Plant Biotechnol Rep (2010) 4:269–280
DOI 10.1007/s11816-010-0147-y

ORIGINAL ARTICLE

Metabolic engineering of *Lilium* × *formolongi* using multiple genes of the carotenoid biosynthesis pathway

Pejman Azadi · Ntui Valentaine Otang · Dong Poh Chin · Ikuo Nakamura · Masaki Fujisawa · Hisashi Harada · Norihiko Misawa · Masahiro Mii

Received: 8 July 2010 / Accepted: 31 July 2010 / Published online: 20 August 2010

Abstract *Lilium* × *formolongi* was genetically engineered by *Agrobacterium*-mediated transformation with the plasmid pCrtZW-N8idi-crtEBIY, which contains seven enzyme genes under the regulation of the CaMV 35S promoter. In the transformants, ketocarotenoids were detected in both calli and leaves, which showed a strong orange color. In transgenic calli, the total amount of carotenoids [133.3 μg/g fresh weight (FW)] was 26.1-fold higher than in wild-type calli. The chlorophyll content and photosynthetic efficiency in transgenic orange plantlets were significantly lowered; however, after several months of subculture, they had turned into plantlets with green leaves that showed significant increases in chlorophyll and photosynthetic efficiency. The total carotenoid contents in leaves of transgenic orange and green plantlets were quantified at 102.9 and 135.2 μg/g FW, respectively, corresponding to 5.6- and 7.4-fold increases over the levels in the wild-type. Ketocarotenoids such as echinenone, canthaxanthin, 3′-hydroxyechinenone, 3-hydroxyechinenone, and astaxanthin were detected in both transgenic calli and orange leaves. A significant change in the type and composition of ketocarotenoids was observed during the transition from orange transgenic plantlets to green plantlets. Although 3′-hydroxyechinenone, 3-hydroxyechinenone, astaxanthin, and adonirubin were absent, and echinenone and canthaxanthin were present at lower levels, interestingly, the upregulation of carotenoid biosynthesis led to an increase in the total carotenoid concentration (+31.4%) in leaves of the transgenic green plantlets.

Keywords Carotenoid · Ketocarotenoid · *Agrobacterium*-mediated transformation · *Lilium* × *formolongi* · Metabolic engineering · Multi-gene construct

Introduction

Carotenoids are a group of natural pigments that furnish attractive colors to fruits, vegetables and flowers, and their compositions are highly valuable (Sandmann 2001). The colors of yellow, orange and red are provided with carotenoid pigments in tissues or organs such as flowers and fruits. They fulfill many functions in plants such as light harvesting and photoprotection (Demmig-Adams and Adams 1996; Frank and Cogdell 1996). Carotenoids play an important role in human nutrition and health as the primary dietary source of provitamin A (Combs 1998) and in reducing the incidence of certain diseases including cardiovascular, cancer and aging-related (Collins 1999; Krinsky et al. 2003). In addition, they are utilized as natural pigments for industrial food preparation, pharmaceutical industry and the cosmetics industry. Color in horticultural crops and their products represents an important quality trait, which enhances the product diversity and marketing opportunities (Clotault et al. 2008).

In order to produce the pigments with increased amounts or altered quality of carotenoids, transgenic technologies

Electronic supplementary material The online version of this article (doi:10.1007/s11816-010-0147-y) contains supplementary material, which is available to authorized users.

P. Azadi · N. V. Otang · D. P. Chin · I. Nakamura · M. Mii (✉)
Laboratory of Plant Cell Technology,
Graduate School of Horticulture, Chiba University,
648 Matsudo, Matsudo, Chiba 271-8510, Japan
e-mail: miim@faculty.chiba-u.jp; azadip22@yahoo.com

M. Fujisawa · H. Harada · N. Misawa
Central Laboratories for Frontier Technology,
i-BIRD, 3-570 Suematsu, Nonoichi-machi,
Ishikawa 921-8836, Japan

are now expected to apply for various plant species. The carotenoid biosynthetic pathway in crops has been modified to increase carotenoid levels by genetic manipulation using key genes in carotenoid biosynthesis such as the phytoene synthase gene (*crtB* or *Psy*) (Shewmaker et al. 1999; Ye et al. 2000; Rosati et al. 2000; Fraser et al. 2002; Ducreux et al. 2005; Paine et al. 2005; Diretto et al. 2006, 2007a, b; Fujisawa et al. 2008; Naqvi et al. 2009; Apel and Bock 2009). Recently, pathway engineering of plants for producing novel ketocarotenoids, that is carotenoids with 4-ketolated β-ring, has been the focus of many researchers of plant biotechnology, since ketocarotenoids including astaxanthin and canthaxanthin confer special benefits on human health (Mann et al. 2000; Stalberg et al. 2003; Ralley et al. 2004; Morris et al. 2006; Gerjets et al. 2007; Zhu et al. 2008; Hasunuma et al. 2008; Harada et al. 2009; Fujisawa et al. 2009). Flower color is also changed from yellow to red by synthesizing ketocarotenoids in the petal (Suzuki et al. 2007). In these plants, the β-carotene ketolase gene (*crtW*, *bkt1* or *bkt2*) has been introduced sometimes along with β-carotene hydroxylase gene (*crtZ*) (reviewed by Misawa 2009, 2010), since higher plants cannot produce ketocarotenoids naturally. One to three genes were introduced to the target plants in most of these reports, while six and seven key genes, involved in ketocarotenoid formation and originating from a soil bacterium *Pantoea ananatis*, marine bacteria *Brevundimonas* sp. strain SD212 and *Paracoccus* sp. strain N81106, were recently successfully introduced into *Arabidopsis* and *Brassica napus* (Harada et al. 2009; Fujisawa et al. 2009). The use of these cassettes resulted in an increase in the total carotenoid amount, which was 19- to 30-fold higher than the untransformed control (Fujisawa et al. 2009).

Lilium is one of the most important floricultural crops because of the large beautiful flowers with varied colorations. Among the *Lilium* species, *L. longiflorum* and *L.* × *formolongi*, which is an interspecific hybrid between *L. longiflorum* and *L. formosannum*, are most commonly used as cut flowers worldwide. However, their cultivars only have white flowers, and hence production of the cultivars with novel flower colors has long been desired. In the present study, therefore, we considered the possibility for the expression of seven multiple enzyme genes from the carotenoid biosynthesis pathway, as shown in Fig. 1, in the transgenic *L.* × *formolongi* by using *Agrobacterium*-mediated transformation method as the first step to create the cultivars with novel flower color, such as yellow to orange by producing carotenoids with orange colorations, and hopefully by producing astaxanthin, which has a red

