

Functional analysis of multiple carotenogenic genes from *Lycium barbarum* and *Gentiana lutea* L. for their effects on β -carotene production in transgenic tobacco

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Abstract Carotenoids are red, yellow and orange pigments, which are widely distributed in nature and are especially abundant in yellow-orange fruits and vegetables and dark green leafy vegetables. Carotenoids are essential for photosynthesis and photoprotection in plant life and also have different beneficial effects in humans and animals (van den Berg et al. 2000). For example, β -carotene plays an essential role as the main dietary source of vitamin A. To obtain further insight into β -carotene biosynthesis in two important economic plant species, *Lycium barbarum* and *Gentiana lutea* L., and to investigate and prioritize potential genetic engineering targets in the pathway, the effects of five carotenogenic genes from these two species, encoding proteins including geranylgeranyl diphosphate synthase, phytoene synthase and δ -carotene desaturase gene, lycopene β -cyclase, lycopene ϵ -cyclase were functionally analyzed in transgenic tobacco (*Nicotiana tabacum*) plants. All transgenic tobacco plants constitutively expressing these genes showed enhanced β -carotene contents in their leaves and

flowers to different extents. The additive effects of co-ordinate expression of double transgenes have also been investigated.

Keywords Carotenoid biosynthesis · β -carotene contents · *Gentiana lutea* L. · *Lycium barbarum* · Transgenic tobacco

Introduction

Carotenoids are C40 isoprenoid polyene compounds that form lipid-soluble yellow, orange and red pigments (Sandmann 2001; Zaripheh and Erdman 2002). In higher plants, carotenoids play an essential role including light-harvesting and protecting photosynthetic apparatuses from excess light energy in green tissues (Siefermann-Harms 1987; Demming-Adam and Adams 1996). In certain non-photosynthetic organs of plants, carotenoids accumulate in large amounts in chromoplast and attract pollinating or seed dispersing animals. Furthermore, dietary carotenoids are essential requirements for human nutrition (DellaPenna 1999; Hirschberg 1999). For example, β -carotene is a precursor of vitamin A and has been proposed to act as an anti-cancer agent (Mayne 1996).

The simplified pathway of carotenoid biosynthesis in plants is illustrated in Fig. 1 (Cunningham and Gantt 1998; Ronen et al. 1999). In higher plants,

