

Overexpression of carotenogenic genes in the Japanese morning glory *Ipomoea (Pharbitis) nil*

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Abstract Japanese morning glory, *Ipomoea nil*, has several coloured flowers except yellow, because it can accumulate only trace amounts of carotenoids in the petal. To make the petal yellow with carotenoids, we introduced five carotenogenic genes (*geranylgeranyl pyrophosphate synthase*, *phytoene synthase*, *lycopene β -cyclase* and *β -ring hydroxylase* from *Ipomoea obscura* var. *lutea* and bacterial *phytoene desaturase* from *Pantoea ananatis*) to white-flowered *I. nil* cv. AK77 with a petal-specific promoter by *Rhizobium (Agrobacterium)*-mediated transformation method. We succeeded to produce transgenic plants overexpressing carotenogenic genes. In the petal of the transgenic plants, mRNA levels of the carotenogenic genes were 10 to 1,000 times higher than those of non-transgenic control. The petal colour did not change visually; however, carotenoid concentration in the petal was increased up to about ten-fold relative to non-transgenic control. Moreover, the components of carotenoids in the petal were diversified, in particular, several β -carotene derivatives, such as zeaxanthin and neoxanthin, were newly synthesized. This is the first report, to our knowledge, of changing the component and increasing the amount of carotenoid in petals that lack ability to biosynthesize carotenoids.

Key words: carotenoid, floral colour modification, *Ipomoea nil*, metabolic engineering.

Introduction

The Japanese morning glory, *Ipomoea nil* (or *Pharbitis nil*), is one of the two traditional horticultural model plants selected for the National BioResource Project by the Agency for Medical Research and Development (AMED) in Japan (Yamazaki et al. 2010). In the eighth century, only one cultivar of *I. nil* had heard to be imported from China to Japan as a medicinal plant (Hoshino et al. 2016). The original cultivar bore pale blue flowers containing cyanidins: anthocyanin compounds. Thereafter, *I. nil* became one of the most important traditional garden plants representing summer flowers. In the seventeenth century, a lot of transposon mutagenesis caused the flower colour of *I. nil* to be varied e.g. red, pink, purple, brown, white (Hoshino et al. 2016). However, there were no bright yellow-flowered cultivars of *I. nil*. Although some cultivars contain a small amount of yellow pigments such as auronones (Saito et al. 1994), these acyanic flowers exhibit pale yellow. Another yellow pigment colouring flower petals is carotenoids, but *I.*

nil accumulates only trace amounts of carotenoids in the petal. Contrary, there is a distant relative species that contains a high amount of carotenoids bearing a vivid yellow flower, *Ipomoea obscura* var. *lutea* (formally *Ipomoea* sp.). Previously, we reported that the transcription levels of carotenogenic genes in petals of *I. nil* were remarkably lower than those in *I. obscura* var. *lutea* (Yamamizo et al. 2010). Because crossbreeding of *I. nil* and *I. obscura* is unsuccessful, we decided to transfer carotenogenic genes of *I. obscura* var. *lutea* to *I. nil* by genetic transformation to produce yellow-flowered *I. nil*.

The carotenoid biosynthesis of higher plants consists of multistep reactions (Figure 1A); isoprene units' polymerization, addition of conjugated double bonds and the conversion of *cis*- to *trans*-configuration, cyclization, hydroxylation, and/or epoxidation (Wise and Hooper 2006). Almost all the genes encoding enzymes that catalyse the core reactions of carotenoid biosynthesis in plants have been identified (Wise and Hooper 2006). Based on these knowledge, a lot of metabolic engineering studies of carotenoid were conducted mainly in food

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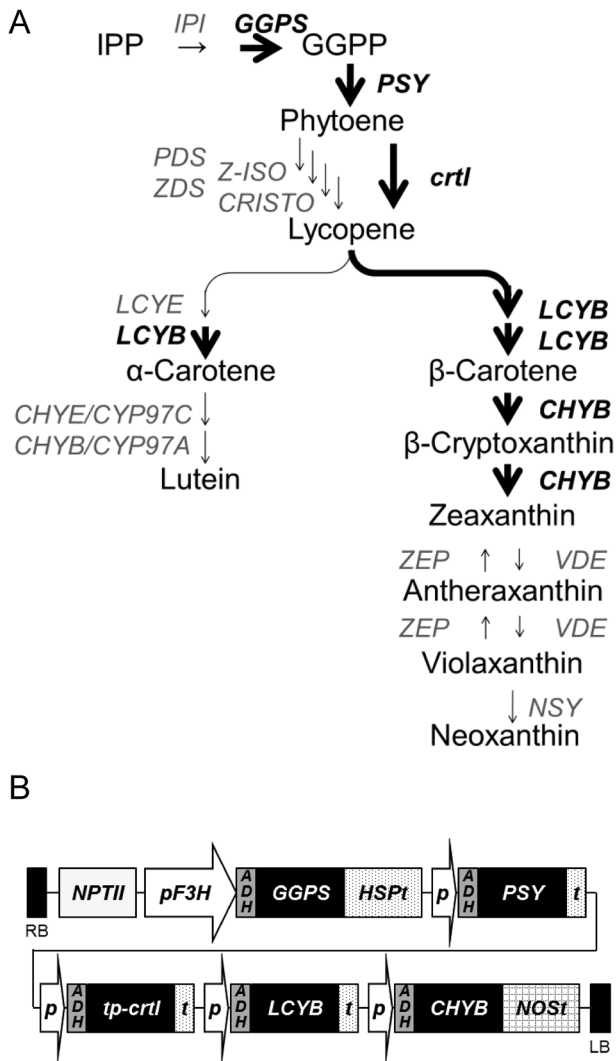