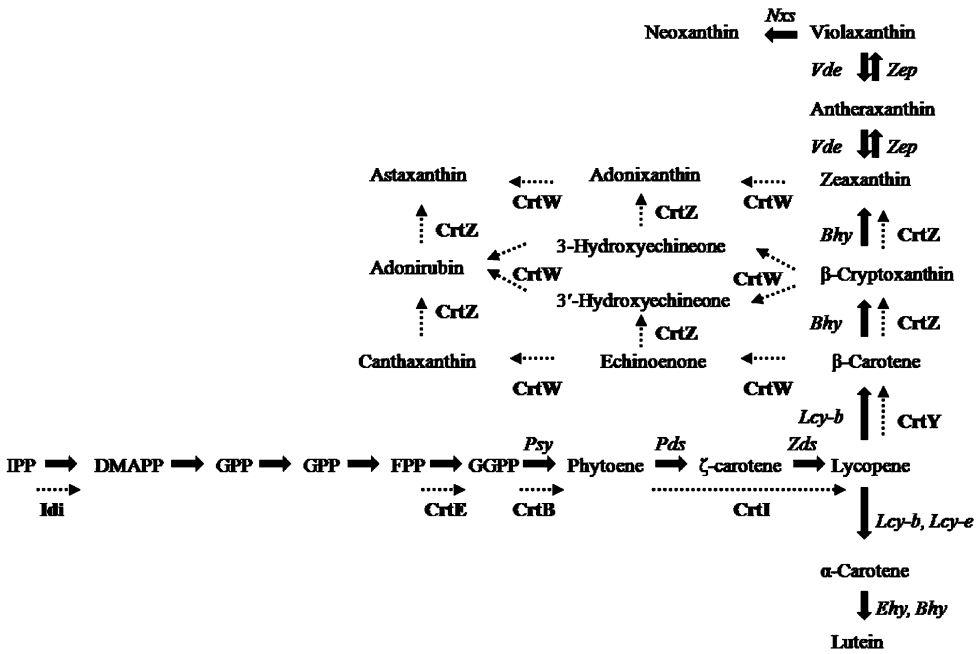

Fig. 1 A schematic representation of the carotenoid biosynthesis pathway for introduction of seven genes cassettes. *Arrows* indicate the reactions of endogenous enzymes (*italics*). *Dashed arrows* indicate the reactions of the bacterial enzymes (*bold*). *IPP* Isopentenyl pyrophosphate (diphosphate), *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *Psy* plant phytoene synthase, *Pds* plant phytoene desaturase, *Zds* plant ζ-carotene desaturase, *Lcy-b* plant lycopene β-cyclase, *Lcy-e* plant lycopene ε-cyclase, *Bhy* plant β-carotene hydroxylase, *Ehy* plant ε-carotene hydroxylase, *Zep* plant zeaxanthin epoxidase, *Vde* plant violaxanthin de-epoxidase, *Nxs* plant neoxanthin synthase, *Idi* bacterial IPP isomerase, *CrtE* bacterial GGPP synthase, *CrtB* bacterial phytoene synthase, *CrtI* bacterial phytoene desaturase/carotene isomerase, *CrtY* bacterial lycopene β-cyclase, *CrtW* bacterial β-carotene ketolase, *CrtZ* bacterial β-carotene hydroxylase

color. This is the first report concerning transforming *Lilium* using multiple genes of the carotenoid biosynthesis pathway.

Materials and methods

Plant material

Meristematic nodular calli were established in *Lilium* × *formolongi* 'Akasu' by using the method described previously (Mii et al. 1994). Calli of 10–14 days after subculture were used for inoculation with *Agrobacterium*.

Bacterial strain and plasmid construct

Plasmid pCrtZW-N8idi-crtEBIY was constructed by inserting the *crtI-crtY* cassette into the pCrtZW-N8idi-crtEBI (Harada et al. 2009), in which the *crtI* cassette had been removed with *Pac*I digestion (Fig. 2). The seven foreign genes originated from a soil bacterium *Pantoea ananatis* (formerly called *Erwinia uredovora* 20D3; *crtE*, *crtB*, *crtI* and *crtY*), and a marine bacteria *Brevundimonas* sp. strain SD212 (*crtZ* and *crtW*) and *Paracoccus* sp. strain N81106 (formerly called *Agrobacterium aurantiacum*; *idi*) (Fujisawa et al. 2009; Harada et al. 2009). For inoculation, *A. tumefaciens* strain EHA101 containing the plasmid was used. Each of these seven genes was driven by the CaMV 35S promoter and their transcriptions were terminated by the terminator of the heat shock protein, the 18.2-kDa gene (*hsp*) from *Arabidopsis thaliana* (Fig. 2). Hygromycin phosphotransferase (*hpt*) gene, flanked between Nos promoter and terminator, was used as a selectable marker.

Transformation

For inoculation and co-cultivation, modified MS (Murashige and Skoog 1962) medium lacking KH_2PO_4, NH_4NO_3, KNO_3 and $CaCl_2$ and containing 100 μM acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA), 10 mM 2-morpholinoethane-sulfonic acid (MES) (Wako Pure Chemical Industries, Osaka, Japan), 30 g/l sucrose and 1 mg/l picloram was used. Hygromycin-resistant calli and transgenic plantlets were obtained by the *Agrobacterium*-mediated transformation system (S1) as described previously (Azadi et al. 2010).

Detection of seven genes in transgenic plants by polymerase chain reaction analysis

Genomic DNA was extracted from leaves (0.5 g) of the control and putatively transgenic plantlets of *Lilium* following the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980), modified by Ogaki et al. (2008). Seven genes were amplified by polymerase chain reaction (PCR) to detect transgenic events. The amplifications were performed in 20 μl reaction mixture containing 0.5 U TaKaRa Ex Taq polymerase and 1× Ex Taq buffer (Takara Shuzo, Shiga, Japan), 0.2 mM each dNTP, 0.5 μM each primer and 50 ng of template DNA. The PCR was carried out under the following conditions: 94°C for 4 min, 30 cycles of three steps [94°C for 30 s (denaturation), 61°C for 30 s (annealing) and 72°C for 1 min (elongation)], and 72°C for 1 min.

Amplification of the genes was done using the primer sets as described in Table 1. After amplification, 4 μl of PCR products were loaded on the gel and detected by

Keys:

35S-crtZ: P35S- tp- crtZ –T*hsp*

35S-crtW: P35S- tp- crtW- T*hsp*

35S-idi: P35S- tp- idi- T*hsp*

35S-crtE: P35S- tp- crtE- T*hsp*

35S-crtB: P35S- tp- crtB- T*hsp*

35S-crtI: P35S- tp- crtI- T*hsp*

35S-crtY: P35S- tp- crtY- T*hsp*

Fig. 2 Schematic representation of plasmid pCrtZW-N8idi-crtEBIY. T-DNA construct of the plasmid pCrtZW-N8idi-crtEBIY harbouring the seven key gene cassettes used for genetic transformation of *Lilium* × *formolongi*. *LB* and *RB* left and right borders, *Pnos* and *Tnos* promoter and terminator of the nopaline synthase gene (*nos*), *hpt* hygromycin phosphotransferase, *P35S* CaMV 35S promoter, *tp* transit peptide sequence from pea RuBisCO small subunit, *Thsp* heat shock protein (HSP18.2) gene terminator from *A. thaliana*. Structure of each gene cassette is indicated in *keys*. The origins of genes are indicated in *parentheses*

Table 1 List of primers used in this study

Gene		Sequence (5′–3′)	PCR product size (bp)
cryZ	F[a]	atgcatggtttcctttggtc	402
	R[b]	tcaagctccgctagaagaaga	
crtW	F	tgctgttgctgagcctagaa	717
	R	cctctccaaagtctccacca	
idi	F	ttgcgctcatcatagaatcg	743
	R	cagcaagatcaccagatcca	
crtE	F	atcagttattgcccgtggag	802
	R	tcaaaccaggcctgaataaaa	
crtB	F	ttgcgacagcctcaaagtta	702
	R	ttttccggtaaacctgcttc	
crtI	F	cccagtgccattgaagaact	1,092
	R	atcaaacggcgtaaacatcc	
crtY	F	ttcggttgaaaaagggtcag	761
	R	ttgcaatgctgctaataccg	

[a] Forward primer

[b] Reverse primer

ethidium bromide staining after electrophoresis on 1% agarose gel at 100 V for 30 min.

