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APPROACHES TO IMPROVE *CrtI* EXPRESSION IN RICE ENDOSPERM FOR INCREASING THE β-carotene CONTENT IN GOLDEN RICE

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ABSTRACT

The invention of Golden Rice, which could produce β-carotene (provitamin A) in its endosperm, offered a new hope to improve diet of millions of people in the world. However, it is necessary to develop lines, which could produce a higher level of β-carotene than the existing golden rice lines to make Golden Rice possible for practical adoption. Working in this direction, we have modified the crtI gene, which encodes the bacterial carotene desaturase CrtI in the pathway of β-carotene synthesis by (a) replacing the CaMV35S promoter with an endosperm specific promoter, GluB1 or (b) using a synthetic crtI gene, which abolishes differences to the codon usage, met in genes for rice storage proteins driven by GluB1 promoter. With these new versions of the crtI gene, we obtained corresponding transgenic plants of which the resulting T_1 rice grains were analyzed for carotenoid contents. Our data indicates that the approaches of modifying crtI gene did not directly result in an increase of β-carotene content in Golden Rice.

Key words: carotenoids, *CrtI*, endosperm codon usage, provitamin A, Golden Rice, rice transformation.

INTRODUCTION

Rice is consumed by nearly half of the world's population. It represents the major source of energy of millions of people in developing countries. However, rice is a poor source of many essential micronutrients and vitamins; many are malnourished due to extreme poverty limiting the access to more diversified food sources. As a result, deficiency of iron, iodine and vitamin A prevails in developing countries. It is estimated that 140 to 250 million people are deficient in vitamin A (Underwood 2000). The United Nations Children's Fund states that improved vitamin A nutrition could prevent 1 to 2 million deaths each year among children aged 1 to 4 years (UNICEF 2000). Vitamin A deficiency (VAD) causes increased morbidity and mortality of infants, children, and pregnant women; poor growth of children; and possibly increased mortality and morbidity of infants infected by HIV. VAD impairs with vision leading to the manifestation of xerophthalmia (zitat). It also contributes to anemia by interfering with iron transport and utilization (Gillespie and Haddad 2001).

All naturally occurring vitamin A derives from carotenoids with provitamin A activity and among these; β-carotene is the most important in mammals. Carotenoids are a group of isoprenoid pigments widely distributed in nature. They are synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. Carotenoids protect the photosynthetic apparatus from photo oxidation and represent structural components of light harvesting antenna and reaction centre complexes.

Rice endosperm does not contain carotenoids but it has its precursor geranylgeranyl-diphosphate (GGDP). Upon transformation with a cDNA coding for phytoene-synthase (PSY), this GGPP can be utilized to form phytoene, the first, but still uncolored carotene in the pathway (Burkhardt et al. 1997). The first Golden Rice lines was produced by Ye et al. (2000) by engineering the full complement of all necessary genes coding for PSY (from *Narcissus pseudonarcissus* L.), phytoene desaturase (*CrtI*, from the bacterium *Erwinia uredovora*) and lycopene cyclase (*LCY,* from *N. pseudonarcissus* L.) resulted in yellow colored rice grains which produced

carotenoids up to 1.6 µg/g in the segregating seeds of the T_0-generation. The Golden Rice technology was subsequently shown to be functional in different cultivars of rice belonging both japonica and indica type (Hoa *et al*. 2003; Datta *et al*. 2003). The additional supplementation with lycopene β-cyclase was found unnecessary because β-carotene also formed in its absence.

To enhance the carotenoid content, in the absence of any knowledge on limiting factors in carotenoid biosynthetic pathway in rice endosperm, the possible interventions are to optimize of the two carotenoid biosynthesis enzymes expressed in golden rice lines. There are (1) phytoene synthase as the first committed step in caroteogenesis and (2) *CrtI*, the bacterial desaturase which, by introducing four double bonds into conjugation, catalyzes the formation of the red-colored lycopene as the substrate for cyclization yielding β-carotene. In this study, we report on our approaches to improve *CrtI* expression in rice endosperm by replacing the constitutive CaMV35S promoter for an endosperm specific promoter, GluB1 or by using a synthetic *crtI* gene which abolishes differences to the codon usage met in genes for rice storage proteins driven by GluB1 promoter. Accordingly, DNA constructs were made and transformed into rice plants. The resulting T1 seeds were measured for carotenoid content.

