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Molecular characterization of marker-free transgenic lines of *indica* rice that accumulate carotenoids in seed endosperm

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Abstract A single *Agrobacterium* strain harbouring two binary plasmids was successfully used for the first time to develop a marker-free transgenic rice of improved nutritional value. Sixty-eight T_0 co-transformants were obtained in three *indica* rice cultivars—two popular high-yielding Bangladeshi varieties (BR28 and BR29), and one high-iron rice cultivar (IR68144). Marker-free lines were obtained from 14 out of 24 selected co-transformants screened in the T_1 generation. The accumulation of total carotenoids in polished T_2 rice seeds of the primary transgenic VPBR29-17-37 reached levels of up to 3.0 μg/g, with the level of β-carotene reaching 1.8 μg/g. In the cultivars BR28 and IR68144, total carotenoid levels in the transformants reached 2.0 μg/g of polished rice seeds. The levels of lutein and other carotenoids in the seeds were also significantly enhanced. T_1 plants obtained from primary transgenics with simple gene-integration patterns tended to have a lower carotenoid content than the original parental lines. This study describes the development of marker-free transgenic rice lines containing high levels of carotenoids, and addresses the relationship between the rearrangement of transgenes and the presence of metabolic end products in transgenic rice.

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Introduction

Carotenoids, a broad class of isoprenoid-derived pigments, serve as photoprotectants, anti-oxidants, and as precursors of abscisic acid. These pigments are synthesized by all photosynthetic, and many non-photosynthetic, organisms (Naik et al. 2003). Vertebrates cannot synthesize carotenoids de novo, and depend entirely on dietary sources for their supply. β-Carotene and lutein are the most important carotenoids in human nutrition. The 40-carbon compound β-carotene (also known as provitamin A) is the main precursor of the retenoids: retinol, vitamin A and retinoic acid. Lutein, a derivative of α-carotene, provides protection against age-related degeneration of the macula lutea in the retina (Krinsky et al. 2003). Although high levels of β-carotene and lutein have been reported in some rice germplasms (Tan et al. 2005), these pigments are found mainly in the aleurone layer of the seed, and are removed by polishing, leaving only negligible amounts in the rice grain. Genetic engineering has tremendous potential to overcome malnutrition and vitamin A deficiency (VAD), especially in developing countries. The reported accumulation of β-carotene in rice endosperm following insertion of *crtI* (phytoene desaturase), *psy* (phytoene synthase) and *lyc* (lycopene cyclase) genes into *japonica* and *indica* rice varieties (Ye et al. 2000; K. Datta et al. 2003a; Hoa et al. 2003) has greatly raised hopes that VAD can be overcome.

Carotenoid biosynthesis is a complex trait and is influenced by environmental stresses such as drought, salinity and high temperature (see Demmig-Adams and Adams 2002, and references therein). To study the carotenoid biosynthetic pathway in transgenic plants would require collection of a considerable number of independent transgenic events, which in turn requires

an effective selection/marker system. However, selectable marker genes are themselves often undesirable, and represent an extra load on the host genome once the final product is developed. To simplify the regulatory process, and to improve consumer acceptance of genetically modified (GM) crops, the ability to dispense with sequences that serve no purpose in the final product is highly desirable. The development of marker-free crops will help to raise confidence in the use of GM crops amongst consumers and farmers, as well as addressing the anti-GMO (Geneticaly modified organisms) stance of environmentalists and consumer organizations.

In recent years, several means of producing marker-free transgenic plants have been developed. Transposon-mediated elimination of selectable markers in transgenic rice expressing *Bt* endotoxin, where the marker is excised in the T_1 generation, was reported by Cotsaftis et al. (2002). Techniques using intrachromosomal homologous recombination (Zubko et al. 2000), and site-specific recombination such as the Cre/LoxP (Odell et al. 1990) and FLP/FRT (Lloyd and Davis 1994) systems have also been developed for the excision of marker genes. In these cases, the marker gene is flanked by the target sequence for a specific DNA recombinase, and can be removed from the genome of the transgenic plant by the introduction of the corresponding recombinase gene by conventional crossing. The main disadvantages of these recombinase-mediated systems are the low efficiency of DNA recombination, and the time-consuming crossing process. Co-transformation of marker and target genes, with subsequent excision of the marker gene in the T_1 generation by genetic segregation, is an alternative way to develop marker-free transgenic plants. Because of its simplicity, co-transformation is the method of choice for excision of selectable markers for seed-bearing, short-duration crops like rice, and is independent of the method of gene delivery. Biolistic transformation (Tu et al. 2003; Rao et al. 2003) and *Agrobacterium*-mediated transformation (Komari et al. 1996) can both be used, although the latter has the advantage of generating transgenics with simple gene-integration patterns and small numbers of transgenes, and is more cost-effective.