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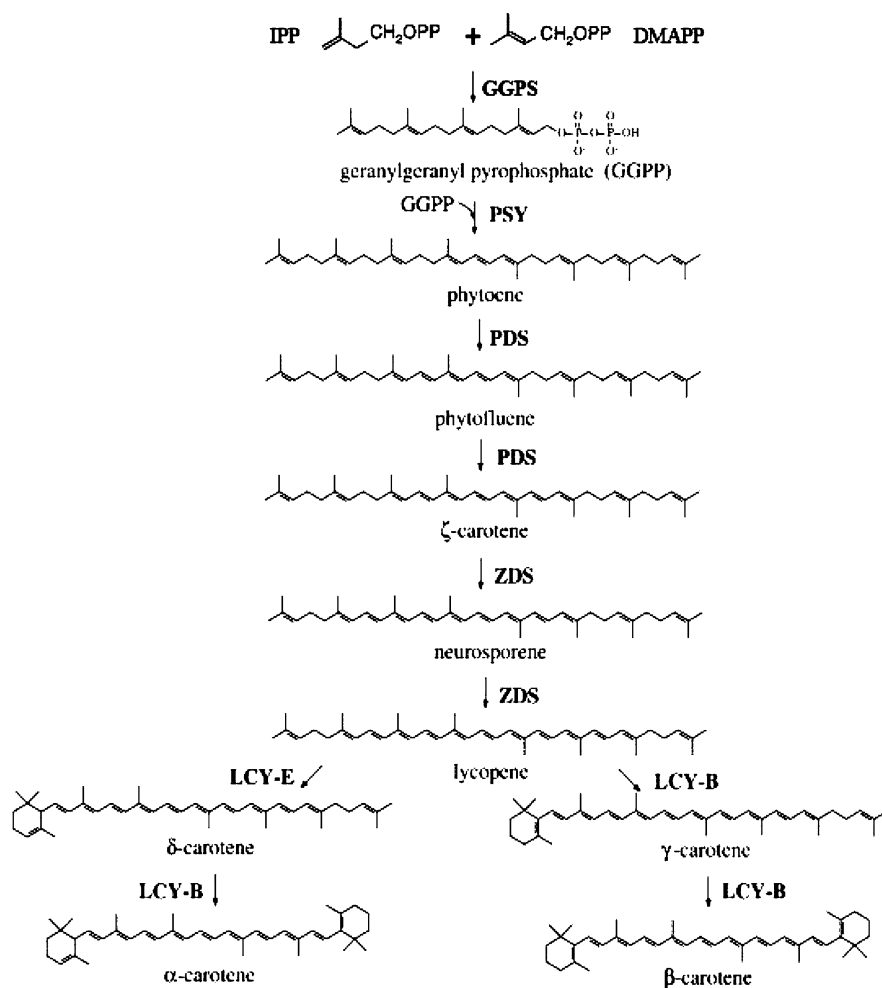
carotenoids are synthesized in the plastids from isopentenylidiphosphate precursors originating from the glyceraldehyde phosphate pyruvate pathway (Cunningham and Gantt 1998). The first committed step of carotenoid biosynthesis is a head-to-head coupling of two molecules of geranylgeranyldiphosphate (GGPP) to yield colorless phytoene by phytoene synthase (PSY). Subsequently, four additional double bonds are introduced by desaturases producing the colored carotenes phytofluene, ζ -carotene, neurosporene, and lycopene. Lycopene is cyclized twice by two individual cyclases, yielding β - and α -carotene, which subsequently are further processed to different xanthophylls, such as lutein, violaxanthin, and zeaxanthin.

Because of the importance of carotenoids, the genes encoding enzymes of carotenoids biosynthesis in

higher plants are potential targets to increase the biosynthesis of carotenoids. β -Carotene levels have been increased in rice (Al-Babili and Beyer 2005; Paine et al. 2005) and canola seeds (Shewmaker et al. 1999a; Ye et al. 2000) and in tomato fruits (Romer et al. 2000) employing genetic transformation with carotenogenic (*crt*) genes.

Lycium barbarum and *Gentiana lutea* L. are both sources of β -carotene, with the largest amount of β -carotene being found respectively, in fruit and flowers. This makes them interesting and useful experimental materials to investigate their endogenous molecular mechanisms regulating β -carotene biosynthesis. In the present study, functions related to β -carotene production of five genes cloned from *L. barbarum* and *G. lutea* L. have been investigated simultaneously in the model system tobacco.

Fig. 1 Biosynthesis of carotenes in plants



The carotenoid biosynthetic genes encode *Gentiana lutea* geranylgeranyl diphosphate synthase (GIGGPS), *Lycium barbarum* phytoene synthase (LbPSY), *Gentiana lutea* δ -carotene desaturase (GIZDS), *Lycium barbarum* lycopene β -cyclase (LbLCY-B) and *Gentiana lutea* lycopene ϵ -cyclase (GILCY-E). Meanwhile, the effects of co-expressing of different double transgene combinations were also investigated. This study provides information on the physiological roles of those five genes involved in β -carotene biosynthesis. On the basis of the comparison of the effect of manipulating these genes, the potential use of these genes in genetic engineering in order to increased β -carotene content in other plants was also discussed.