MATERIALS AND METHODS

Construction of the plasmids

Two plasmids, namely pCarNew (Fig .1) and pFun3 (Fig. 2) were constructed as described below

Construction of pCarNew: To isolate the endosperm specific promoter GluB1 (Takaiwa *et al*., 1991) from pUC18-GluB, the vector was digested with *Bam*HI, filld-in using T4-DNA polymerase and treated with *Hin*dIII. The obtained GluB1-fragment was then ligated into the filled-in *Xba*I- and the *Hin*dIII-site of pUCET4 (Misawa *et al*., 1993) replacing the original CaMV35S promoter in the *CrtI* expression cassette and yielding the vector pGCrtI. pCarNew was constructed in two steps. First, the tissue specific CrtI-expression cassette was isolated from pGCrtI using *Eco*RI and *Hind*III and ligated into the corresponding sites of pMCA1380, a derivative of pCAMBIA1380 (Cambia, Canberra, Australia) encoding a phosphomannose isomerase as a selection marker, yielding the binary vector pBCrtI. Finally, pCarNew was then constructed by ligating a *Hind*III-fragment encoding the *PSY*-expression cassette from pCaCar (Hoa *et al*., 2003) into the corresponding site of pBCrtI.

Construction of pFun3: The modified *TP-CrtI* gene was synthesized by Entelechon GmbH, Regensburg; Germany. It was excised from pPCR-CrtI using *Not*I and *Eco*RI. Prior *Eco*RI digestion, a T4-DNA polymerase treatment was performed to fill-in the *Not*1-site. The obtained fragment was then cloned into *Sma*I/*Eco*RI treated pUC18-GluB yielding pFun1. A *Sal*I/*Eco*RI fragment encoding the endosperm specific promoter GluB1 and the synthetic *TP-CrtI* was isolated from pFun1 and ligated into the corresponding sites of pMCA1390, a derivative of pCAMBIA1390 (Cambia, Canberra, Australia) encoding a phosphomannose isomerase as a selection marker, yielding the binary vector pFun2. Finally, pFun3 was constructed by ligating a *Sal*I-fragment encoding the *PSY* expression cassette from pBaal2 (Ye *et al*., 2000) into the corresponding site of pFun2.

Plant materials and transformation

Embryogenic calli-derived from immature embryos of the cultivar Taipei 309 were inoculated with *Agrobacterium tumefaciens* strain *LBA 4404* (Hoekema *et al*., 1984). The transformation experiments were performed according to Hoa et al. (2002), Hoa and Bong (2003). Two weeks after in the rooting medium, root of the putative transformants were tested for phosphomannose isomerase activity using the chlorophenol-red (CR) assay as described by Hoa and Bong (2003). Only the plants showed positive reaction were transferred to soil and grown in the greenhouse at 28°C (day) and 21°C (night) and 80% relative humidity.

DNA isolation and Southern blot analysis

Genomic DNA was isolated from rice leaves following the method of McCouch et al.

(1988). Ten micrograms of genomic DNA were digested with *Eco*RI to detect *psy* or *crtI* and with *Kpn*I for copy number analysis. Southern blot analysis was carried out following standard protocols (Sambrook et al. 1989). PCR-amplified, DIG-labeled (Boehringer, Rotkreuz, Switzerland) fragments of *psy* (primers: 5' TACGTAGCAGGAACTG 3'; 5' CAAACAGGCCACCTGCTAGC 3') and *crtI* (primers: 5' GAGTGGGGCGT 3'; 5' TAACTGCCGCAACCTT 3') were used as probes. Hybridization, washing and detection were performed following the procedures as given in Wünn et al. (1996).

Segregation analysis of the transgenes

The self-pollinated seeds of T1 generation were first phenotypically selected by visible color after polishing (Table 2) and then grown in MS (Murashige and Skoog 1962) medium supplemented with 3% mannose. The resistant plants scored 2 weeks after culture were transferred and grown in the greenhouse for further Southern blot analysis. In the same way, generations were advanced to T2, T3 and T4.