Figure 1. Schematic of the carotenoid biosynthesis pathway in plants and the vector construct used in the present study. A: Carotenoid biosynthesis pathway. Bold arrows indicate the introduced transgenes in this study. IPP: isopentenyl pyrophosphate; IPI: IPP isomerase; GGPP: geranylgeranyl pyrophosphate; GGPS: GGPP synthase; PSY: phytoene synthase; PDS: phytoene desaturase; Z-ISO: 15-*cis*- ξ -CRTISO; ZDS: ξ -carotene desaturase; CRTISO: carotenoid isomerase; crtI: phytoene dehydrogenase from plant pathogen (*P. ananatis*); LCYE: lycopene ϵ -cyclase; LCYB: lycopene β -cyclase; CHYE: ϵ -ring hydroxylase; CHYB: β -ring hydroxylase; ZEP: zeaxanthin epoxidase; VDE: violaxanthin deepoxidase; NSY: neoxanthin synthase. B: T-DNA region of the construct (GpLC). RB: right border, NPTII: neomycin phosphotransferase II, pF3H [p]: promoter of flavanone 3-hydroxylase of *Chrysanthemum morifolium* (petal specific promoter); ADH: 5'UTR region of alcohol dehydrogenase (translational enhancer); HSPt [t]: terminator of heat shock protein; tp: transit peptide; NOST: terminator of nopaline synthase; LB: left border.

crops for improved nutrition, because carotenoids are precursors for vitamin A and have antioxidant function; for examples, canola, *Brassica napus* (Shewmaker et al. 1999); rice, *Oryza sativa* (Paine et al. 2005; Ye et al. 2000); tomato, *Solanum lycopersicum* (Römer et al. 2000), maize, *Zea mays* (Zhu et al. 2008) and so on [reviews (Cazzonelli and Pogson 2010; Giuliano et al. 2008;

Nisar et al. 2015; Yuan et al. 2015)]. On the other hands, carotenoid metabolic engineering studies for floral colour modification were less conducted than ones for food crops. The studies on *Lotus japonicus*, *Iris germanica* and *Chrysanthemum morifolium* were notable examples of carotenoid metabolic engineering for floral colour modification (Jeknić et al. 2014; Ohmiya et al. 2006, 2009; Suzuki et al. 2007). By overexpression of bacterial β -carotene ketolase gene *CrtW* in *L. japonicus* (Suzuki et al. 2007), the petal accumulated red ketocarotenoids and the colour changed from yellow to red. By overexpression of bacterial *phytoene synthase* gene *crtB* in *Iris germanica* (Jeknić et al. 2014), the petal accumulated red lycopene and the colour changed from yellow to pink-orange. In case of white petals of *C. morifolium*, carotenogenic genes are expressed at similar levels to yellow petals, suggesting that carotenoid biosynthesis takes place even in the white petals (Kishimoto and Ohmiya 2006). Subsequently, Ohmiya et al. (2006) found that a gene encoding carotenoid cleavage dioxygenase 4 (CCD4) is specifically expressed in white chrysanthemum petals and cleaves the synthesized carotenoids. Suppression of *CCD4* expression by RNAi converted the petal colour from white to yellow (Ohmiya et al. 2006, 2009). These studies have been conducted in the petals that carotenogenic genes are substantially expressed. There was report about constitutive overexpression of multiple carotenogenic genes in the *Lilium \times formolongi* which lacked yellow flower cultivars (Azadi et al. 2010). In the report, the carotenoid levels increased in the callus and leaves but there was no description about endogenous carotenogenic genes expressions and flower of transgenic plants (Azadi et al. 2010). Hence, as far as we know, there is no report about the manipulation of carotenoid level in petals that the expression levels of carotenogenic genes are extremely low.

In this study, to increase the accumulation of carotenoid in the petal of *I. nil*, four carotenogenic genes (*geranylgeranyl pyrophosphate synthase*: GGPS, accession number: AB499049; *phytoene synthase*: PSY, AB499050; *lycopene β -cyclase*: LCYB, AB499055 and β -ring hydroxylase: CHYB, AB499056) from *I. obscura* var. *lutea* and one [*phytoene desaturase* (*crtI*, accession number: D90087)] from bacteria, *Pantoea ananatis* (formally *Erwinia uredovora*), were introduced to *I. nil* with *Agrobacterium*-mediated transformation method (Figure 1B; Kikuchi et al. 2005). The former four genes are highly expressed in the flower of *I. obscura* var. *lutea* and encode enzymes that would catalyse carotenoid biosynthesis in the chromoplast (Yamamizo et al. 2010). In higher plants, four enzymes are involved in the biosynthesis from phytoene to lycopene, however in the plant pathogen *P. ananatis*, it is catalysed by only one enzyme, *crtI* (Figure 1A; Misawa et al. 1990, 1994). The carotenoid biosynthesis pathway from isopentenyl

pyrophosphate (IPP) to zeaxanthin would be catalysed by the translational products of the five genes (hereinafter called 'GPcLC' from five initials of transgenes; Figure 1A, B). The previous study reported that constitutive overexpression of *PSY* in tomato caused gibberellin less and showed a dwarf phenotype because the biosynthesis pathway of carotenoid shares geranylgeranyl pyrophosphate (GGPP) with that of gibberellin (Fray et al. 1995). To prevent the detrimental effect, a tissue-specific promoter is essential for metabolic engineering of carotenoid. In addition, because master regulator (or transcription factor) of carotenoid biosynthesis is still unknown, un-expectable regulation would occur when using carotenogenic promoter. Therefore we used petal-specific *flavanone 3-hydroxylase* promoter from *C. morifolium* (*pCmF3H*; Noda et al. 2013). To modify petal colour of *I. nil*, we made a *GPcLC* overexpression construct under the control of petal-specific *pCmF3H* and introduced it to the white-flowered *I. nil*. Here we report the comparison of carotenoid composition and carotenogenic gene expression between transgenic and non-transgenic plants.