Depicker et al. (1985) demonstrated the generation of marker-free plants by using the simplest method of co-transformation, i.e. using two *Agrobacterium* strains with one binary plasmid in each. Although simple, this co-transformation method has its limitations, such as lower efficiency compared to single-strain transformation (Depicker et al. 1985). The high frequency with which the two T-DNAs become linked is also a disadvantage (Poirier et al. 2000). An advanced system of co-transformation using multiple T-DNAs in one binary plasmid, with segregation of marker gene and gene of interest in the succeeding generation, was described by Komari et al. (1996). Although this method gives a high frequency of co-transformation and segregation of the selectable marker, the vector used is very large, and is not suitable for routine cloning of genes of interest. A single binary vector with two right borders in one T-DNA (Lu et al. 2001; Huang et al. 2004) has also been used to achieve a high efficiency of co-transformation and marker segregation. However, these systems require specialized cloning vectors, and the size of the vector could be a limiting factor, especially when two or more genes of interest need to be cloned.

The 'two binary plasmids in one *Agrobacterium* strain' approach, which requires no specialized binary vector for co-transformation, and allows segregation of the selectable marker, is the method of choice when more than two genes need to be transferred for product development. Here we report the first use of this system in rice for the development of a genetically engineered product of commercial value. The goal of this study was the development of marker-free transgenic lines of *indica* rice (starting from two popular varieties as well as a high-iron rice cultivar) with enhanced accumulation of β-carotene, lutein, and other carotenoids in polished rice endosperm. Two genes coding for enzymes of the carotenoid biosynthetic pathway were inserted into the rice genome: *psy* for phytoene synthase (from *Narcissus pseudonarcissus*; Schledz et al. 1996), driven by the endosperm-specific *Glutelin* promoter, and *crtI* for phytoene desaturase (from *Erwinia uredovora*; Misawa et al. 1993), linked to the CaMV 35S promoter and fused to the coding sequence for the transit peptide of Rubisco.

Materials and methods

Construction of transformation vectors

The binary plasmid pCacar was obtained from Dr. Peter Beyer (University of Freiburg, Germany). The selection marker on pCacar, *pmi* (phosphomannose isomerase), is flanked by recognition sequences for the restriction enzyme *Xho*I. The binary plasmid pCacar has four recognition sequences for *Xho*I. To excise the *pmi* gene from pCacar, the plasmid was partially digested with *Xho*I, religated, and transformed into the *Escherichia coli* strain XL1Blue (Invitrogen, Carlsbad, CA, USA). Colonies were selected on LB medium containing chloramphenicol (25 mg/l). Plasmid DNAs was isolated from 40 colonies and digested with *Xho*I to identify clones that lacked the 1,186-bp coding sequence of the *pmi* gene. The vector lacking the *pmi* gene was designated pNCacar (see Fig. 1). The following genes are present in the T-DNA of the pNCacar plasmid: *psy* (phytoene synthase) under the control of the endosperm-specific *Glutelin* promoter, and *crtI* (phytoene desaturase) fused to the Official Reading Frame (ORF for the Rubisco transit peptide sequence under the control of the CaMV 35S promoter.

pTok233 (Hiei et al. 1994) is a super-binary vector with an extra copy of the virulence genes with the T-

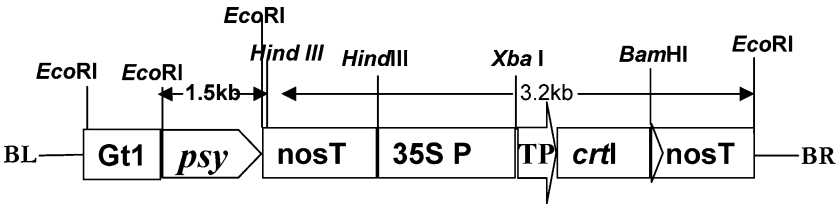

Fig. 1 Partial map of the binary plasmid pNCacar containing the phytoene synthase (*psy* gene under the control of the endosperm-specific *Glutelin* promoter (*Gt1*) and the *nos* terminator (*nosT*), and the phytoene desaturase (*crtI*) gene with 35S CaMV promoter (*35S P*), *nos* terminator and pea Rubisco transit peptide sequence (*TP*). Restriction sites used for cloning are indicated. *BL* Left border, *BR* right border

DNA. The T-DNA carries three genes: two selectable marker genes, *nptII* and *hph* (which confer resistance to kanamycin and hygromycin, respectively) and one scorable marker gene (reporter gene), *gus*.