Carotenoid extraction and analysis

Polished rice grains (0.5-1 g) were ground for 1 min to a fine powder using a Micro-Dismembrator (Braun, Melsungan, Germany). The powder in the Teflon capsules was resuspended in 2 ml of acetone at room temperature and transferred to a glass test tube. This suspension was sonicated for 10-20 s (until it gave temperature), centrifuged for 10 minutes at 10,000 rpm and the supernatant collected. The extraction procedure was repeated with another 2 ml to ensure complete carotenoid extraction. The combined extracts were dried in a Vortex-Evaporator (Haake-Buchler; Saddle Brook, NJ, USA) and resuspended in 500 µl of acetone. Carotenoid amounts were determined by HPLC using the internal plus an external standard (echinenone, kindly provided by Hoffmann-La Roche, Basel, Switzerland). Samples were taken to dryness and dissolved in 70 µl of chloroform, of which 50 µl was injected into the HPLC system according to Hoa et al. (2003).

RESULTS AND DISCUSSION

Codon optimization of TP-CrtI

Based on sequences of 6 different rice storage proteins (Table 1), we determined the codon probabilities and compared them with those of *TP-CrtI*. The *TP-CrtI* codon usage differed markedly from the one met in rice genes. For instance, the 20 codons for histidin in *TP-CrtI* are represented 7 times by CAC and 13 times by CAU corresponding to a frequency of 35% and 65%, respectively. In contrast, the inverse ratio was found in the selected rice genes. Similar results were obtained by comparing codon-ratios of lysine and phenylalnine. Therefore, a synthetic *TP-CrtI* was made exhibiting 26.2% in homology to the original version (data not shown).

Table 1. Selected storage genes used for determination of the codons frequency in rice endosperm

Storage protein	Accession Nr.
10 kDa prolamin	X84649
13 kDa prolamin	S39468
glutelin	X14568
glutelin	AB016501
Gt2	M17513
glutelin 1 (Gt22)	M28159

Rice transformation with the vector pCarNew or pFun3

Different sets of *A. tumefaciens* mediated transformation experiments were performed using immature embryos of japonica rice variety "Taipei 309", using the vector, pCarNew or pFun3 haboring *pmi* (phosphomannose isomerase) selective marker gene (Fig.1, Fig.2). The resulted transgenic T_0 plants were confirmed by the presence of both the *psy* and *crtI* gene shown in Southern blot analysis and the positive reaction in CR assay (Table 2) as well. For the transformation using pCarNew, we have obtained 44 independent transformants (Fig.3, Fig 4 and Fig. 5). Meanwhile, for the transformation using pFun3, 48 independent transgenic lines were obtained (Fig.6 and Fig.7). The event E1-10 transformed with pFun3 was an exception because it did not show the DNA band of *crtI* gene (Fig.6).

As earlier revealed by Hoa et al. (2003), the transgenic lines generated by *A. tumefaciens* mediated transformation had simple integration of the transgene with low copy number, hence "clean" events could be identified and selected. Such observations were also seen in the transformation experiments of this study. The advantage of *A. tumefaciens* mediated transformation over other transformation methods was well recognized. Using this transformation method along with mannose selection system would be an option to lighten the concerns on the biosafety of genetically modified plants (Hoa et al., 2003).

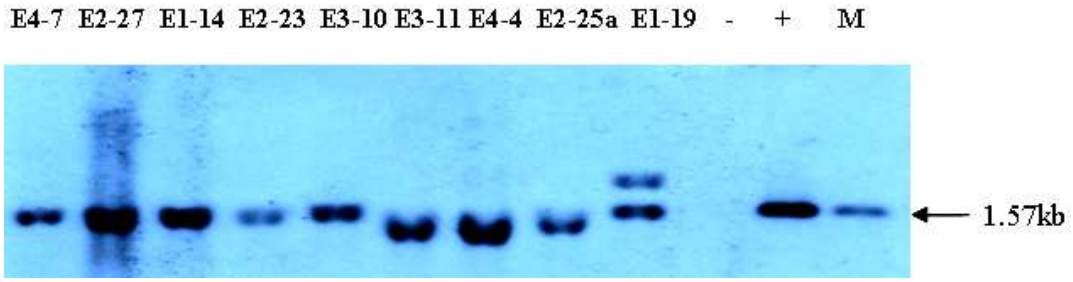

Fig 3. Southern blot analysis of T_0 plants Taipei 309 transformed with pCarNew, DNA digested with *Eco*RI + *Hin*d III and probed with *psy*. The expected size ~ 1.57kb.
(+): Plasmid DNA (-): Untransformed