Materials and methods

Plant materials and growth conditions

The seed of *I. nil* cv. AK77 (obtained from the NBRP "Morning glory") was used for the experiments. The seedlings were grown on vermiculite fertilized with 1,000-fold diluted Hyponex 6-10-5 solution (Hyponex Japan, Tokyo, Japan) once a week under continuous light ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$, FL40SW lamps; NEC Lighting Ltd., Tokyo, Japan) at 25°C for two weeks. Those plants were transferred under short-day conditions (8 h light: 16 h dark) at 25°C. For transformation, immature embryos of *I. nil* cv. AK77 were collected two weeks after flower opening (Ono et al. 2000).

Vector construction

The backbone of the binary vector was pBI121 (Jefferson et al. 1987). The fragments of the expression constructs were synthesized using overlapping PCR (Higuchi et al. 1988). Five-prime untranslated region of *alcohol dehydrogenase* (*ADH*) from *Nicotiana tabacum* (Matsui et al. 2015; Satoh et al. 2004) were added to all transgenes for translational enhancer. The sequence of the transit peptide: *tp* from Rubisco subunit of *Pisum sativum* (Misawa et al. 1993; Schreier et al. 1985) was also added to bacterial *crtI* for plastid localization. The PCR products were digested with *XbaI* and *SalI*, and then ligated to pMCE entry vector [modified pSPORT2 vector (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with *pF3H* and *NOS*] individually. The DNA fragments were cut with *AscI* and *StuI* to release the *pF3H::ADH::(Trans gene)::NOS* expression boxes, and then inserted into the multiple cloning sites of pBI121 (Jefferson et al. 1987) one fragment each. The vector was verified by DNA sequencing.

Plant transformation

Previously described *Agrobacterium*-mediated transformation using an immature embryo-derived secondary embryo was performed (Kikuchi et al. 2005). For transformation, *Rhizobium radiobacter* (formally *Agrobacterium tumefaciens*) strain LBA4404 harbouring a ternary plasmid for *virG* N54D (Van Der Fits et al. 2000) was used for raising transformation efficiency. As the transgenic plants directly germinated from kanamycin-resistant secondary embryos, we described these plants as the T1 generation. The validity of transformation was confirmed by PCR using total DNA extracted from young leaves and primers for *NPTII* (Forward: 5' GAG GCT ATT CCG CTA TGA CT 3', Reverse: 5' TCC CGC TCA GAA GAA CTC GT 3'). Total DNA was extracted from the young leaves of plants using a previously described method (Edwards et al. 1991). PCRs were performed with GoTaq[®] Green Master Mix (Promega, Madison, WI, USA) on a thermal cycler, with initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min and a subsequent extension step at 72°C for 5 min. The progeny of the transformation plants (T2 and T3 generation) were also confirmed by the same DNA extraction and PCR condition using *NPTII* primers.

Quantitative real-time PCR (RT-qPCR) and reverse transcriptional PCR analysis

Total RNAs were isolated from petals of fully opened flowers by using the Get pure RNA Kit (Dojindo, Kumamoto, Japan). Then, cDNAs were synthesized from total RNA (1.0 μg) by the use of the SuperScriptIII First-Strand Synthesis System (Invitrogen) with oligo (dT)₂₀.

Transcript levels of *GGPS*, *PSY*, *LCYB*, and *CHYB* were analysed via RT-qPCR with Power SYBR[™] Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and Applied Biosystems 7900HT Fast Real Time PCR System (Applied Biosystems), according to the manufacturers' instructions. Each reaction (final volume, 10 μl) consisted of 5 μl 2x Power SYBR[™] Green PCR Master Mix, 0.4 μM each of the forward and reverse primers, and 0.1 μl of the cDNA template (corresponding to 1 ng of total RNA). The reaction mixtures were heated to 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. The transcript levels were calculated according to the $\Delta\Delta\text{Ct}$ method using the *Ubiquitin* and *Actin* gene for reference (Bustin et al. 2009; Livak and Schmittgen 2001). The primer sets were designed as which could amplify both transgenes and internal genes (Supplemental Table S1). The Student's *t*-test was used for the statistical analysis. The results are presented as the standardized mean of three independent experiments with the SE.

Transcript levels of bacterial *crtI* transgene were analysed using reverse transcription PCR (semi-quantitative RT-PCR) with BIOTAQ[™] (Bioline Reagents Ltd., London, UK). Reactions were carried out on a Takara PCR thermal cycler Dice (Takara

Bio Inc., Shiga, Japan), starting denaturation at 95°C for 2 min followed by 25 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a subsequent extension step at 72°C for 5 min. The *Ubiquitin* gene was used as an internal standard. The PCR products were separated on 1.5% agarose gels and then stained with ethidium bromide solution.