Southern hybridization

Approximately 20 µg of genomic DNA was digested overnight with the enzyme *Pac*I for *crtW* gene and the enzyme *Mlu*I for *crtE* and *crtI* genes. DNA fragments were separated by electrophoresis on a 0.8% agarose gel, and subsequently transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore, Billerica, MA, USA). The *crtW*, *crtE* and *crtI* probes were generated from plasmid DNA of plasmid pCrtZW-N8idi-crtEBIY by labeling with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany), and the primer sets as described in Table 1 (Hamill et al. 1991). Prehybridization and hybridization were carried out using high-SDS hybridization buffer containing 50% deionized formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 2% blocking solution, 0.1% *N*-lauroylsarcosine and 7% SDS. Washing and detection were performed according to the instruction manual of the DIG labeling and Detection System (Roche Diagnostics). For detection of hybridization signals, membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics) for 1 h.

RNA extraction

Total RNA was extracted from 200 mg of leaves of *Lilium* × *formolongi* transgenic orange and green and wild-type plantlets using Krapp et al. (1993) method with slight modification. Leaf from each sample was ground in liquid nitrogen in pre-cooled mortar. The samples were then homogenized in RNA extraction buffer [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 7.2 µl β-mercaptoethanol]. After adding phenol and chloroform, the mixture was centrifuged at 12,000*g* for 20 min; the supernatant was washed with equal volume of isopropanol and incubated at −20°C for 1 h. Total nucleic acid was precipitated with 150 µl extraction buffer and 150 µl isopropanol at −20°C overnight. The pellet after centrifugation (12,000*g*, 10 min) was washed with 500 µl 70% ethanol and centrifuged again at 10,000*g* for 10 min at 4°C. The pellet was then dissolved in 15 µl DEPC-treated water.

Northern blot analysis

To detect the expression of the carotenoid biosynthesis genes at the messenger RNA (mRNA) level, 20 µg of denatured total RNA of each sample was resolved by electrophoresis on a 1.5% (w/v) agarose gel containing formaldehyde in 1× morphpropane sulphonic acid (MOPS) buffer and transferred onto a nylon membrane (Immobilon-Ny + Transfer Membrane; Millipore) in 20× SSC buffer. The membrane was dipped in 2× SSC and cross-linked by UVC 500 Cross-linker (Amersham Bioscience, UK). Northern hybridization was carried out using the DIG-labeled DNA probes for all seven genes of the carotenoid biosynthesis, following the same procedure used in Southern blot analysis, with slight modification where necessary. Prehybridization (2 h), hybridization (overnight) and washing were performed at 50°C. For detection of hybridization signals, membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics) for 30 min.

Herbicide resistance assay

In order to determine the bleaching effect of herbicide, six leaves each of wild-type and transgenic plantlets (one leaf in each Petri dish) were placed on MS media containing 3% sucrose, 2.5% Gelrite and 15 µM of the herbicide norflurazon (AccuStandard, New Haven, USA), under the 16/8 h photoperiod at 25°C for 4 weeks.

Pigment extraction and analysis

Carotenoid pigments were extracted from calli and leaves with methanol–chloroform as described by Fraser et al. (2000). The total pigment extracts were then saponified with methanol containing 18 mM sodium hydroxide as described by Yuan and Chen (1999), and eluted in

methanol–chloroform. High-performance liquid chromatography (HPLC) was conducted using a 2695 separation module (Waters, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector (Waters). HPLC–PDA analysis was carried out as described previously (Choi et al. 2005). Carotenoids were monitored at its maximum absorption wavelength between 280 and 500 nm. Authentic standard of carotenoids were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemical (Tokyo, Japan), and used to quantify each carotenoid.

Chlorophyll was extracted from leaves [100 mg fresh weight (FW)] following the method of Shabala et al. (1998). Chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*) were quantitated from the absorbance at 644 and 662 nm, respectively. The Chl*a* and Chl*b* concentrations in the leaf tissue were calculated according to the following equation:

$$[\text{Chl}a] = 9.784D_{662} - 0.99D_{644}$$

$$[\text{Chl}b] = 21.42D_{644} - 4.65D_{662}$$

where D_i is the optical density at wavelength i.

Determination of photosynthetic efficiency

Chlorophyll fluorescence was measured with a chlorophyll fluorometer (PAM-2000; Waltz, Germany) with leaves attached to the plantlets, at room temperature. After 5 h of dark adaptation of plantlets, the initial florescence yield (F) in weak modulated light was recorded, followed by the maximum fluorescence yield (F_m) after a saturating light pulse (4,000 μmol/m^2 s). Photosynthetic efficiency F_v/F_m (where $F_v = F_m - F$) ratio of dark-adapted leaves was calculated automatically.

Results

Production of transgenic *Lilium* × *formolongi*

In this study, the plasmid pCrtZW-N8idi-crtEBIY which contains seven bacterial enzyme genes was used. A transit peptide is needed to target a bacterial gene product into plant chloroplasts. We used the transit peptide sequence of RuBisCO small subunit from pea plants. Each gene surrounded with the CaMV 35S promoter (P35S) and the terminator of heat shock protein 18.2 kDa gene (*hsp*) from *Arabidopsis thaliana* (T*hsp*), the resulting construct (Fig. 2) was used for transformation experiments. Using the *Agrobacterium*-mediated method, five Hygromycin (Hm)-resistant calli from 400 co-cultured calli of *L.* × *formolongi* were generated (Fig. 3a). Among the five Hm-resistant calli, one callus showed a strong orange color (Fig. 3b). Plantlets regenerated 8 weeks after transfer to the regeneration medium. In total, 7 plantlets were recovered on MS medium without growth regulators but containing 25 mg/l Hm. Two types of plantlets were observed, green

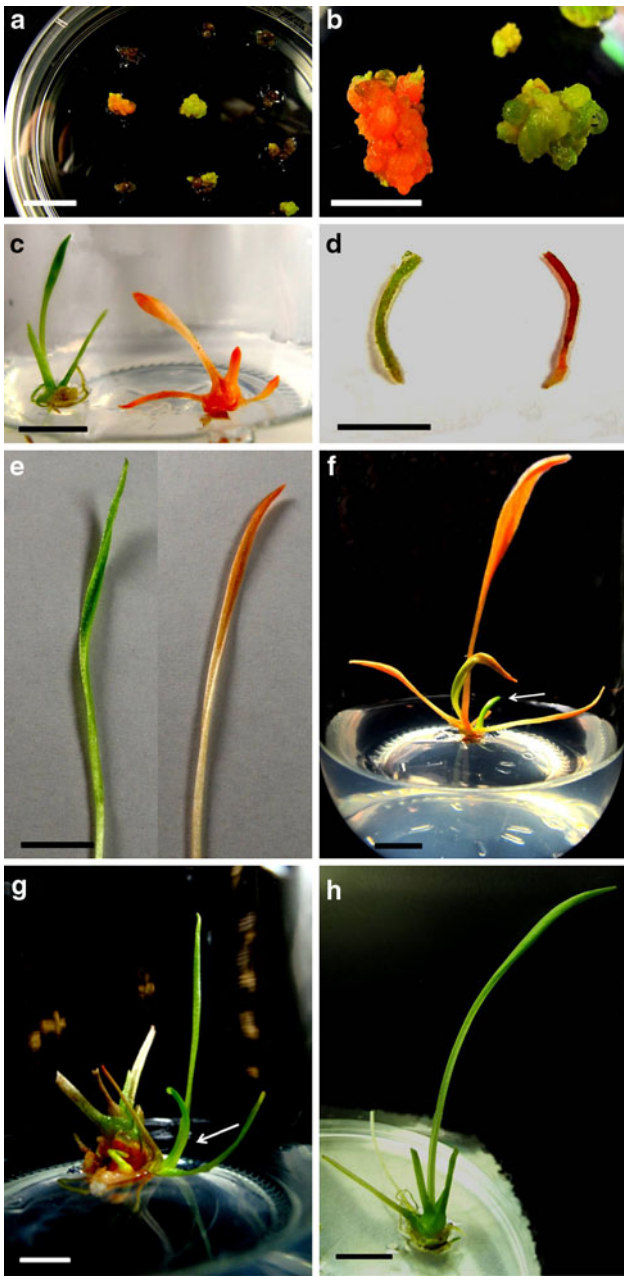

Fig. 3 Production of transgenic calli and plant of *Lilium* × *formolongi* from meristematic nodular calli and expression of seven key gene cassettes. **a** Hygromycin resistant calli 4 months after selection medium, **b** hygromycin resistant calli 2 months after transfer to regeneration medium, **c** transformed plantlets after 4 months on regeneration medium. **d** Expression of seven key gene cassettes on root and **e** leaf of transgenic plant (*right*) and wild-type plant (*left*), respectively. **f** Green leaves appeared from a transgenic orange plant 6 months after transfer to regeneration medium. **g** Transgenic green shoots (*arrow*) emerged from transgenic orange (TOr) plantlet (9 months after transfer to regeneration medium). **h** Transgenic green (TGr) plantlet. *Bars* 1 cm