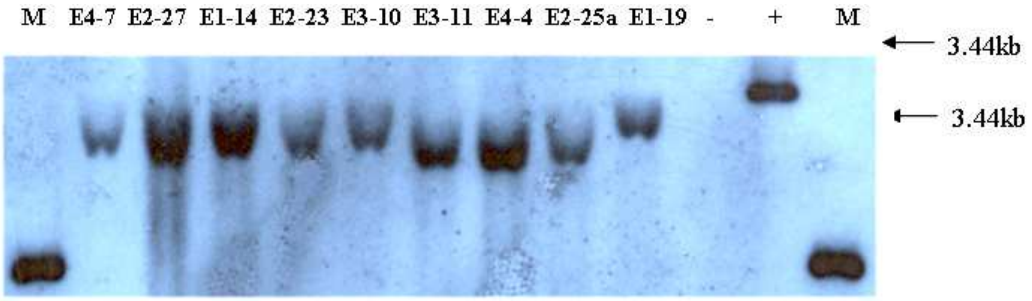

Fig 4. Southern blot analysis of T_0 plants Taipei 309 transformed with pCarNew, DNA digested with *Eco*RI + *Hin*d III and probed with *crtI*. The expected size ~3.44kb
(+): Plasmid DNA (-): Untransformed

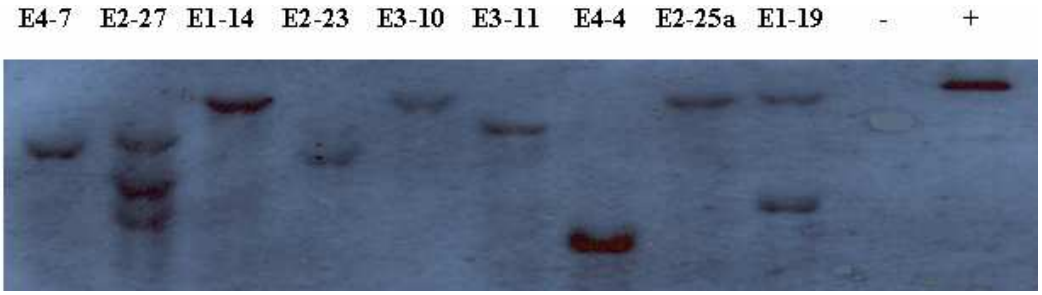

Fig 5. Southern blot analysis of T_0 plants Taipei 309 transformed with pCarNew, DNA digested with *Eco*RI + *Hin*d III and probed with *pmi*. To detect copy number (+): Plasmid DNA (-): Untransformed

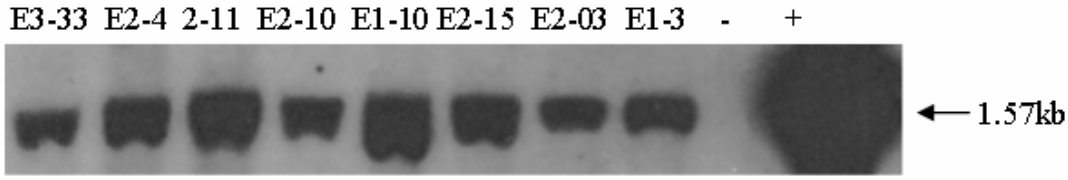

Fig 6. Southern blot analysis of T_0 plants Taipei 309 transformed with pFun3 probed with *psy.* DNA digested with *Eco*RI plus *Hin*d III
(+): Plasmid DNA (-): Untransformed

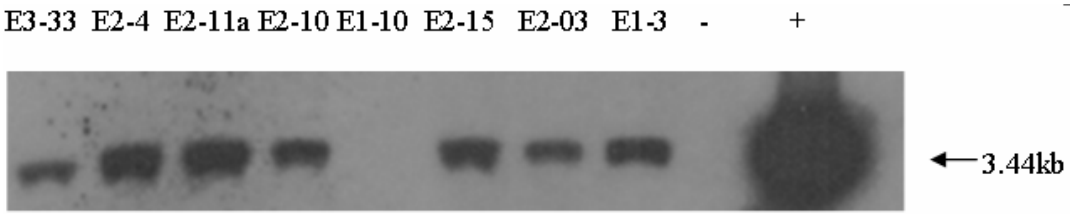

Fig 7. Southern blot analysis of T_0 plants Taipei 309 transformed with pFun3 probed with *Crt*I, DNA digestedwith *Eco*RI plus *Hin*d III
(+): Plasmid DNA (-): Untransformed