Carotenoid extraction and HPLC analysis

Carotenoids were extracted from petals of fully opened flowers and were analysed by HPLC, according to previously described method (Kishimoto et al. 2007) with slight modification. An acetone extract of frozen petals (0.5 g) was partitioned between diethyl ether and aqueous NaCl. The organic layer was washed with 5.0 mM Tris-HCl (pH 8.0), and the residue was saponified with equivalent 10% KOH-MeOH for 1 h at room temperature. The saponified matter was then extracted with diethyl ether and washed with water. The organic layer was dried and dissolved in 125 μ l MeOH and subjected to HPLC analyses. The non-saponified carotenoid extract was prepared with the same method except saponification step. Each carotenoid extract was analyzed by HPLC with a Jasco MD-915 photodiode array detector (Jasco, Tokyo, Japan) under the following conditions: column, YMC Carotenoid (250 mm \times 4.6 mm i.d., 5 μ m; YMC, Kyoto, Japan); solvent A, methanol (MeOH)/methyl *tert*-butyl ether (MTBE)/H₂O=95:1:4 (v/v/v); solvent B, MeOH/MTBE/H₂O=25:71:4; gradient, 0/100, 12/100, 96/0 (min/% A); flow rate, 1.0 ml \cdot min⁻¹; column temperature, 35°C; UV/visible monitoring range, 200–600 nm. To identify carotenoids, the following carotenoid standards have been used: violaxanthin, neoxanthin (DHI lab products, Hørsholm, Denmark), lutein, β -carotene (Sigma-Aldrich, St. Louis, MO, USA), zeaxanthin and β -cryptoxanthin (Extrasynthese, Genay, France). The total content of carotenoids was estimated from the absorbance at absorption maxima using the E^{1%} value of lutein (2550) (Britton 1995), which was defined as the theoretical absorbance of a 1% solution in a cell of 1 cm pathlength. The content of each carotenoid was calculated according to the total peak area of HPLC chromatograms at a wavelength of 450 nm, using program ChromNAV ver. 2 (Jasco). Measurements were performed in triplicate.

Results

Transgenic lines

More than ten lines of transgenic plants were produced from independent transformation events by *Agrobacterium*-mediated transformation method. All transgenic plants grew normally and had fertility. No morphological differences between transgenic and non-transgenic (NT) plants were visually distinguished, even in the colour of the opened flower (Figure 2A). The T3 generations of the homozygous individuals of three lines (#1–2, #5–9 and #13–11) with highest expression of *PSY* mRNA in the petals of opened fully flowers were selected for further study.

Analysis of carotenogenic gene expression in transgenic plants

Standard curves for PCR efficiency of each endogenous gene and transgene were independently examined. Because the PCR efficiency in all amplicons of each genes showed almost same value (Supplemental Table S1), we calculated expression levels without distinction of endogenous gene and transgene. The expressions of *GGPS*, *PSY*, *LCYB*, and *CHYB* in the petals of fully opened flowers after normalization using *Ubiquitin* and *Actin* as the reference gene were almost similar (Figure 2B and Supplemental Figure S1). Both The expression levels in the petals of fully opened flowers of the three lines were significantly increased in transgenic *GpLC* plants. The expression levels of all the genes tested were about 10 to 1,000 times higher than those of NT (Figure 2B). Bacterial *crtI* was also highly expressed in the petal of the three transgenic lines but was not present in the NT control (Figure 2C).

HPLC analysis of carotenoids

HPLC chromatograms of the carotenoid extracts obtained from the petals of fully opened flowers of the three transgenic lines differ from that obtained from NT. Representative chromatograms of saponified and non-saponified carotenoid extract of NT and transgenic line #1–2 are shown in Figure 3A and Supplemental Figure S2, respectively. These analyses allowed us to identification of carotenoid composition and existence of esterified carotenoids, respectively (Yamamizo et al. 2010). In the petal of NT, only trace amounts of lutein and violaxanthin were detected and the other carotenoid components were below the detection limit (Table 1). On the other hand, zeaxanthin and neoxanthin were detected in the petals of three transgenic lines. Moreover, β -cryptoxanthin and β -carotene were also detected in the two transgenic lines (#1–2 and #13–11). These carotenoid components are β -carotene derivatives (Figure 1A right side) which biosynthetic pathway is supposed to be enhanced by the transgenes. Total carotenoids in the petals of fully opened flowers of the three lines were significantly increased (Figure 3B). Especially, carotenoid levels in #1–2 and #13–11 were about ten times higher than that in NT. Although we could not observe visually yellowish petals, novel *I. nil* flowers that contain various carotenoids were established. The HPLC chromatogram of the non-saponified carotenoid extract was almost the same as that of the saponified extract, indicating that carotenoids contained in the petals of opened fully flowers of transgenic plants were not esterified (Supplemental Figure S2).