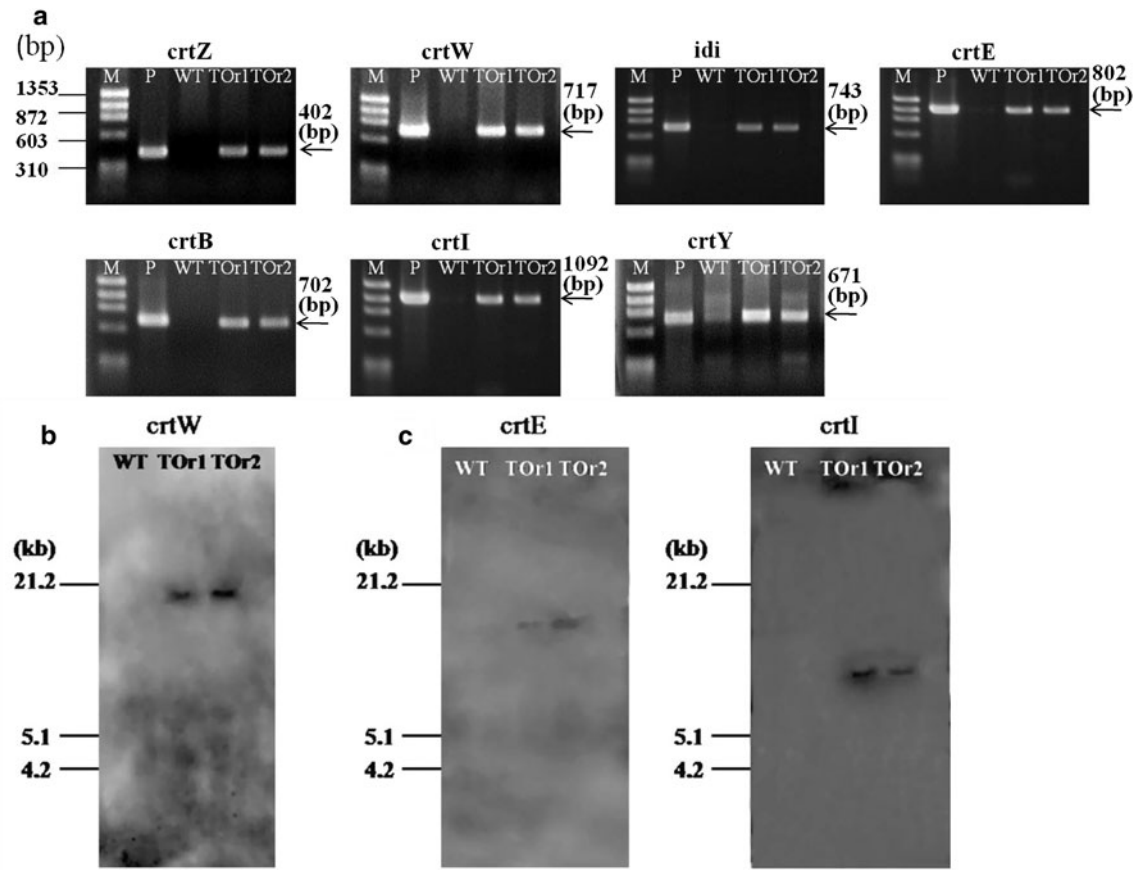

Fig. 4 Confirmation of presence of key gene cassettes in two transgenic orange plantlets of *Lilium* × *formolongi* (TOr1 and TOr2). **a** PCR analysis of transgenic orange plantlets, *Lanes TOr1 and TOr2* PCR amplified product of 7 genes (which is mentioned above of each one) showed presence of PCR amplified fragment of all seven genes in the genomic DNA, *Lane M* molecular size marker (*λ*/*Hin*dIII, Ø/X174/*Hae*III), *lane P* Plasmid DNA of pCrtZW-N8idi-crtEBIY as positive controls, *lane WT* wild-type (negative control). **b**,**c** Southern blot analysis; DNA samples were digested with *Pac*I and hybridized to *crtW* probe (**b**). DNA samples were digested with *Mlu*I and hybridized to *crtE*, and *crtI* probes (**c**). Molecular markers are indicated on the *left*

color (5 plantlets) and orange color (2 plantlets) (Fig. 3c). The two orange plantlets which regenerated from the orange callus showed strong orange color in roots and leaves (Fig. 3d, e). Although all the 7 Hm-resistant plantlets were PCR positive for the *hpt* gene (data not shown), only the 2 transgenic orange plantlets (TOr1 and TOr2) were PCR positive for all the seven gene cassettes (Fig. 4a) and the other 5 plantlets originating from green calli were not positive for any of the seven gene cassettes (data not shown). Unexpectedly, after several subcultures green leaves started appearing from 6-month-old transgenic orange (TOr) plantlets (Fig. 3f). By continuing the subculture and multiplication of the TOr plantlets (15 plantlets multiplied), new transgenic green (TGr) plantlets emerged 9 months after transfer to regeneration medium (Fig. 3g), and later turned to be completely green plantlets (Fig. 3h). Micropropagated plantlets from TGr plantlets showed similar phenotype.

Integration and expression of the multiple genes in the transgenic plants

For Southern blot analysis, three genes (*crtW, crtE* and *crtI* as probes) were selected at different positions of the cassette. The integration of these genes is shown in Fig. 4b, c. Hybridization of *Pac*I digested genomic DNA for *crtW* gene and *Mlu*I digested genomic DNA for *crtE* and *crtI* genes implies that the transgenic orange 1 (TOr1) and transgenic orange 2 (TOr2) plantlets are the same line with a single gene copy insertion. Consequently, transformation efficiency obtained in the present study using the plasmid pCrtZW-N8idi-crtEBIY was 0.25% (1/400). In the wild-type plantlets which served as a negative control, no *crtW*, *crtE* or *crtI* signal was detected. In order to investigate whether all seven genes were expressed in the TOr and TGr plantlets, northern blot analysis was performed. The expression of *crtW* was higher in the leaves of TOr plantlet

compared to the leaves of TGr plantlet, whereas in the TGr plantlets the expression of *crtZ* and *crtY* was slightly higher than in the TOr plantlets. For the other genes, the expression levels were equal, or slightly higher in transgenic orange plantlets than in transgenic green plantlets (Fig. 5).

Herbicide resistance of the transgenic plantlets

Norflurazon is known as a non-competitive inhibitor of phytoene desaturase enzyme in higher plants by direct interference, but not that of CrtI which is bacterial phytoene desaturase, because of differences in function and structure (Misawa et al. 1993, 1994). The leaves of the transgenic plantlets showed resistance to bleaching, indicating the expression of the *crtI* gene under the control of the CaMV 35S promoter. The wild-type leaves (Fig. 6a) bleached after 4 weeks of incubation (Fig. 6c), whereas the leaves of transgenic orange plantlets (Fig. 6b) did not show any visible change in color (Fig. 6d).