Materials and methods

Isolation and sequence analysis of *L. barbarum* and *G. lutea* L. carotenogenic genes

Total RNA was extracted from the petal (for *Ggps*, *Zds*, *Lcy-e* of *G. lutea*) or green leaves (for *Psy* and *Lcy-b* of *L. barbarum*) according to the method described by (Sambrook et al. 1989) and mRNA was purified by the Straight A's mRNA isolation system (Novagen, Madison, WI, USA). The first strand cDNA was synthesized with the cDNA cloning kit (Stratagene, La Jolla, CA, USA). *G. lutea* *Ggps*, *Zds*, *Lcy-e* and *L. barbarum* *Psy*, *Lcy-b* were amplified by PCR with primers shown in Table 1 based on the sequences published previously. The amplified cDNAs were cloned with a TA Cloning Kit (Invitrogen, San Diego) and the sequences were determined using the standard Big Dye Terminator (PE Applied Biosystems, Foster City, CA) on the

ABI PRISM™ 310 genetic analyzer (ABI, Foster City, California, USA).

Vectors, bacterial strains for cloning and transgenic tobacco construction

Sequenced PCR amplification products were ligated to the pGEM T-Easy vector (Promega, Madison, USA). After electroporation into DH5 α competent cells, transformants were selected by blue/white screening using IPTG and X-Gal on ampicillin LB agar plates. The encoding sequences of each transgene were fused with CaMV35S promoter and nopaline synthase (Nos) terminator and inserted in EBis-KH02 binary vector with hygromycin as selective marker in plants. The modified pEBis-KH02 vectors were introduced into *Agrobacterium tumefaciens* EHA101 by electroporation (Sambrook et al. 1989).

Agrobacterium tumefaciens carrying the binary vector was incubated in liquid YEB medium containing 30 mg kanamycin/l and 100 mg spectinomycin/l for 48 h at 28°C with shaking. The bacterial suspension was pelleted by centrifugation at 3,500 g for 30 min, then resuspended in liquid inoculation media consisting of MS medium supplemented with 100 mM acetosyringone. The ordinary tobacco (*Nicotiana tabacum*) cultivar leaves were cut transversely into segments approx. 2 mm long and immersed in the inoculation media. Leaf tissues were then transferred onto MS medium containing 0.1 mg α -naphthaleneacetic acid/l (NAA), 1 mg 6-benzyladenine (BA)/l and cultured for 3 days at 25°C in the dark. For selection of transformed calluses, the segments were transferred onto MS medium containing 0.1 mg NAA/l, 1 mg 6-BA/l, 250 mg Claforan/l and 30 mg hygromycin/l, and were cultured at 25°C in the dark. After 9–10 days, transformed calluses were transferred under fluorescent light

Table 1 PCR primers for reverse transcriptase-PCR of carotenoids biosynthesis genes from *Lycium barbarum* and *Gentiana lutea*

cDNA	Accession #	Forward primer	Reverse primer
<i>Ggps</i>	<u>AB028667</u>	ATGGTGGATTCATGGGTGGTTCAA	CTAATTCTGCCTATACGCAATATA
<i>Zds</i>	<u>AB017370</u>	ATGCCCTTTCACCTTCAACTTAGTGAA	TCAGTTTGGAATGCTTGCTTCTGC
<i>Lcy-e</i>	<u>AB017373</u>	ATGGAGGTGTTCAAAGTTCGAAAC	CTACATTCCAGATAAGTTCCTTAC
<i>Psy</i>	<u>AY986508</u>	ATGTCTATTTGTACGCTATGGGTTGTT	TCATGTTTGGGGTATCCATAAAAGA
<i>Lcy-b</i>	<u>AY906864</u>	ATGGATACTTTAGTGAAAACCTCCA	TTATTCTTTGTCCCGCAATAAGTT

[16/8-h (day/night) cycle] until shoots were induced on the surviving green calluses. Regenerated shoots were excised from the calluses and transferred to MS medium supplemented with 250 mg Claforan/l and 30 mg hygromycin/l. Ten to fifteen independent transgenic tobacco plants were acclimatized and transferred to the greenhouse. After self-fertilization and harvesting capsules from selected transgenic lines, seeds were germinated with hygromycin to generate homozygous plants overexpressing respective transgenes. Apparent changes in phenotypes were observed and recorded in transgenic tobacco plants with each construct.