Discussion

In the present study, we succeeded to produce

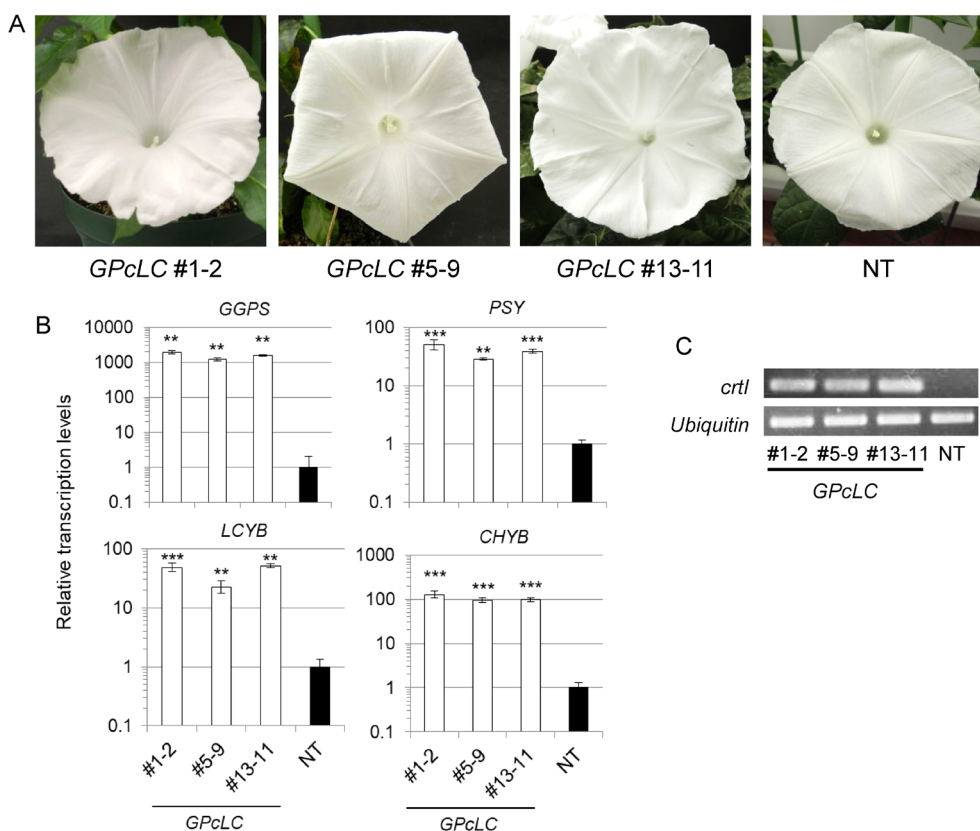


Figure 2. Carotenogenic genes expression levels in the transgenic plants. A: Appearance of *GPcLC* and non-transgenic (NT) flowers. B: Relative expression levels of the botanical transgenes in opened flower of the transgenic *GPcLC* and NT plants detected by RT-qPCR analyses (white bars and a black bar, respectively). The primer sets used for the analysis were designed as which could amplify both transgenes and internal genes. The expression levels were normalized against mRNA levels of *Ubiquitin*. Student's *t*-test was used to determine statistical significance. Asterisks indicate significant difference (** $p < 0.01$, *** $p < 0.001$) to NT. Error bars indicate standard error (SE, $n = 3$). C: Expression levels of the bacterial *crtI* genes in opened flower of the transgenic *GPcLC* plants detected by Semi-quantitative reverse transcriptional PCR analysis. The constitutively expressed gene for the *Ubiquitin* in *I. nil* was used as an internal control.

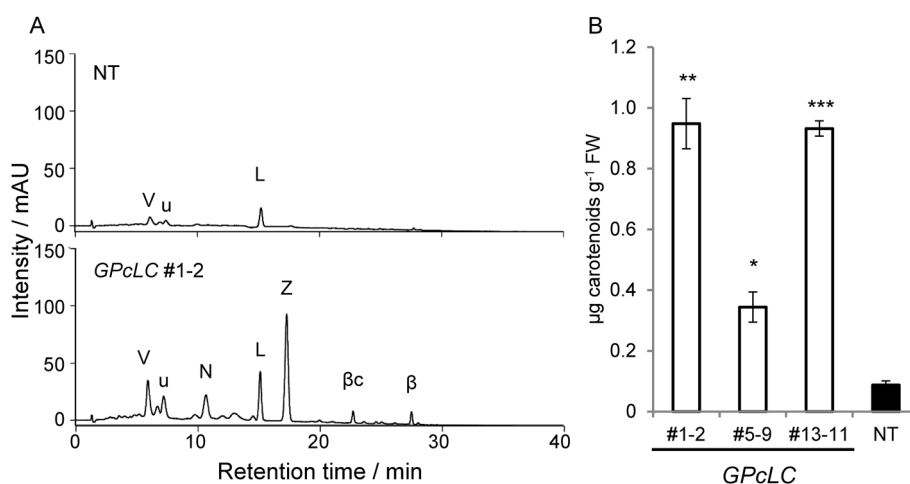


Figure 3. HPLC chromatograms and total carotenoid concentration of flowers of non-transgenic (NT) and transgenic *GPcLC* plant. A: HPLC elution profiles of saponified carotenoids extracted from petals of opened flower. Upper: NT; lower: *GPcLC* #1-2. V: violaxanthin; u: un-identified carotenoid; N: neoxanthin; L: lutein; Z: zeaxanthin; βc: β-cryptoxanthin; β: β-carotene. B: Total carotenoid contents in open flower of the NT and *GPcLC*, as determined by HPLC analysis after saponification. All experiments were biologically repeated three times. Student's *t*-test was used to determine statistical significance. Asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) to NT. Error bars indicate standard error (SE, $n = 3$).