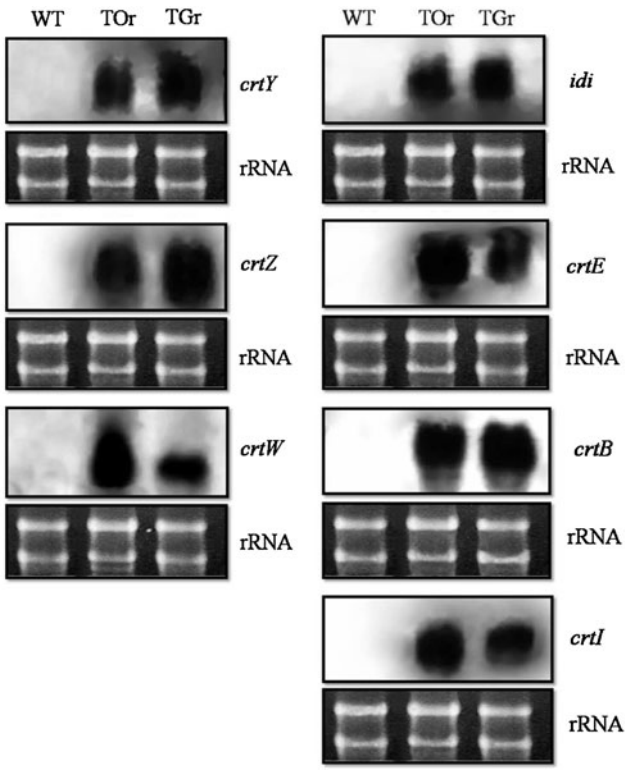

Fig. 5 Northern blot analysis with seven different probes (*idi*, crtE, crtB, crtI, crtY, crtZ and crtW) to monitor transgene expression in wild-type (*WT*), transgenic orange (*TOr*) and transgenic green (*TGr*) plantlets of *Lilium* × *formolongi*. Staining of rRNA with ethidium bromide was used as reference

Pigment amount and composition in the transgenic plantlets

HPLC–PDA analysis was used to determine the carotenoid profiles of the wild-type and transgenic *L.* × *formolongi* calli and leaves (TOr and TGr plantlets). The leaves of transgenic green plantlets were collected from the first and second generations of micropropagated plantlets.

The wild-type calli contained only phytoene, lutein, β-carotene and α-carotene, whereas, the transgenic calli showed some novel peaks such as β-cryptoxanthin, lycopene, echinenone, canthaxanthin, 3′-hydroxyechinenone, 3-hydroxyechinenone and astaxanthin beside these compounds. During the transition from the TOr plantlets to the TGr plantlets, we also examined the composition of the carotenoids, chlorophyll *a* and *b*, and photosynthetic efficiency. In the wild-type leaves, lutein, β-carotene, α-carotene, violaxanthin and neoxanthin were identified, while 9 novel peaks beside those compounds were detected in the leaves of transgenic orange plantlets. Interestingly, in the leaves of transgenic green plantlets only 5 novel peaks were detected. Astaxanthin, an important ketocarotenoid, was not detected in the leaves of transgenic green plantlets (Table 2).

Quantitative data showing the contents and compositions of the carotenoids in the calli and leaves of the wild-type and transgenic orange and green plantlets are summarized in Table 2. In the transgenic calli, the total amount of the carotenoids (133.3 μg/g FW) was increased 26.1-fold compared to wild-type calli (5.1 μg/g), and the

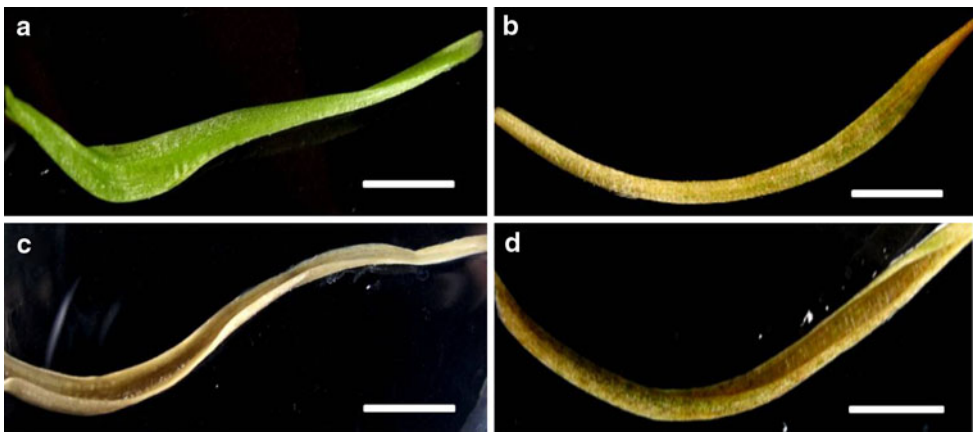

Fig. 6 Herbicide resistance assay to consider for crtI expression on leaves of transgenic plants of *Lilium* × *formolongi*. **a**,**c** Control wild-type. **b**,**d** Transgenic orange leaves, first day (*upper*) and 4 weeks (*lower*) after transferring to MS medium containing 15 μM norflurazon. *Bars* 1 cm

Table 2 Pigments and photosynthetic efficiency of wild-type and transgenic *L.* × *formolongi* tissues

	Pigments content μg/g FW (% composition)				TGr
	Callus		Leaf		
	WT	T	WT	TOr	
Phytoene	0.3 ± 0.3 (5.8)	50.5 ± 5.0 (37.9)	–	15.1 ± 8.4 (14.7)	8.5 ± 4.5 (6.3)
Lutein	3.9 ± 0.4 (76.4)	7.0 ± 0.1 (5.3)	11.5 ± 1.9 (62.5)	31.4 ± 5.4 (30.5)	39.3 ± 14.1 (29.1)
β-Carotene	0.3 ± 0.2 (5.8)	17.7 ± 1.5 (13.3)	5.2 ± 0.2 (28.3)	25.0 ± 10.5 (24.3)	44.6 ± 16.9 (33.0)
α-Carotene	0.6 ± 0.3 (12)	32.6 ± 23.1 (24.4)	0.28 ± 0.28 (1.5)	7.7 ± 3.8 (7.5)	12.1 ± 6.2 (8.9)
β-Cryptoxanthin	–	3.6 ± 0.9 (2.7)	–	1.3 ± 0.5 (1.3)	0.7 ± 0.7 (0.5)
Lycopene	–	0.8 ± 0.4 (0.6)	–	5.0 ± 3.6 (4.8)	4.6 ± 3.9 (3.4)
Violaxanthin	–	–	0.4 ± 0.1 (2.3)	4.9 ± 2.6 (4.7)	14.0 ± 7.4 (10.4)
Neoxanthin	–	–	1.0 ± 0.3 (5.4)	4.5 ± 2.3 (4.4)	10.1 ± 4.7 (7.5)
Echinenone	–	7.9 ± 0.9 (5.9)	–	1.9 ± 1.1 (1.8)	0.6 ± 0.7 (0.4)
Canthaxanthin	–	3.5 ± 2.5 (2.6)	–	2.0 ± 0.7 (1.9)	0.6 ± 0.5 (0.4)
3′-Hydroxyechinenone	–	7.0 ± 4.3 (5.3)	–	3.9 ± 2.3 (3.8)	–
3-Hydroxyechinenone	–	2.1 ± 0.7 (1.6)	–	0.1 ± 0.2 (0.1)	–
Adonirubin	–	–	–	<0.1 (<0.1)	–
Astaxanthin	–	0.5 ± 0.4 (0.4)	–	0.2 ± 0.5 (0.2)	–
Total	5.1 ± 0.1	133.3 ± 9.4	18.4 ± 1.6	102.9 ± 29.9	135.2 ± 55.0
Fold	1	26.1	1	5.6	7.4
Chlorophyll *a*			314.5 ± 10.9	94.5 ± 7.0*	263.0 ± 29
Chlorophyll *b*			146.0 ± 10.0	59.0 ± 8.0*	160.0 ± 22
Photosynthetic efficiency			0.7558 ± 0.029	0.2818 ± 0.098	0.6243 ± 0.030