Transfer of both binary vectors into one *Agrobacterium* strain

The *Agrobacterium* strain LBA4404 was transformed with the super-binary vector pTok233 using a freeze–thaw transformation method (Hellens et al. 2000). Kanamycin (*nptII*) was used for transformant selection. The LBA4404 (pTok233) strain was then transformed with the second binary vector, pNCacar, using the same freeze–thaw method, and applying chloramphenicol (25 mg/l) and kanamycin (50 mg/l) as selection agents. A single doubly resistant colony was selected and plasmids were isolated to confirm the presence of both plasmids in this strain. All genes present on pTok233 and pNCacar could be amplified using stringent PCR conditions (data not shown).

Plant transformation

Embryogenic calli were generated on MS + 2,4-D (2 mg/l) medium (Murashige and Skoog 1962) from scutellum of immature embryos. Embryogenic calli (3–4 weeks old; 3–4 mm^2) of the *indica* rice varieties BR28, BR29 and IR68144 were incubated for 30 min with an overnight culture ($OD_{600} = 0.5–0.8$) of *Agrobacterium* (LBA4404/pNCacar/pTok233). Calli were then blotted on sterile filter paper and transferred to co-cultivation medium [MS + 2,4-D (2 mg/l) and acetosyringone (200 μM)] and incubated in the dark at 28°C for 3 days. This was followed by three successive selection cycles of 3 weeks each on MS + 2,4-D (2 mg/l) + hygromycin (50 mg/l). The calli were then transferred to regeneration medium [MS with BAP (2.5 mg/l)] as described earlier (Datta et al. 1990, 2000; Vasconcelos et al. 2003) with some modifications. Regenerated rice plants were transferred to a special greenhouse for transgenics (containment facilities). GUS staining of T_0 plants was carried out using the method described by Jefferson et al. (1987).

Polymerase Chain Reaction

DNA was extracted from leaf fragments (2 cm^2) using the modified protocol described by Edward et al. (1991). Polymerase chain reactions (PCRs) were carried out for *crtI*,*psy*, *hph*, and *gus* genes using standard PCR conditions as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. A final extension at 72°C for 10 min was included to polish the ends of PCR products. The primer sequences used were: *psy* F (5′-TGG TGGTTGCGA-TATTACGA-3′), *psy* R (5′-ACCTTCC CAGTGAA-CACGTC-3′), *crtI* F (5′-GGTCGGGCTT ATGTCTACGA-3′), *crtI* R (5′-ATACGGTCGCGTA GTTTGG-3′), *hph* F (5′-CCTGAACTCACCGCGA CG-3′), *hph* R (5′-AAGACCAATGCGGAGCATA-TAC-3′), *gus* F (5′-AATTGATCAGCGTTGGTGG-3′) and *gus* R (5′-GGTGTAGAGCATTACGCTGC-3′).

Southern hybridization

High-molecular-weight genomic DNA was extracted using the procedure described by Datta et al. (2003a, 2003b). Genomic DNA was digested with *Eco*RI and fractionated on a 0.8% agarose gel by electrophoresis in TAE-buffer at 20 V for 20 h. Southern hybridization was carried out as described by Sambrook et al. (1989). PCR probes of 1 and 0.8 kb were used for hybridization to the *crtI* and *psy* genes, respectively. The blots were then stripped with 0.5% SDS and rehybridized with an 0.8-kb PCR probe specific for the *hph* gene.

Transgene inheritance and screening for marker-free plants

Of 68 transgenic lines containing both marker and target genes, 24 were selected for segregation analysis in T_1. Lines were selected based on a carotenoid content of more than 0.5 μg/g. T_1 progenies of selected lines were screened by histochemical staining for *gus* expression, and by PCR to detect the *hph, crtI*, and *psy* genes. All plants that were negative for GUS staining but PCR-positive for *hph* were analyzed for *gus* by PCR. Marker-free T_1 progenies were confirmed by Southern analysis.