Semiquantitative RT-PCR, Northern blot for transgenic analysis

This was performed by conducting parallel reactions on each RNA sample: one using specific primers for transgene and the other using primer for a house-keeping gene (18S rRNA). Total RNA from 150 mg leaf tissue was extracted as described above and used as templates to synthesize the first-strand cDNA as described previously. Second-strand cDNA amplifications were performed with gene-specific primers as: *Ggps* (forward: 5'-CAGCTTGTGCGGTGGAGA TGA-3'; reverse: 5'-CTACTTGGCCAGCTACTAA GCC-3'); *Psy* (forward: 5'-AGTTTAATGTACA ATGGAAGA-3'; reverse: 5'-CTACTTGGCCAGCT ACTAAGCC-3'); *Zds* (forward: 5'-TGGTTCCTATC CAAAGGCGG-3'; reverse: 5'-CACCTAAGATGAA ACCTGCC-3'); *Lcy-b* (forward: 5'-GAGAATCTTG AATTTGAGCTC-3'; reverse: 5'-ATGTAGCATCA AGGCAATCTA-3'); *Lcy-e* (forward: 5'-ATGTAAA GGCTGGTGGATCTGAG-3'; reverse: 5'-ACA CGTCCATAAGCACGACCAA-3'). For semiquantitative RT-PCR, the PCR products from various experiments had confirmed that it was in the linear range. For Northern hybridization, total RNA was prepared as described above and then separated on a denaturing 1.5% agarose/formaldehyde gel and transferred to nitrocellulose filters according to the method of Sambrook et al. (1989). As probes, fragments amplified in semiquantitative RT-PCR were labeled by a random primed labeling kit (Stratagene La Jolla, CA) to a specific activity $>8 \times 10^8$ cpm/ μ g DNA. Hybridization was for 16 h at 42°C, and washes were at 50°C in $0.1 \times$ SSC and 0.1% SDS. X-ray films

were exposed for 3 days at -70°C using intensifier screens.

Extraction and determination of β -carotene by HPLC

Approx. 500 mg fresh leaf material was ground to a fine powder and extracted with 1 ml acetone/methanol (2:1 v/v) overnight in dark. The extracts were evaporated and the residue redissolved in 1 ml acetone. A sample of each extract was then analyzed by HPLC using a standard C-18 reverse-phase column (3.9 mm $\times$ 300 mm). β -Carotene peaks were identified from on-line spectra and retention times relative to commercial trans β -carotene standards (Sigma-Aldrich) and the concentration was determined from peak areas in a chromatogram recorded at 450 nm. For each transgene, 15 transgenic tobacco plants were used and the number 5 leaf from the top and petals with color was pooled and used for pigment extraction. All data were subject to analysis of variance using a statistical package (SAS, Research Triangle Park, NC), and mean differences were analyzed using the Bonferroni method ($P = 0.05$).

Results and discussion

Cloning the carotenogenic genes from *G. lutea* and *L. barbarum*

The coding sequences of *G. lutea Ggps*, *Zds*, *Lcy-e* and *L. barbarum Psy* and *Lcy-b* were cloned with PCR-based methods from petal and leaf tissues. Following PCR reactions, PCR products were amplified and sequenced. The sizes of their respective full length cDNA and ORF together with the molecular weight of each encoded peptide are shown in Table 2. The first three homologues from other plant species with the highest similarity in amino acid sequences with the five encoded proteins are also listed in Table 2.