Table 1. Concentrations of carotenoid compounds in the petals

	Violaxanthin	Un-identified	Neoxanthin	Lutein	Zeaxanthin	β -cryptoxanthin	β -carotene
NT	0.028 \pm .003	0.015 \pm .002	ND	0.042 \pm .007	ND	ND	ND
<i>GPcLC</i> #1–2	0.188 \pm .032	0.091 \pm .005	0.108 \pm .011	0.109 \pm .007	0.306 \pm .040	0.020 \pm .004	0.022 \pm .008
#5–9	0.110 \pm .025	0.041 \pm .011	0.032 \pm .011	0.073 \pm .004	0.051 \pm .010	ND	ND
#13–11	0.216 \pm .033	0.107 \pm .002	0.075 \pm .005	0.212 \pm .005	0.154 \pm .004	0.007 \pm .004	0.020 \pm .005

Values are expressed as $\mu\text{g g}^{-1}$ FW; ND, not detectable; FW, fresh weight. Each value represents the mean result from triplicate \pm SE

transgenic plants overexpressing carotenogenic genes. In the petals of fully opened flowers of the transgenic plants, transcription levels of all the transgenes were increased. The carotenoid compositions in the petal of the transgenic plants were dramatically changed. The lutein (α -carotene derivative; Figure 1A left side) and the violaxanthin (β -carotene derivative, Figure 1A right side) were increased as compared with NT. Moreover, zeaxanthin and β -cryptoxanthin (β -carotene derivatives) were newly detected. The possible explanation for the result is that high expression of *GGPS*, *PSY*, and *crtI* enhanced the activity of the whole biosynthetic pathway, and both α - and β -carotene derivatives were increased. Moreover, overexpression of *LCYB* and *CHYB* enhanced activity of biosynthetic pathway, in particular β -carotene derivative branch. These two genes encode the enzymes that catalyze β -ring cyclization and hydroxylation, respectively, so β -carotene derivatives would be specifically produced.

In general, *PSY* is a rate-limiting enzyme of carotenoid biosynthesis in higher plant (Wise and Hooper 2006). For example, in marigold, *Tagetes erecta*, different levels of lutein accumulation cause differences in petal colour, from pale-yellow to dark orange. The previous study found that the lutein content correlates well with the transcript level of *PSY* and *1-deoxy-D-xylulose 5-phosphate synthase* (Moehs et al. 2001). It has also been demonstrated that *PSY* catalyses the rate-limiting step in the carotenoid pathway of ripening tomato fruit (Fraser et al. 1994; Giuliano et al. 1993). In addition, previous study showed that *CHYB* is mainly responsible for the regulation of chromoplast-specific carotenoid accumulation in petals of tomato (Galpaz et al. 2006), because the *CHYB* catalyses the addition of hydroxyl residues required for carotenoid esterification and esterification is an important event in carotenoid accumulation in the chromoplast. Therefore, we expected that overexpressing the genes encoding key enzymes of carotenogenesis would increase the carotenoid levels sufficient for yellow pigmentation. However, the carotenoid levels in the petal of the *GPcLC* transgenic plants were increased up to about $1 \mu\text{g g}^{-1}$, which was not sufficient to make petals yellow visually. Carotenoid level of the petal of the fully opened flower of *I. obscura* var. *lutea* is about $100 \mu\text{g g}^{-1}$ (Yamamizo et al. 2010), so hundred-fold of carotenoid accumulation would be

needed to make petal colour visually yellow. The previous studies on carotenoid metabolic engineering in crops reported that simple overexpressing the biosynthesis genes could increase carotenoid amount in the tissue which is almost carotenoid-free. Overexpressing only bacterial *PSY*, *crtB*, in the seeds of *B. napus* made 50-fold increase in carotenoids (Shewmaker et al. 1999) and overexpressing *PSY* and *crtI* in endosperm of *O. sativa* made total carotenoids up to $37 \mu\text{g g}^{-1}$ (Paine et al. 2005). In contrast to these previous studies (Paine et al. 2005; Shewmaker et al. 1999), in the petal of *I. nil*, only overexpressing the carotenogenic genes could not increase the level of carotenoids high enough to express yellow colour. In our case, three possible reasons were postulated; esterification would not occur, carotenoids would be degraded, or the petal of *I. nil* would not work as a sink organ of carotenoid.

The esterified carotenoids are the dominant chemical form for storage of this compounds within chromoplasts. In the petal of *I. obscura* var. *lutea*, β -carotene, β -cryptoxanthin, and zeaxanthin occupy about 85%. Among them, xanthophylls such as β -cryptoxanthin and zeaxanthin existed in the esterified form (Yamamizo et al. 2010). However, in our case, carotenoids contained in the petals of transgenic plants were not esterified and existed in the free form. Absence of esterification activity would be the reason why total amount of carotenoid was not high enough to express yellow colour in spite of carotenogenic genes were overexpressed. Promoting esterification activity by, for example, overexpressing the *Pale yellow petal 1* which encodes the enzyme that esterifies carotenoids (Ariizumi et al. 2014) would increase the carotenoid accumulation.

Carotenoid degradation are caused by the action of carotenoid cleavage dioxygenases 4 (*CCD4*) in petals of some higher plants (Ahrazem et al. 2016; Ohmiya 2009). In *Brassica* species, transposon mutagenesis of a *BnaCCD4*, one of the orthologue of *CCD4*, converts petal colour from white to yellow (Zhang et al. 2015). The cultivars of *C. morifolium* have white or yellow coloured petals, which rely on existence of *CmCCD4a* (Ohmiya et al. 2006). In *I. nil*, substantial amount of *InCCD4* are also expressed in the petal (Yamamizo et al. 2010), so it would cleave carotenoids. Suppressing the *InCCD4* would increase carotenoid accumulation. However, in the previous study of *C. morifolium*, to

suppress the *CmCCD4a* expression, a single *CmCCD4a* RNAi construct were introduced, but the carotenoid amount in the petal of the transformant was below the detection limit (Ohmiya et al. 2009). Introducing two separate *CmCCD4a* RNAi constructs made petal colour from white to yellow, and its carotenoid amount was increased up to $102 \mu\text{g g}^{-1}$ but was still much less than that of yellow-flowered cultivars (Kishimoto et al. 2007; Ohmiya et al. 2009). The completely knocking out the *CmCCD4* expression might make the yellow petal colour much deeper. Also in *I. nil*, completely knocking out the *InCCD4* with the targeted mutagenesis might lead to the yellow petal.