HPLC analysis of carotenes

Polished rice seeds were ground to a fine powder, and 500 mg of rice powder was soaked in 1 ml water and incubated at 50°C for 20 min, followed by the addition of 2 ml of acetone. The mixture was then vortexed and incubated for a further 20 min. The samples were centrifuged for 5 min at 1,000 rpm, and the supernatants were transferred to clean tubes. This procedure was repeated three times, or until the rice-seed powder became completely white. Half a volume of petroleum ether was added to the total supernatant, mixed thoroughly, and water was then added for phase separation. The upper layer was removed and dried in a Speed vac (Maxi Dryer Plus, Heto, Allerod, Denmark). Carotenoids were dissolved in 1.0 ml of ether, and the absorbance was measured at 450 nm in a spectrophotometer. The solution was evaporated to dryness, and the solute was re-dissolved in 100 µl of acetone; a 40-µl aliquot of this sample was then used for HPLC analysis. HPLC analysis was performed using a Waters Alliance 2690 Separation Module (Waters Corporation, Milford, MA, USA) equipped with a Waters 996 photodiode array detector, Waters 474 scanning fluorescence detector, and Waters Millennium32 Chromatography Manager. Samples were loaded on a Waters YMC Carotenoid column (4.6′250 m, 5 µm) after passing them through a guard column containing the same material (4.0′10 mm, 5 µm), and eluted using solvents A (acetonitrile-tetrahydrofuran-water, 10 : 4 : 6) and B (acetonitrile–tetrahydrofuran–water, 10 : 8.8 : 1.2). The column was developed with 100% solution A for the first 3 min, then a linear gradient to 100% solution B was applied over a period of 7 min, then 100% solution B was pumped through the column for 20 min. Peak identification was based on the retention time, the main absorption maxima, and spectrum shape in comparison with the corresponding standards subjected to the same chromatographic conditions. Lutein and β-carotene standards were obtained from Sigma (St. Louis, Mo, USA). All other chemicals were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Results

Vector construction and development of a single *Agrobacterium* strain carrying two binary plasmids

The T-DNA of the binary plasmid pNCacar carries two genes for carotenoid biosynthesis, i.e. *psy* (under the control of the endosperm-specific *Glutelin* promoter) and *crtI* (with the 35S CaMV promoter and the Rubisco transit peptide) (Fig. 1). The *Agrobacterium* strain LBA4404 was first transformed with the binary plasmid pTok233 carrying the selectable marker gene *hph* and the *gus* reporter gene. pNCacar was then transferred into the LBA4404 (pTok233 strain), resulting in an *Agrobacterium* strain carrying two binary plasmids, i.e. LBA4404 (pTok233, pNCacar).

Transformation of rice and analysis of T_0 transformants

The popular high-yielding Bangladeshi *indica* rice varieties BR28 and BR29, and the high-iron *indica* rice cultivar IR68144, were transformed using *Agrobacterium* strain LBA4404 (pTok233, pNCacar). Of the 124 transformants obtained, 56 contained only the marker genes *hph* and *gus*, while 68 carried the carotenoid genes *psy* and *crtI* together with the marker genes (Table 1). The presence of the marker genes was confirmed by staining for GUS activity, and the presence of the genes *psy* and *crtI* was confirmed by PCR and Southern analysis. All putative transformants that grew on hygromycin-containing selection medium were found to contain both *hph* and *gus* transgenes.

Segregation analysis and identification of marker-free transgenic plants in the T_1 generation

The T_1 progenies of 24 independent transgenic (T_0) founders (selected based on the total carotenoid content in polished seeds) were analyzed in order to identify the plants that had lost the marker genes; 14 of the 24 lines showed segregation of marker genes (*hph* and *gus*). The carotenoid genes *crtI* and *psy* could be amplified by PCR from the marker-free progeny, while no amplification of the marker gene *hph* was observed (Fig. 2). All PCR-positive, marker-free transgenic T_1 progenies were confirmed by Southern analysis (Fig. 3). Four types of linkage were found in this analysis (H represents the marker genes *hph* and *gus*; C represents carotenoid biosynthetic genes, *psy* and *crtI*): (H–C), (H, H–C, C), (H, H–C) and (H–C, C). The H–C class was rare, while 50% of transgenic events showed the (H, H–C, C) pattern (Table 2). Various patterns of gene integration were found, both simple and re-arranged. Based on the analysis of T_1 plants, most of the transgenic founders had integrated the marker genes at multiple loci, whereas the T-DNA with the carotenoid genes of interest showed single-locus integration in most transgenics (Table 3).