The T_1 seeds of selective transgenic events (T_0) were polished. Segregation of yellow endosperm vs white endosperm was visible following Mendelian pattern with nearly 3:1 ratio (yellow:white) (Table 3). Endosperms of advancing generations (T_2, T_3, T_4) also expressed yellow color indicating the inheritance of the trait was stable. It was observed that the yellow color was more deep with the advanced generations (Fig. 8), this may be due to the increase of carotenoid content in homozygous lines of advanced generations.

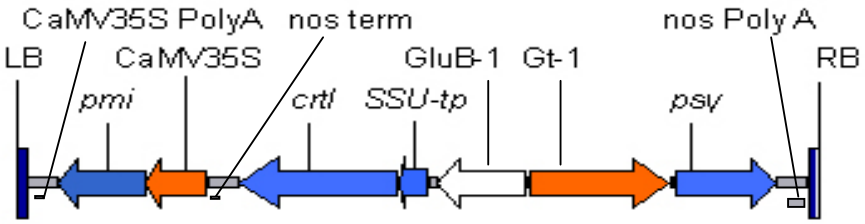

Fig 1. Schemes of the vector pCarNew. LB and RB, left and right T-DNA boders; Gt1, glutelin promoter; GluB1, endosperm specific promoter; *pmi*, phosphomannose insomerase; *psy*, phytoene synthase; *crtI*, bacterial phytoene desaturase; SSU-tp, transit peptide of the ribulose-bis-phosphate carboxylase small subunit

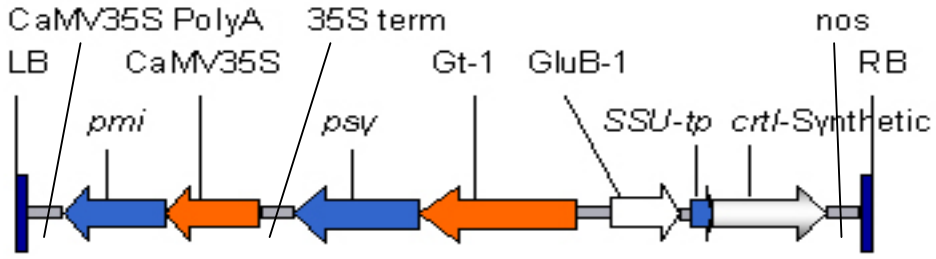

Fig 2. Schemes of the vector pFun3. LB and RB, left and right T-DNA boders; Gt1, glutelin promoter; GluB1, endosperm specific promoter; *pmi*, phosphomannose insomerase; *psy*, phytoene synthase; *crtI*- synthetic (codon optimized); SSU-tp, transit peptide of the ribulose-bis-phosphate carboxylase small subunit

Fig 8. Seeds of transgenic line E2-11a transformed with pFun3.
A: T_1 seeds; B: T_2 seeds; C: T_3 seeds; D: T_4 seeds and F: Taipei 309 wild type

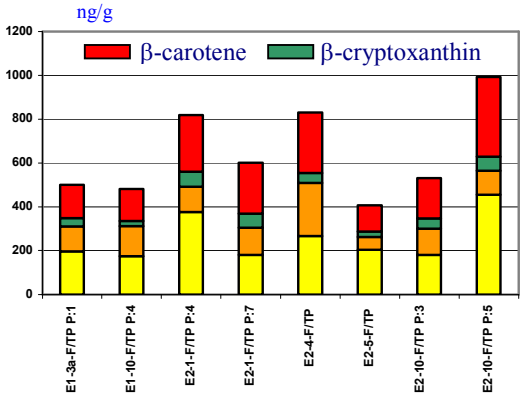

Fig 9 A. Carotenoid analysis of Taipei 309 T_1 seeds (transformed with pFun3)

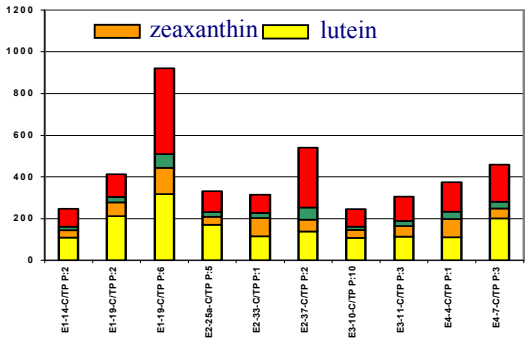

Fig 9 B. Carotenoid analysis of Taipei 309 T_1 seeds (transformed with pCarNew)

Table 2. Overview on T0 transgenic lines transformed with pCarNew and pFun3.