Each value in carotenoids is the mean result from three different samples ± standard deviation (SD), except for that of WT leaves, which is the average of two samples. The values in chlorophylls and photosynthetic efficiency represent the means of eight measurements from three plantlets. In TGr plantlets samples were collected from the two generations of micropropagated plantlets. Values in parentheses represent the ratio of the carotenoid composition to the total amount (%). Values in the row titled fold represent the total amount of carotenoids relative to that of wild-type tissues

WT Wild-type, *T* transgenic, *TOr* transgenic orange plantlets, *TGr* transgenic green plantlets, *FW* fresh weight, – not detected

* Significant differences compared to the value of the wild-type (*P* value <0.05)

ketocarotenoids were 19.1% of the total carotenoid content. A strong increase in phytoene, *β*-carotene, α-carotene and lutein (+79%) was observed in the transgenic calli as compared to the wild-type (Table 2).

The total amount of carotenoid in the leaves of TOr and TGr plantlets was quantified at 102.9 and 135.2 μg/g FW, respectively, corresponding to 5.6- and 7.4-fold increases over the levels in the leaves of wild-type (18.4 μg/g FW). Novel carotenoids were 28.6 and 11% of the total carotenoid content in the leaves of TOr and TGr plantlets, respectively. Since *Lilium* is propagated vegetatively, we checked the stability through subsequent generations of the micropropagated plantlets. The carotenoid content in the two generations of the micropropagated TGr plantlets showed minor variations (the mean ± standard deviation of these generations is shown in Table 2), suggesting the stability of the transgenes in the transgenic green plantlets. The wild-type leaves contained lutein, *β*-carotene, α-carotene, violaxanthin and neoxanthin but not lycopene, zeaxanthin and antheraxanthin, which are synthesized naturally in the leaves of plants. However, a strong increase in violaxanthin and neoxanthin was observed in the leaves of transgenic orange and green plantlets as compared to the leaves of wild-type plantlets, but zeaxanthin and antheraxanthin, which are the precursors of these two compounds, were not detected (Table 2).

In the leaves of transgenic orange and green plantlets, lutein and *β*-carotene were the most accounted carotenoids (54.8 and 62.1%, respectively) while the ketocarotenoids of the total carotenoid content in the leaves of TOr plantlets was 7.8 and 0.8% in the leaves of TGr plantlets. Interestingly, in the leaves of transgenic green plantlets only echinenone and canthaxanthin (0.6 μg/g FW in both) were detected (Table 2).

The amount of chlorophyll *a* and *b* in the transgenic orange leaves (94.5 and 59 μg/g FW) was less than half the amount in the wild-type leaves. On the other hand, the leaves of transgenic green plantlets showed a significant increase in chlorophyll *a* and *b* as compared to the leaves of transgenic orange plantlets (Table 2). Also, photosynthetic

efficiency of the transgenic orange leaves significantly decreased (0.282). However, the photosynthetic efficiency of the transgenic green leaves was lower than the wild-type but significantly greater than the photosynthetic efficiency of the transgenic orange leaves (Table 2).

Discussion

The limited knowledge of the regulation of carotenoid biosynthesis is the main barrier to engineering carotenoid metabolism. Isolation of the genes that encode pathway enzymes, and modifying their expression, constitutes one approach to overcome this challenge, but modulating single enzymes is often unhelpful because the pathways are regulated at multiple points. It has been shown that the simultaneous expression of multiple enzymes is the most desirable way to study and modulate complex pathways such as carotenoid biosynthesis (Zhu et al. 2008; Harada et al. 2009; Fujisawa et al. 2009; Naqvi et al. 2009).

In this study, *Lilium* × *formolongi* was successfully transformed by the introduction of seven bacterial enzyme genes. The transformants showed increased carotenoid contents and the production of ketocarotenoids. This is the first time that seven gene cassettes (18.9 kb T-DNA) have been introduced into *Lilium* and expressed successfully. Fujisawa et al. (2009) found no significant decrease in transformation efficiency using the same seven key genes (fused to a seed-specific promoter) in *Brassica napus*. However, the efficiency of transformation in *L.* × *formolongi* using these cassettes (0.25%) was extremely lower than that of the T-DNA (7.8 kb) of plasmid pIG121-Hm containing the *β*-glucuronidase (GUS) gene (25%) in our previous study (Azadi et al. 2010). This suggests that the large size of the cassettes might be a barrier to their introduction into *Lilium* cells. Significantly lower transformation efficiencies were also observed with the introduction of a larger T-DNA (8.4 kb) into tobacco, cotton, and rice, as compared to the introduction of a 4.3-kb T-DNA (Park et al. 2000).

The use of a strong constitutive promoter, CaMV 35S, in this study resulted in strong expression of the inserted genes in transgenic orange and green plantlets (Fig. 5). Although the cassettes did not significantly affect the growth habit of the orange transgenic *L.* × *formolongi* plants in MS medium containing 3% sucrose, the growth rate of TOr plantlets was lower than that of the wild-type plantlets. Moreover, in contrast with the TGr plantlets, the TOr plantlets were not able to grow on sucrose free medium (data not shown). There have been other reports that the growth habits of tobacco (Misawa et al. 1993, 1994; Ralley et al. 2004), tomato (Ralley et al. 2004), and *Lotus japonicus* (Suzuki et al. 2007) are not affected by the overexpression of *crtI*, *crtW*, or *crtZ* using the CaMV 35S promoter.

Plants generally lack the biosynthetic potential for ketocarotenoid formation. In this study, transgenic lily calli produced five ketocarotenoids, including low amounts of astaxanthin (0.5 μg/g FW). For comparison, expression of the *bkt1* gene driven by a double CaMV 35S promoter showed 124 μg/g FW of astaxanthin in transgenic carrot calli (Jayaraj et al. 2008).

The production of phytoene at 50.5 μg/g FW in the transgenic calli (37.9% of the total carotenoids), and 15.1 and 8.5 μg/g FW in transgenic orange and green leaves, respectively, suggest that *idi*, *crtE*, and *crtB* were expressed at high levels. It was reported that the increase in the total carotenoids in *B. napus* seeds was mainly due to the activity of *crtB* and not those of *idi* and *crtE*. The use of only the *crtB* gene caused a similar increase in total carotenoids, compared to the use of all three genes together (Fujisawa et al. 2009). A similar result was observed in *Arabidopsis* calli, suggesting the importance of *crtB* in increasing the total carotenoid content (Harada et al. 2009). The overexpression of *crtB* in *B. napus* seeds resulted in up to a 50-fold increase in total carotenoids (Shewmaker et al. 1999).