Integration of truncated T-DNAs and reduced *gus* expression in the T_1 generation

Integration of truncated T-DNA was observed in the case of the vectors carrying carotenoid biosynthesis

Table 1 Molecular evaluation of T_0 transformants

Cultivar	Number of transformants obtained	Genotype	
		hphgus	*hphgus, psy crtI*
BR28	11	5	6
BR29	59	25	34
IR68144	54	26	28
Total	124	56	68

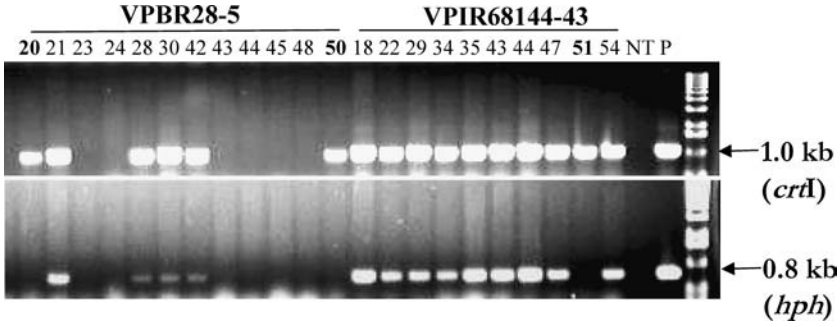

Fig. 2 Polymerase chain reaction PCR screening of T_1 progenies of the founders VPBR28-5 and VPIR68144-43 for marker-free transgenic rice. The 1.0 and 0.8-kb bands corresponding to amplified products of the *crtI* and *hph* genes, respectively, are indicated. Progeny designated by *bold numbers* are marker-free

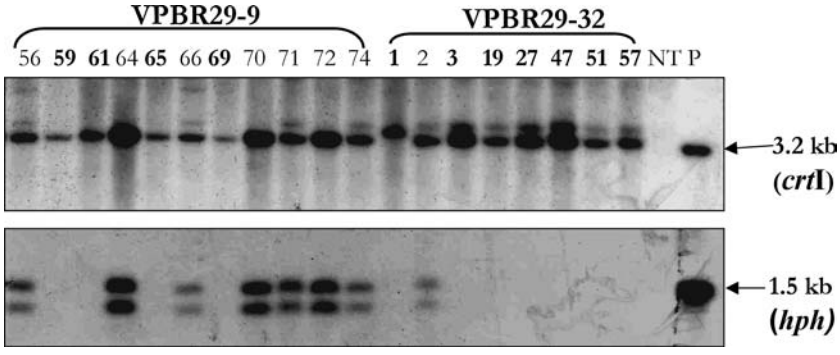

Fig. 3 Southern analysis of electrophoretically fractionated, *Eco*RI-digested genomic DNA isolated from T_1 transgenic progenies of transgenic founders VPBR29-9 and VPBR29-32 identified as *crtI*-positive by PCR, using a *crtI* probe (*upper panel*). The same blot re-probed with *hph* is shown in the lower panel. Progeny designated by *bold numbers* are marker-free

genes as well as marker genes. In one IR68144- and two BR29-derived plants, the *crtI* gene, but not *psy*, was detected (Fig. 4). A different transgenic BR29 plant had retained the *gus* gene but lost *hph* (Table 4). Fewer plants showed GUS activity (as assessed by staining) than integration of the *gus* gene. This was found to be the case in 13 out of 24 transgenic plants analyzed in the T_1 generation (Table 2).