Plasmids	Number of T_0 plants	T_1 selected by visual inspection and carotenoid analysis	Integration (n)	T_2, T_3, T_4 Seeds
pCarNew	44	E1-14	1	yes
		E1-19, P:2	2	yes
		E1-19, P:6	2	yes
		E2-25a	1	yes
		E2-23	1	yes
		E2-33	1	yes
		E2-27	1	yes
		E2-37	3	yes
		E3-10	1	yes
		E3-11	1	yes
		E4-4	1	yes
		E4-7	1	yes
		ZB1-07	1	no
		ZB1-11	2	no
		ZB1-13	1	no
		ZB3-04	1	no
		ZB3-14	2	no
		ZB4-09	1	no
pFun3	48	E1-3	1	yes
		E1-10	1	yes
		E2-1, P:4	2	yes
		E2-1, P:7	2	yes
		E2-03	1	yes
		E2-4	4	yes
		E2-5	3	yes
		E2-10	1	yes
		E2-11a	1	yes
		E3-33	1	yes
		E2-15	1	yes
		ZA2-03	1	no
		ZA2-11	2	no
		ZA2-14	2	no
		ZA2-15	1	no
		ZA2-23	1	no
		ZA2-36	1	no
		ZA3-16	3	no
		ZA3-22	1	no
		ZA3-25	2	no
		ZA3-59	1	no

Table 3. T_1 seeds obtained from T_0 transgenic plants transformed with pCarNew and pFun3.

Plasmids	Transgenic lines (plant ID)	Color of T_1 seeds after polishing (yellow : white)	Total polished grains
pCarNew	E 1-3	19 y: 6 w	25
	E 1-4	15 y: 7 w	22
	E 1-5	13 y: 6 w	19
	E 1-9	17 y: 7 w	24
	E 1-10	26 y: 8 w	24
	E 1-11	27 y: 8 w	35
	E 1-13	16 y: 7 w	23
	E 1-14	22 y: 8 w	30
	E 1-17	20 y: 6 w	26
	E 1-19	19 y: 6 w	25
	E 2-10	13 y: 7 w	20
	E 2-16	26 y: 11 w	37
	E 2-16a	12 y: 6 w	18
	E 2-16b	23 y: 11 w	34
	E 2-23	27 y: 9 w	36
	E 2-25a	28 y: 10 w	38
	E 2-33	25 y: 8 w	33
	E 2-37	25 y: 3 w	28
	E 3-4	17 y: 4 w	21
	E 3-10	26 y: 9 w	35
	E 3-11	26 y: 9 w	35
	E 4-4	29 y:11 w	40
pFun3	E 1-3a	21 y: 6 w	27
	E 1-10	23 y: 8 w	31
	E 2-1	36 y: 8 w	44
	E 2-4	25 y: 2 w	27
	E 2-5	13 y: 15 w	28
	E 2-7b	15 y: 6 w	21
	E 2-9	25 y: 15 w	40
	E 2-10	28 y: 8 w	36
	E 2-11a	18 y: 5 w	23
	E 2-11b	19 y: 3 w	22
	E 2-12	9 y: 3 w	12
	E 2-13	10 y: 5 w	15
	E 2-13b	14 y: 14 w	28
	E 2-18	12 y: 8 w	20
	E 3-16	26 y: 18 w	44
	E 3-19c	8 y: 3 w	11
	E 3-20	20 y: 19 w	39
	E 3-23	25 y: 15 w	40
	E 3-25	26 y: 15 w	31
	E 3-33	17 y: 8 w	25
	E 3-35	14 y: 7 w	21
	E 3-41b	16 y: 9 w	25
	E 3-43	23 y: 8 w	31
	E 3-55	12 y: 4 w	16
	E 3-58	26 y: 17 w	43