Production and analysis of transgenic tobacco plants

Using the *Agrobacterium*-mediated method, 15–30 independent transgenic lines of tobacco were generated for each transgene. Transcript levels of transgenic plants expressing each transgene were

Table 2 The size of full length cDNA and ORF of each carotenogenic gene together with the molecular weight and number of amino acids of their encoded peptide

Gene	cDNA		Peptide	
	Full-length (bp)	ORF (bp)	MW (kDa)	Similarity with homologues
<i>Ggps</i>	1482	1103	40.1	<i>Arabidopsis thaliana</i> (63%), <i>Eucommia ulmoides</i> (62%), <i>Sinapis Alba</i> (61%)
<i>Psy</i>	2085	1280	48.6	<i>Tagetes erecta</i> (86%), <i>Lycoper esculentum</i> (83%), <i>Arabidopsis thaliana</i> (77%)
<i>Zds</i>	2257	1763	65.0	<i>Citrus paradise</i> (85%), <i>Zea Mays</i> (84%), <i>Oryza Sativa</i> (83%)
<i>Lyc-b</i>	2165	1526	57.2	<i>Daucus carota</i> (87%), <i>Lycoper esculentum</i> (79%), <i>Capsicum annum</i> (79%)
<i>Lyc-e</i>	1988	1586	59.6	<i>Daucus carota</i> (84%), <i>Solanum tuberosum</i> (76%), <i>Tagetes erecta</i> (71%)

The peptide similarities with homologues from other plant species are also shown

determined using semi-quantitative reverse transcriptase (RT)-PCR and Northern blots and the results showed a strong and de novo expression of transgene on the transcriptional level (Fig. 2).

Transgenic tobacco overexpressing different heterologous carotenogenic genes exhibited increased β -carotene biosynthesis to various extents in comparison to control plants (Table 3). GGPS catalyzes the biosynthesis of GGPP via sequential adding three IPP molecules to a DMAPP molecule (Hirschberg 2001). Overexpression of *Ggps* should supply more precursors for carotenogenesis. However, in this study, overexpression of *glGgps* gene in tobacco only caused slight increase (<20%) in leaf β -carotene content. This indicated that there is only little increase in carbon flux into the carotenoid pathway caused by *glGgps* overexpression. *Ggps*-overexpressing tobacco did not result in any abnormal phenotype compared with control plants and they can flower normally without obvious change in morphology and color.

Phytoene synthase is a key enzyme in carotenoids biosynthesis and it is the branching enzyme that determines the flux of carbon source towards carotenoids (Bramley et al. 1992; Shewmaker et al. 1999b); therefore, it has been the target in several studies. Increasing the activity of phytoene synthase leads to substantial increases in carotenoid accumulation and/or result in carotenoid accumulation in plant tissues that do not normally produce carotenoid pigments in tobacco, tomato, rice and rapeseed (Shewmaker et al. 1999a). However, *Psy*-overexpressing plants have been shown to be reduced in stature because of changes in the gibberellic and abscisic acid contents due to the competition for prenylpyrophosphates by both pathways (Fray et al. 1995). In this work, dwarf

phenotype was observed with *lbPsy*-overexpressing tobacco in which about 100% and 50% increase of β -carotene content could be found in leaf and flower tissues, respectively (Fig. 3).

Phytoene desaturase has also been the target of genetic manipulation in plants. Manipulation of the desaturation activity in tomato has resulted in a three-fold increase in the β -carotene content in transgenic tomato fruit (Romer et al. 2000). In the present study, overexpression of the *glZds* gene in tobacco resulted in 91% increase of β -carotene in leaf and 49% increase in flower (Fig. 3).

In the carotenogenic pathway, there are two downstream routes after the generation of lycopene for the formations of acyclic and cyclic carotenoids respectively (Schmidt-Dannert 2000). Two types of LYC have been found in plants: lycopene β -cyclase (LCY-B) can introduce a β ring into each end of lycopene to form γ -carotene (monocyclic) or β -carotene (dicyclic), while LYC-E can only introduce one ε ring into one end of lycopene to produce monocyclic δ -carotene (Cunningham et al. 1996). Larger increases (>7-fold) in β -carotene have been seen with overexpression of LCY-B in tomato fruit (Rosati et al. 2000; D'Ambrosio et al. 2004). In this study, overexpression of either *glLyc-e* or *lbLyc-b* resulted in a similar and moderate increase ($\sim 50\%$) in β -carotene content in transgenic tobacco leaves.