In yellowish tissues of higher plants, such as flowers and fruits, carotenoid is stored in the chromoplast. The edible tissues of crops, such as endosperms and/or tubers, are full filled with amyloplasts which are one of the plastid forms that store starch (Solymosi and Keresztes 2012). Because plastids can convert each other, amyloplast to chromoplast conversion would naturally occur in amyloplast-rich tissues. Hence the simple overexpression of the carotenogenic genes in the seeds of *B. napus* and endosperm of *O. sativa* can increase the carotenoids accumulation (Paine et al. 2005; Shewmaker et al. 1999). On the other hand, in the petal of *I. nil*, over-expressions of carotenogenic genes could not cause plastid to chromoplast conversion in floral tissue, unlike amyloplast to chromoplast conversion in crops. Otherwise the plastids might be absent in the petal. Induction of the chromoplast differentiation would increase carotenoids sufficient to express yellow colour. Although little is known about the mechanisms of chromoplast differentiation, some factors that affect the event have been reported. One of the factor is *Orange* (*Or*) which is firstly isolated in mutant of cauliflower, *Brassica oleracea* var. *Botrytis* (Crisp et al. 1975; Li et al. 2006; Lu et al. 2006) and recent studies showed that *Or* is post-translational stabilizer of PSY (Park et al. 2016; Zhou et al. 2015). Overexpression of *Or* would cause potential of chromoplast differentiation and facilitate more accumulation of carotenoids.

Recent studies reported that high-quality genome sequence (Hoshino et al. 2016) and the success of targeted mutagenesis by CRISPR/Cas9 system (Watanabe et al. 2017) in *I. nil*. These information and technology would enable advanced carotenoid metabolic engineering so more progressive studies using *I. nil* will be conducted. If carotenoid accumulation will be enabled in a petal of *I. nil*, the range of colour variation of the flower will get almost no limitation with crossbreeding with other anthocyanin pigmented flowers, because varied colours of anthocyanin-pigmented flowers of *I. nil* were already produced in the seventeenth century. It would be the fusion of a modern genetic engineering technology and a past developing traditional breeding technique, in other

words, the connection between innovation and tradition.

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References

- Ahrazem O, Gómez-Gómez L, Rodrigo M, Avalos J, Limón M (2016) Carotenoid cleavage oxygenases from microbes and photosynthetic organisms: Features and functions. *Int J Mol Sci* 17: 1781
- Ariizumi T, Kishimoto S, Kakami R, Maoka T, Hirakawa H, Suzuki Y, Ozeki Y, Shirasawa K, Bernillon S, Okabe Y, et al. (2014) Identification of the carotenoid modifying gene *PALE YELLOW PETAL 1* as an essential factor in xanthophyll esterification and yellow flower pigmentation in tomato (*Solanum lycopersicum*). *Plant J* 79: 453–465
- Azadi P, Otang NV, Chin DP, Nakamura I, Fujisawa M, Harada H, Misawa N, Mii M (2010) Metabolic engineering of *Lilium × formolongi* using multiple genes of the carotenoid biosynthesis pathway. *Plant Biotechnol Rep* 4: 269–280
- Britton G (1995) UV/visible spectroscopy. In: Britton G, Liaen-Jensen S, Pfander H (eds) *Carotenoids, Volume 1B: Spectroscopy*. Basel, Birkhäuser Verlag, pp 13–62
- Bustin SA, Benes V, Garson JA, Hellems J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al. (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55: 611–622
- Cazzonelli CI, Pogson BJ (2010) Source to sink: Regulation of carotenoid biosynthesis in plants. *Trends Plant Sci* 15: 266–274
- Crisp P, Walkey DGA, Bellman E, Roberts E (1975) A mutation affecting curd colour in cauliflower (*Brassica oleracea* L. var. *Botrytis* DC). *Euphytica* 24: 173–176
- Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res* 19: 1349
- Fraser PD, Truesdale MR, Bird CR, Schuch W, Bramley PM (1994) Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiol* 105: 405–413
- Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J* 8: 693–701
- Galpaz N, Ronen G, Khalfa Z, Zamir D, Hirschberg J (2006) A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato *white-flower* locus. *Plant Cell* 18: 1947–1960