Analysis of T_1 seeds for carotenoid content

Randomly selected endosperms of each segregating T_1 population derived from T_0 independent events transformed by pCarNew (the bacterial *TP-CrtI* under the control of the endosperm specific promoter, GluB1) or pFun3 (*TP-Synthetic CrtI* under the control of the GluB1 promoter) were subjected to carotenoid analysis separately and the carotenoid amount and pattern were determined by HPLC. The results (Fig.9A, Fig.9B) showed that the carotenoid amounts in the T_1 seeds of pCarNew and pFun3 transformants were in a similar range from 0.2 to about 0.9-µg g^{-1} (pCarNew) and 0.2 to about 1 µg g^{-1} (pFun3). These observed values did not surpass the amounts obtained from seeds transformed with pCaCar that contained the original *TP-CrtI* under the control of the constitutive CaMV35S promoter (Hoa *et al*., 2003). In addition, we did not observe significant changes in the carotenoid pattern of the pCarNew and pFun3 seeds in comparison to the one found in the endosperm of pCaCar transformants.

The results of this study sugested that the improved expression of the *crtI* gene by replacing its promoter is less relevant to increase the β-carotene content in rice endosperm. This is further corroborated by recent studies that show the rate limiting step is evidently represented by phytoene synthase (Paine *et al*. 2005). However, there are reasons other than the expression level suggesting the endosperm specific expression of CrtI to be the preferred option. Constitutive expression of CrtI leads to a decrease in lutein, partially compensated by an increase of β-carotene-derived xanthophylls in leaves of tomato (Romer *et al*. 2000) and rice (Schaub *et al*. 2005). This alteration is not mirrored by a change in the expression of the respective lycopene ε or β-cyclases but may reside at the level of the cis-trans isomerism of lycopene (Schaub *et al*., 2005) and may have a negative impact on the photosynthetic capacity under high-light conditions (Lokstein *et al*., 2002).

The modification of bacterial genes towards plant gene codon usage increased their expression *in planta*. For instance, such modification of the *Bacillus thuringiensis* genes *cryI(A)b* and *cryI(A)c* dramatically increased their protein levels in tomato, tobacco and rice plants (Perlak *et al*. 1991; Cheng *et al*. 1998) as well as in maize endosperm cell cultures (Sardana *et al*. 1995). Similarly, the codon optimization of a bacterial (1, 3-1, 4)-β-glucanase resulted in a significant increase of its protein level in barley (Jensen *et al*., 1996). Nevertheless, in the case of carotenoid content in rice, although a synthetic *TP-CrtI* with codon optimization was introduced, we did not observe any significant increase in the carotenoid content; the obtained amounts remained in the range determined for Golden Rice lines expressing the original *TP-CrtI* constitutively.

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Thử nghiệm các phương pháp chỉ thị sự biểu hiện CrtI để nâng cao hàm lượng β-carotene ở hạt của giống lúa vàng

Sự phát minh ra giống Lúa Vàng có khả năng tạo ra β carotene (tiền vitamim A) trong hạt gạo đã mở ra triển vọng cải thiện dinh dưỡng trong bữa ăn của hàng triệu người trên thế giới. Tuy nhiên, để Lúa Vàng có giá trị áp dụng thực tiễn, chúng ta cần phải tạo ra các dòng lúa chứa hàm lượng β-carotene cao hơn các dòng Lúa Vàng đã có. Nghiên cứu theo hướng này, chúng tôi tiến hành cải tiến gen *crtI* có chức năng tạo ra enzyme tác động vào chu trình tổng hợp β-carotene bằng 2 cách: (1) thay đổi CaMV35S promoter bằng GluB1 promoter, có tính chuyên biệt đối với phôi nhũ, (2) tổng hợp gen *crtI* nhân tạo với GluB1 promoter, gen nhân tạo này có những codon được tối hảo hóa theo hướng tương ứng với các codon điều khiển sinh tổng hợp protein dự trữ trong cây lúa. Vectơ tương ứng đã được chuyển nạp vào cây lúa. Các hạt T_1 của cây lúa được chuyển gen (khẳng định qua phân tích Southern) đã được xét nghiệm về hàm lượng và thành phần carotenoids. Kết qủa cho thấy, hướng cải thiện sự biểu hiện gen *crtI* không có tác động trực tiếp đến hàm lượng carotenoid ở các dòng Lúa Vàng.