Successful alteration of flower color through genetic engineering of the biosynthetic pathways of the flavonoids has already been achieved (Tanaka et al. 1998; Forkmann and Martens 2001). Similar efforts have also been made for carotenoid pigments. The light yellow petals has been altered to reddish in *Lotus japonicus* by modification of the carotenoid biosynthetic

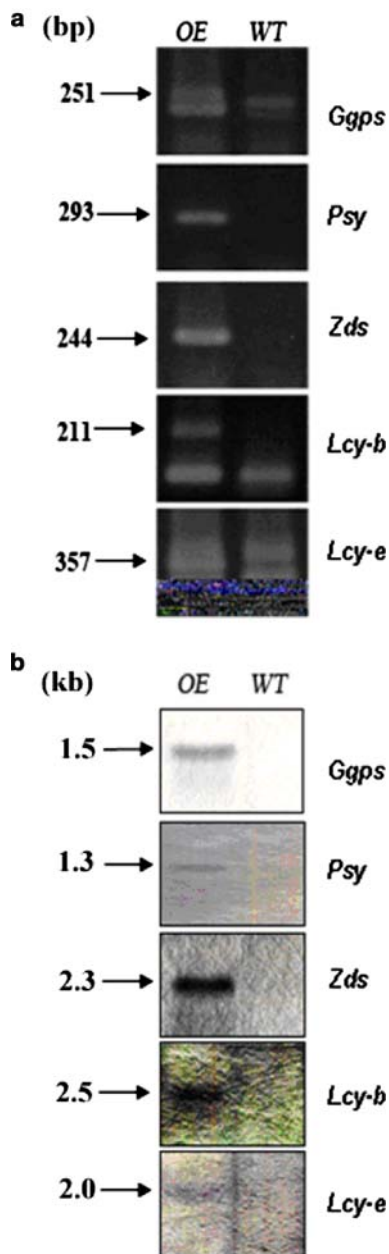


Fig. 2 Transcript analysis of heterologous carotenogenic genes in tobacco leaves of transformed plants and non-transformed controls by semi-quantitative reverse transcriptase (RT)-PCR (a) and Northern blots (b). OE, Overexpressing transgenic plants; WT, wild-type plants

pathway using the marine bacteria *crtW* gene (Suzuki et al. 2007). In this study, apparent changes in flower color intensity were observed in transgenic tobacco plants with *Psy*, *Zds*, and *Lcy-b* genes. High β -carotene content ($\sim 150\%$) makes the flowers display fainter

coloration owing to the characteristic absorption spectrum of β -carotene in *Psy*- and *Zds*-overexpressing transgenic tobaccos. Slight (15%) and no visible pigmentation alternations in flower color were observed for the *Lcy-e* transgenic tobacco plants.

Co-ordinate expression

In addition to the effects of single gene overexpression on β -carotene accumulation in tobacco leaves and flowers, the effects of different combinations of double transgenes have also been investigated by crossing the transgenic tobacco plants. As the most effective transgene in terms of increasing β -carotene content in tobacco leaves and flowers, the *lbPsy*-overexpressing tobacco plant was crossed with *glZds*, *glLcy-b*, and *lbLcy-e* transgenic plants and the hybrids showed a further increase in β -carotene content by 62%, 40% and 19%, respectively in leaf tissues (Fig. 4). When *glZds*-overexpressing tobacco was crossed with *glLcy-b* and *lbLcy-e* overexpressing transgenics, it resulted a 47% and 33% increase in β -carotene content in tobacco leaf tissue (Fig. 4). When *glLcy-b* overexpressing tobacco was crossed with *lbLcy-e* overexpressing transgenics, it resulted in another 36% increase in β -carotene content in tobacco leaf tissue. Although no dramatic increase of β -carotene has been observed in all these combinations, it indicated an additive effect existed for all these transgenes (Fig. 4). Meanwhile, this demonstrated that there might be more unknown metabolic cross-talk between the carotenoid pathway and other pathways, and the interplays between endogenous carotenogenic genes and the introduced foreign genes also need to be determined to precisely modifying carotenoid metabolism by introducing a or several *trans* genes.