- Giuliano G, Bartley GE, Scolnik PA (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* 5: 379–387
- Giuliano G, Tavazza R, Diretto G, Beyer P, Taylor MA (2008) Metabolic engineering of carotenoid biosynthesis in plants. *Trends Biotechnol* 26: 139–145
- Higuchi R, Krummel B, Saiki R (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. *Nucleic Acids Res* 16: 7351–7367
- Hoshino A, Jayakumar V, Nitasaka E, Toyoda A, Noguchi H, Itoh T, Shin-I T, Minakuchi Y, Koda Y, Nagano AJ, et al. (2016) Genome sequence and analysis of the Japanese morning glory *Ipomoea nil*. *Nat Commun* 7: 13295
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6: 3901–3907
- Jeknić Z, Jeknić S, Jevremović S, Subotić A, Chen THH (2014) Alteration of flower color in *Iris germanica* L. ‘Fire Bride’ through ectopic expression of phytoene synthase gene (*crtB*) from *Pantoea agglomerans*. *Plant Cell Rep* 33: 1307–1321
- Kikuchi R, Sage-Ono K, Kamada H, Ono M (2005) Efficient transformation mediated by *Agrobacterium tumefaciens* with a ternary plasmid in *Pharbitis nil*. *Plant Biotechnol* 22: 295–302
- Kishimoto S, Ohmiya A (2006) Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium*). *Physiol Plant* 128: 436–447
- Kishimoto S, Sumitomo K, Yagi M, Nakayama M, Ohmiya A (2007) Three routes to orange petal color via carotenoid components in 9 Compositae species. *J Jpn Soc Hortic Sci* 76: 250–257
- Li L, Lu S, Cosman KM, Earle ED, Garvin DF, O’Neill J (2006) β -Carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. *Phytochemistry* 67: 1177–1184
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* 25: 402–408
- Lu S, van Eck J, Zhou XJ, Lopez AB, O’Halloran DM, Cosman KM, Conlin BJ, Paolillo DJ, Garvin DF, Vrebalov J, et al. (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β -carotene accumulation. *Plant Cell* 18: 3594–3605
- Matsui T, Sawada K, Takita E, Kato K (2015) Compatibility of translational enhancers with various plant species. *Plant Biotechnol* 32: 309–316
- 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
- Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J Bacteriol* 172: 6704–6712
- 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
- Moehs CP, Tian L, Osteryoung KW, Dellapenna D (2001) Analysis of carotenoid biosynthetic gene expression during marigold petal development. *Plant Mol Biol* 45: 281–293
- Nisar N, Li L, Lu S, Khin NC, Pogson BJ (2015) Carotenoid metabolism in plants. *Mol Plant* 8: 68–82
- Noda N, Aida R, Kishimoto S, Ishiguro K, Fukuchi-Mizutani M, Tanaka Y, Ohmiya A (2013) Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins. *Plant Cell Physiol* 54: 1684–1695
- Ohmiya A (2009) Carotenoid cleavage dioxygenases and their apocarotenoid products in plants. *Plant Biotechnol* 26: 351–358
- Ohmiya A, Kishimoto S, Aida R, Yoshioka S, Sumitomo K (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. *Plant Physiol* 142: 1193–1201
- Ohmiya A, Sumitomo K, Aida R (2009) “Yellow Jimba”: Suppression of carotenoid cleavage dioxygenase (*CmCCD4a*) expression turns white chrysanthemum petals yellow. *J Jpn Soc Hortic Sci* 78: 450–455
- Ono M, Sage-ono K, Kawakami M, Hasebe M, Ueda K, Masuda K, Inoue M, Kamada H (2000) *Agrobacterium*-mediated regeneration and transformation of *Pharbitis nil*. *Plant Biotechnol* 17: 211–216
- Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, et al. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotechnol* 23: 482–487
- Park S, Kim HS, Jung YJ, Kim SH, Ji CY, Wang Z, Jeong JC, Lee H-S, Lee SY, Kwak S (2016) Orange protein has a role in phytoene synthase stabilization in sweetpotato. *Sci Rep* 6: 33563
- Römer S, Fraser PD, Kiano JW, Shipton CA, Misawa N, Schuch W, Bramley PM (2000) Elevation of the provitamin A content of transgenic tomato plants. *Nat Biotechnol* 18: 666–669
- Saito N, Cheng J, Ichimura M, Yokoi M, Abe Y, Honda T (1994) Flavonoids in the acyanic flowers of *Pharbitis nil*. *Phytochemistry* 35: 687–691
- Satoh J, Kato K, Shinmyo A (2004) The 5′-untranslated region of the tobacco *alcohol dehydrogenase* gene functions as an effective translational enhancer in plant. *J Biosci Bioeng* 98: 1–8
- Schreier PH, Seftor EA, Schell J, Bohnert HJ (1985) The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J* 4: 25–32
- 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
- Solymosi K, Keresztes A (2012) Plastid structure, diversification and interconversions II. Land plants. *Curr Chem Biol* 6: 187–204
- 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
- van der Fits L, Deakin EA, Hoge JHC, Memelink J (2000) The ternary transformation system: Constitutive *virG* on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. *Plant Mol Biol* 43: 495–502
- Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M (2017) CRISPR/Cas9-mediated mutagenesis of the *dihydroflavonol-4-reductase-B* (*DFR-B*) locus in the Japanese morning glory *Ipomoea (Pharbitis) nil*. *Sci Rep* 7: 10028
- Wise RR, Hooper JK (2006) *The Structure and Function of Plastids*.

- Springer Netherlands, Dordrecht
- Yamamizo C, Kishimoto S, Ohmiya A (2010) Carotenoid composition and carotenogenic gene expression during *Ipomoea* petal development. *J Exp Bot* 61: 709–719
- Yamazaki Y, Akashi R, Banno Y, Endo T, Ezura H, Fukami-Kobayashi K, Inaba K, Isa T, Kamei K, Kasai F, et al. (2010) NBRP databases: Databases of biological resources in Japan. *Nucleic Acids Res* 38(Database): D26–D32
- Ye X, Al-Babili S, Klöti 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 H, Zhang J, Nageswaran D, Li L (2015) Carotenoid metabolism and regulation in horticultural crops. *Hort Res* 2: 15036
- Zhang B, Liu C, Wang Y, Yao X, Wang F, Wu J, King GJ, Liu K (2015) Disruption of a *CAROTENOID CLEAVAGE DIOXYGENASE 4* gene converts flower colour from white to yellow in *Brassica* species. *New Phytol* 206: 1513–1526
- Zhou X, Welsch R, Yang Y, Álvarez D, Riediger M, Yuan H, Fish T, Liu J, Thannhauser TW, Li L (2015) Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis. *Proc Natl Acad Sci USA* 112: 3558–3563
- Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell 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