The high accumulation of phytoene in calli as compared to leaves suggests that *crtI* does not play a significant role in calli but makes a strong contribution in leaves. Furthermore, the increases in the levels of lutein (+79.4%) and lycopene (0.8 μg/g FW) in transgenic calli were much lower than those of lutein (+173%, +242%) and lycopene (5 and 4.6 μg/g FW) in orange and green leaves, respectively, indicating that *crtI* is expressed differently in calli and leaves. The resistance of transgenic orange leaves to bleaching in the presence of 15 μM norflurazon also indicates the high activity of *crtI* in transgenic plantlets. However, the endogenous enzymes lycopene *β*-cyclase, lycopene *ε*-cyclase, *β*-carotene hydroxylase and *ε*-carotene hydroxylase might have played a critical role in the production of lutein in both transgenic calli and leaves. Fujisawa et al. (2009) reported that the contribution of *crtI* was not sufficient in transgenic seeds of *B. napus*. However, white maize overexpressing *Zmpsy1* (*Zea mays* phytoene synthase 1) and *crtI* simultaneously showed a further tripling in the total carotenoid levels in endosperm, compared with the overexpression of *Zmpsy1* alone (Zhu et al. 2008). This suggests that the contribution of *crtI* expression varies in different plant tissues.

The expression of many endogenous carotenogenic genes in transgenic *B. napus* seeds has been reported (Fujisawa et al. 2009). In the present study, the relatively high amount of lutein and the low amount of α-carotene in leaves of both orange and green transgenic plantlets suggest high activities of two endogenous enzymes, plant

ε-carotene hydroxylase and plant β-carotene hydroxylase, both of which require α-carotene as a precursor. The dramatic increase in β-carotene observed in all transgenic tissues also suggests that the contribution of a bacterial enzyme, CrtY (bacterial lycopene β-cyclase), was sufficient for carotenoid biosynthesis in lily. In contrast, Apel and Bock (2009) recently showed that the expression of CrtY did not strongly alter the carotenoid composition in tomato, as compared to the plant enzyme (Lyc, lycopene β-cyclase gene), which efficiently converted lycopene into β-carotene. Also, the overexpression of *crtY* along with *crtB* and *crtI* in potato did not cause a further increase in total carotenoids (Diretto et al. 2007a).The production of the ketocarotenoids echinenone, canthaxanthin, 3′-hydroxyechinenone, 3-hydroxyechinenone, and astaxanthin in calli, and these compounds plus adonirubin in leaves of TOr plantlets, suggests that *crtW* and *crtZ* were functionally expressed under the control of the 35S promoter. In calli, echinenone was the major ketocarotenoid (37.6% of the ketocarotenoids), which coincided with the results in potato leaves transformed with *crtO* β-carotene ketolase gene (Gerjets and Sandmann 2006) and *B. napus* seeds transformed using seven similar gene cassettes under the control of a seed-specific promoter (Fujisawa et al. 2009). However, in leaves of TOr plantlets, 48.1% of the ketocarotenoids were 3′-hydroxyechinenone, which was the major ketocarotenoid observed in the present study.

A difference in the ketocarotenoid composition in young and older leaves of tobacco was reported by Zhu et al. 2007. In the present study, a significant change in the type and composition of ketocarotenoids was also observed during the transition of transgenic plantlets from orange to green. Interestingly, in the leaves of the TGr plantlets, upregulation of carotenoid biosynthesis led to an increase in the total carotenoid concentration (+31.4%), although 3′-hydroxyechinenone, 3-hydroxyechinenone, astaxanthin, and adonirubin were absent and the echinenone and canthaxanthin levels decreased to 0.6 μg/g FW.

The detection of β-cryptoxanthin in transgenic calli (3.6 μg/g FW) and orange (1.3 μg/g FW) and green leaves (0.7 μg/g FW) of transgenic plantlets, but not in wild-type plantlets, indicates that *crtZ* was expressed. The use of β-cryptoxanthin as a precursor could lead to the synthesis of 3′-hydroxyechinenone and 3-hydroxyechinenone in transgenic plantlets through the expression of *crtW*. The expression of the *crtW* gene under the regulation of the CaMV 35S promoter in *Lotus japonicus* caused the accumulation of ketocarotenoids such as echinenone, canthaxanthin, adonixanthin, and astaxanthin in flower petals (Suzuki et al. 2007).

In addition, the expression of *crtZ* along with the endogenous plant enzymes zeaxanthin epoxidase and neoxanthin synthase resulted in a strong increase in violaxanthin and neoxanthin in both TOr and TGr plantlets (Table 2; Fig. 1). Furthermore, the production of astaxanthin in leaves of TOr plantlets (0.2 μg/g FW) along with a high expression of *crtW* and *crtZ* (Fig. 5) shows that *crtZ* and *crtW* functioned effectively, although the low amount of astaxanthin may be caused by a shortage of adonirubin or the absence of adonixanthin, which are the precursors for the production of astaxanthin. Fujisawa et al. (2009) also found that the insufficient expression of *crtZ* might be the reason for the low amount of hydroxylated carotenoids in transgenic *B. napus* seeds. Zhu et al. (2008) demonstrated that the avoidance of adonixanthin accumulation was crucial for astaxanthin production in transgenic maize endosperm.

It is reasonable that the low photosynthetic efficiency in leaves of TOr plantlets soon after regeneration from orange calli is associated with the low content of chlorophylls (Table 2). Since the secondary shoots regenerated during the subcultures of TOr plantlets turned green to produce TGr plantlets, it requires some time to express the primary functions of leaves, i.e., the recovery of normal photosynthetic ability. Since the transition of TOr to TGr plantlets was associated with the absence of four ketocarotenoids (3′-hydroxyechinenone, 3-hydroxyechinenone, astaxanthin, and adonirubin), it is possible that some of these ketocarotenoids temporarily interfered with the chlorophyll formation in leaves of TOr plantlets although the detailed mechanism involved is unclear. The absence of ketocarotenoids in TGr plantlets might be due to the low expression level of *crtW* (Fig. 5), which might also be a reason for the significantly elevated level of the violaxanthin and neoxanthin in TGr plantlets (Table 2). Since both violaxanthin and neoxanthin have a role to protect photosynthesis against oxidative stresses (Demmig-Adams and Adams 1996, 2002; Dall'Osto et al. 2007), it is expected that TGr plants have higher tolerance to the various stresses than the wild-type plants.

Although it requires some more time to produce the flowers in the TGr plants, it is highly possible that the flowers will show at least the orange to yellow colorations because of the following reasons: (1) perianths of *Lilium* flowers are known to contain carotenoids even in white-colored flowers (Yamagishi et al. 2010), (2) all the carotenoids detected in TOr callus are expected to express in the flower of TGr plants since perianth of *L.* × *formolongi* has almost no chlorophyll like as the callus used in the present study, and (3) even if the biosynthetic ability of flowers may not convert into callus-like conditions, carotenoids with orange colorations such as β-carotene, violaxanthin and lutein, which were detected at comparatively high amount in TGr leaves, will at least be expressed more or less in perianths.

Acknowledgments The authors thank to Dr. Masaharu Kuroda (Hokuriku Research Center, National Agricultural Research Center) for kindly providing binary vector pZH2B, which was used in plasmid pCrtZW-N8idi-crtEBIY. This work was supported by Ministry of Education, Culture, Sport, Science and Technology of the Japanese Government (MONBUKAGAKUSHO) and as a part of the project P02001 by the New Energy and Industrial Technology Development Organization (NEDO). P. Azadi is the recipient of a PhD fellowship from Ministry of Education, Science and Culture of Japan.