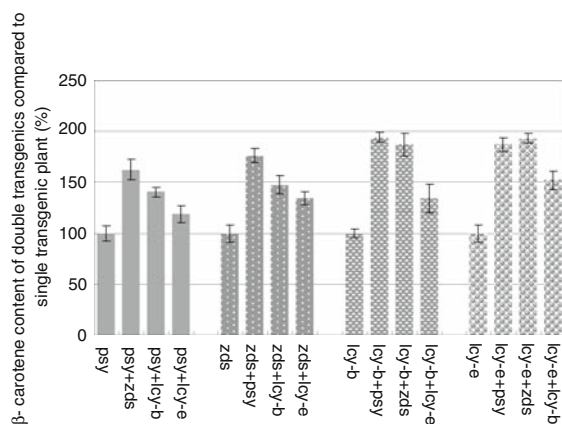
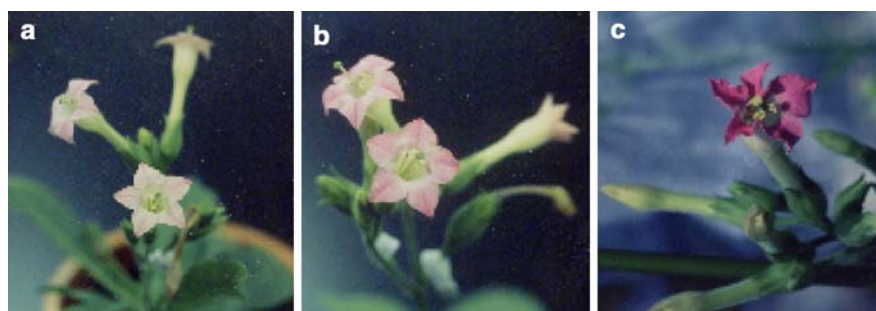
In conclusion, we cloned carotenogenic genes from two plant species accumulating large amount of β -carotene, *Lycium barbarum* and *Gentiana lutea*, including *glGgps*, *lbPsy*, *glZds*, *lbLcy-b*, and *glLcy-e*. These genes correspond to each carotenoids biosynthetic step and we demonstrated that they are all effective and useful for β -carotene enhancement and flower color modification in heterologous expression systems. In the future, up- and down-regulation of these genes in other crop and ornamental plants will give more insight into their individual physiological functions involved in β -carotene biosynthesis.

Table 3 β -Carotene accumulation of different transgenic tobacco plants expressing carotenogenic genes in comparison with control plant and phenotype data

Gene	^a β -carotene in leaf		^a β -carotene in flower	^b Phenotype
	mg gDW ⁻¹	% of WT	% of WT	Compared to WT
<i>Ggps</i>	2.6 $\pm$ 0.2	118 $\pm$ 9	N/A	Normal
<i>Psy</i>	4.6 $\pm$ 0.2	209 $\pm$ 11	151 $\pm$ 8	ly, co
<i>Zds</i>	4.2 $\pm$ 0.2	191 $\pm$ 10	149 $\pm$ 5	dw, co, fr
<i>Lyc-b</i>	3.3 $\pm$ 0.1	150 $\pm$ 8	142 $\pm$ 6	ly, fw, co
<i>Lyc-e</i>	3.2 $\pm$ 0.2	145 $\pm$ 11	116 $\pm$ 5	ly, fw, co
WT	2.2 $\pm$ 0.1	100	100	Normal

^a Mean value of the 15 plants shown above

^b ly, Leaf yellow; dw, dwarf; fw, flowers white; fr, flower development reduced; co, capsules orange

Fig. 3 Change in flower coloration of carotenogenic gene-overexpressing tobacco lines. *Psy*-OE (a), *Zds*-OE (b) compared to control plants (c)**Fig. 4** Addictive effects of different double transgene combinations in leaf β -carotene content of transgenic tobacco plants

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