifuged at 6000 g for 10 min. Supernatants were collected and protein quantified by BCA assay (Pierce). Approximately 10 μg of protein were electrophoresed through a pre-cast 12% acrylamide gel (Novex). The proteins were electroblotted to Nytrocellulose and blocked using 5% non-fat dry milk in $1 \times$ TBST [1 mM Tris pH 8.0, 150 mM NaCl, 1.5% Tween-20] for 30 min at room temp. The membranes were incubated overnight at room temperature with a 1 : 1000 dilution of primary antibody in blocking buffer. The Western was developed using Pierce's chemiluminescent system (Super Signal ULTRA) according to the manufacturer's instructions.

2.4.5. Analysis of carotenoids, tocopherols and chlorophylls

The analysis of carotenoids, tocopherols and chlorophylls was described in detail previously (16). Approximately 100 mg of canola seeds (a total of ~25 seeds) was extracted in 3 ml hexane/acetone/ethanol (50/25/25, v/v) using a mortar and pestle. The extracted tissue was back-extracted two additional times with 2 ml extraction solvent until no color was present. All extracts were combined and centrifuged at medium speed for 3 min using a tabletop centrifuge. The supernatant was removed and evaporated under nitrogen gas. The residue was dissolved in 3 ml hexane and 1 ml methanol and 1 ml saturated NaCl was added to the tube and mixed. The sample was centrifuged again for 3 min to allow for a better phase separation. The top layer, containing isoprenoids and hexane, was removed and transferred to a new glass tube. The bottom layer was back-extracted twice with 2 ml hexane and the resulting top layers combined. All hexane extracted layers were dried under nitrogen gas. The residue was dissolved in 2 ml acetonitrile/methylene chloride/methanol (50/40/10, v/v) and centrifuged for 3 min. Approximately 1 ml of supernatant was filtered through a 0.45 μm filter and collected in a brown auto-sampler vial and capped immediately. The isoprenoid recovery during this extraction procedure was usually >95%, using β-apo-8′-carotenal as an internal standard for quantification. High performance liquid chromatography (HPLC) of carotenoids, tocopherols and chlorophylls was performed using a Hewlett Packard 1100 machine with a Spherisorb ODS2 reverse phase C18 column (5u, 4.6 mm × 25 cm) for separation and two detectors for quantification. The mobile phase consisted of acetonitrile/dioxane/methanol containing 150 mM ammonium acetate/triethylamine (82/10/8/0.1, v/v). The flow rate was 1 ml min^{-1} and the injection volume was 20 μl. Colored carotenoids were detected using a photodiode array detector at 450 nm, phytoene at 280 nm, chlorophyll at 663 nm, and chlorophyll b at 645 nm. Tocopherols were detected using a fluorescence detector set at 290 nm for excitation and 330 nm for emission.

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