References

Apel W, Bock R (2009) Enhancement of carotenoid biosynthesis in transplastomic tomatoes by induced lycopene-to-provitamin A conversion. Plant Physiol 151:59–66

Azadi P, Chin DP, Kuroda K, Khan RS, Mii M (2010) Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium*. Plant Cell Tissue Organ Cult 101:201–209

Choi SK, Nishida Y, Matsuda S, Adachi K, Kasai H, Peng X, Komemushi S, Miki W, Misawa N (2005) Characterization of β-carotene ketolases, CrtW, from marine bacteria by complementation analysis in *Escherichia coli*. Mar Biotechnol (NY) 7:515–522

Clotault J, Peltier D, Berruyer R, Thomas M, Briard M, Geoffriau E (2008) Expression of carotenoid biosynthesis genes during carrot root development. J Exp Bot 59:3563–3573

Collins AR (1999) Oxidative DNA damage, antioxidants, and cancer. Bioessays 21:238–246

Combs GF Jr (1998) Vitamin A, chapter 5 in the vitamins: fundamental aspects in nutrition and health. Academic, New York, pp 107–153

Dall'Osto L, Cazzaniga S, North H, Marion-Poll A, Bassi R (2007) The Arabidopsis aba4–1 mutant reveals a specific function for neoxanthin in protection against photooxidative stress. Plant Cell 19:1048–1064

Demmig-Adams B, Adams WW (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci 1:21–26

Demmig-Adams B, Adams WW (2002) Antioxidants in photosynthesis and human nutrition. Science 298:2149–2153

Diretto G, Tavazza R, Welsch R, Pizzichini D, Mourgues F, Papacchioli V, Beyer P, Giuliano G (2006) Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. BMC Plant Biol 6:13

Diretto G, Al-Babili S, Tavazza R, Papacchioli V, Beyer P, Giuliano G (2007a) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial minipathway. PLoS ONE 2:e350

Diretto G, Welsch R, Tavazza R, Mourgues F, Pizzichini D, Beyer P, Giuliano G (2007b) Silencing of β-carotene hydroxylase increases total carotenoid and β-carotene levels in potato tubers. BMC Plant Biol 7:11

Ducreux LJ, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MA (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. J Exp Bot 56:81–89

Frank HA, Cogdell RJ (1996) Carotenoids in photosynthesis. Photochem Photobiol 63:257–264

Fraser PD, Pinto ME, Holloway DE, Bramley PM (2000) Technical advance: application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. Plant J 24:551–558

Fraser PD, Romer S, Shipton CA, Mills PB, Kiano JW, Misawa N, Drake RG, Schuch W, Bramley PM (2002) Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit specific manner. Proc Natl Acad Sci USA 99:1092–1097

Fujisawa M, Watanabe M, Choi SK, Teramoto M, Ohyama K, Misawa N (2008) Enrichment of carotenoids in flaxseed (*Linum usitatissimum*) by metabolic engineering with introduction of bacterial phytoene synthase gene *crtB*. J Biosci Bioeng 105:636–641

Fujisawa M, Takita E, Harada H, Sakurai N, Suzuki H, Ohyama K, Shibata D, Misawa N (2009) Pathway engineering of *Brassica napus* seeds using multiple key-enzyme genes involved in ketocarotenoid formation. J Exp Bot 60:1319–1332

Gerjets T, Sandmann G (2006) Ketocarotenoid formation in transgenic potato. J Exp Bot 57:3639–3645

Gerjets T, Sandmann M, Zhu C, Sandmann G (2007) Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species. Biotechnol J 2:1263–1269

Hamill JD, Rounsley S, Spencer A, Todd G, Rhodes MJC (1991) The use of the polymerase chain reaction in plant transformation studies. Plant Cell Rep 10:221–224

Harada H, Fujisawa M, Teramoto M, Sakurai N, Suzuki H, Shibata D, Misawa N (2009) Simple functional analysis of key genes involved in astaxanthin biosynthesis using *Arabidopsis* cultured cells. Plant Biotechnol 26:81–92

Hasunuma T, Miyazawa SI, Yoshimura S, Shinzaki Y, Tomizawa KI, Shindo K, Choi SK, Misawa N, Miyake C (2008) Biosynthesis of astaxanthin in tobacco leaves by transplastomic engineering. Plant J 55:857–868

Jayaraj J, Devlin R, Punja Z (2008) Metabolic engineering of novel ketocarotenoid production in carrot plants. Transgenic Res 17:489–501

Krapp A, Hofmann B, Schafer C, Stitt M (1993) Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the "sink-regulation" of photosynthesis? Plant J 3:817–828

Krinsky NI, Landrum JT, Bone RA (2003) Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu Rev Nutr 23:171–201

Mann V, Harker M, Pecker I, Hirschberg J (2000) Metabolic engineering of astaxanthin production in tobacco flowers. Nat Biotechnol 18:888–892

Mii M, Yuzawa Y, Suetomi H, Motegi T, Godo T (1994) Fertile plant regeneration from protoplasts of a seed-propagated cultivar of *Lilium* × *formolongi* by utilizing meristematic nodular cell clumps. Plant Sci 100:221–226

Misawa N (2009) Pathway engineering of plants toward astaxanthin production. Plant Biotechnol 26:93–99

Misawa N (2010) Carotenoids. In: Mander L, Lui HW (eds) Comprehensive natural products II chemistry and biology, vol 1. Elsevier, Oxford, pp 733–753

Misawa N, Yamano S, Linden H, de Felipe MR, Lucas M, Ikenaga H, Sandmann G (1993) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. Plant J 4:833–840

Misawa N, Masamoto K, Hori T, Ohtani T, Boger P, Sandmann G (1994) Expression of an *Erwinia* phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. Plant J 6:481–489

Morris WL, Ducreux LJ, Fraser PD, Millam S, Taylor MA (2006) Engineering ketocarotenoid biosynthesis in potato tubers. Metab Eng 8:253–263

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Perez Conesa D, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767

Ogaki M, Furuichi Y, Kuroda K, Chin DP, Ogawa Y, Mii M (2008) Importance of co-cultivation medium pH for successful *Agrobacterium*-mediated transformation of *Lilium* × *formolongi*. Plant Cell Rep 27:699–705

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487

Park SH, Lee BM, Salas MG, Srivatanakul M, Smith RH (2000) Shorter T-DNA or additional virulence genes improve *Agrobactrium*-mediated transformation. Theor Appl Genet 101:1015–1020

Ralley L, Enfissi EM, Misawa N, Schuch W, Bramley PM, Fraser PD (2004) Metabolic engineering of ketocarotenoid formation in higher plants. Plant J 39:477–486

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of betacarotene and lycopene content in tomato fruit. Plant J 24:413–419

Sandmann G (2001) Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. Trends Plant Sci 6:14–17

Shabala SN, Shabala SI, Martynenko AI, Babourina O, Newman IA (1998) Salinity effect on bioelectric activity, growth, Na+ accumulation and chlorophyll fluorescence of maize leaves: a comparative survey and prospects for screening. Australian J Plant Physiol 25:609–616

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

Stalberg K, Lindgren O, Ek B, Hoglund AS (2003) Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. Plant J 36:771–779

Suzuki S, Nishihara M, Nakatsuka T, Misawa N, Ogiwara I, Yamamura S (2007) Flower color alteration in *Lotus japonicus* by modification of the carotenoid biosynthetic pathway. Plant Cell Rep 26:951–959

Yamagishi M, Kishimoto S, Nakayama M (2010) Carotenoid composition and changes in expression of carotenoid biosynthetic genes in tepals of Asiatic hybrid lily. Plant Breed 129:100–107

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287:303–305

Yuan JP, Chen F (1999) Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. J Agric Food Chem 47:31–35

Zhu C, Gerjets T, Sandmann G (2007) *Nicotiana glauca* engineered for the production of ketocarotenoids in flowers and leaves by expressing the cyanobacterial *crtO* ketolase gene. Transgenic Res 16:813–821

Zhu C, Naqvia S, Breitenbach J, Sandmann G, Christoua P, Capella T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci USA 105:18232–18237