Estimation of the carotenoid content of polished rice seeds in the T_0 generation

Carotenoid accumulation in polished rice seeds could be assessed directly by visual examination of the seeds (Fig. 5). The carotenoids were identified by HPLC analysis (Fig. 6) and quantified spectrophotometrically at 450 nm (Fig. 7). Most of the transgenic plants con-

Table 2 Linkage analysis of carotenoid biosynthetic genes and marker genes and GUS expression in the T_1 generation

T_0 plant	Number of T_1 plants analyzed	*hph*$^+$ *crtI*$^-$	*hph*$^-$ *crtI*$^+$	*hph*$^+$ *crtI*$^+$	*Hph*$^-$ *crtI*$^-$	*gus*$^+$	GUS$^+$	Linkage pattern[a]
VPBR29-2	80	0	0	61	19	61	61	H–C
VPBR29-9	76	10	4	52	10	62	62	H, H–C, H
VPBR29-16	65	15	0	50	0	65	26	H, H–C
VPBR29-17	71	22	5	42	2	64	54	H, H–C, C
VPBR29-18	58	14	0	40	4	54	33	H, H–C
VPBR29-19	50	5	3	35	7	40	28	H, H–C, C
VPBR29-20	50	12	0	33	5	45	25	H, H–C
VPBR29-28	42	3	3	35	1	38	38	H, H–C, C
VPBR29-29	50	4	5	40	1	44	44	H, H–C, C
VPBR29-31	50	9	4	37	0	46	46	H, H–C, C
VPBR29-32	50	8	3	39	0	47	47	H, H–C, C
VPBR29-33	50	0	17	33	0	47	47	H–C, C
VPBR29-35	46	7	0	32	7	39	19	H, H–C
VPBR29-40	50	2	1	38	9	40	35	H, H–C, C
VPBR29-41	50	14	3	33	0	47	47	H, H–C, C
VPBR28-3	31	8	0	23	0	31	8	H, H–C
VPBR28-4	30	9	0	19	2	28	15	H, H–C
VPBR28-5	48	3	3	30	12	33	33	H, H–C, C
VPBR28-8	40	8	2	28	2	36	36	H, H–C, C
VPIR68-1	50	12	0	34	4	46	46	H, H–C
VPIR68-6	50	38	0	6	6	45	0	H, H–C
VPIR68-14	41	0	8	31	2	31	8	H–C, C
VPIR68-16	41	6	0	35	0	41	31	H, H–C
VPIR68-43	56	5	1	50	0	55	10	H, H–C, C

[a] H indicates the marker genes *hph* and *gus*, C, the carotenoid biosynthesis genes *psy* and *crtI* The *hyphen* indicates linkage

Table 3 Segregation analysis of *crtI* and *hph* genes in the T_1 generation, and estimation of the number of *crt*I and *hph* loci

T_0 plant	No. of T_1 plants analyzed[a]	*crtI*+	*crtI*-	X^2 value[b]	Predicted number of loci	*hph*+	*hph*-	X^2 value[b]	Predicted number of loci
VPBR29-2	80	61	19	0.0166	1	61	19	0.0166	1
VPBR29-9	76	56	20	0.0175	1	62	14	1.4210	1
VPBR29-16	65	50	15	0.0461	1	65	0	3.3323	≤ 2
VPBR29-17	71	47	24	2.4835	1	64	7	1.0225	≤ 2
VPBR29-18	58	40	18	0.8275	1	54	10	0.0045	≤ 2
VPBR29-19	50	38	12	0	1	40	5	0.4266	1
VPBR29-20	50	33	17	1.7066	1	45	4	0.6453	≤ 2
VPBR29-28	42	38	4	0.3111	2/<	38	6	0.3111	≤ 2
VPBR29-29	50	45	5	0.6453	2/	44	4	1.9253	≤ 2
VPBR29-31	50	41	9	0.96	1	46	3	0.048	≤ 2
VPBR29-32	50	42	8	1.7066	1	47	17	0.048	≤ 2
VPBR29-33	50	50	0	2.352	2/	33	6	1.7066	1
VPBR29-35	46	32	4	2.7536	2/	40	10	2.8985	1
VPBR29-40	50	39	11	0.1066	1	40	3	0.4266	1
VPBR29-41	50	36	14	0.1066	1	47	0	0.048	≤ 2
VPBR28-3	31	23	8	0.0107	1	31	2	1.1376	≤ 2
VPBR28-4	30	19	11	1.6	1	28	15	0.08	≤ 2
VPBR28-5	48	33	15	0.6944	1	33	4	0.6944	1
VPBR28-8	40	30	10	0.0333	1	36	4	0.4266	≤ 2
VPIR68-1	50	34	16	0.96	1	46	5	0.048	≤ 2
VPIR68-6	50	6	44	102.5067	-	44	10	0.7253	≤ 2
VPIR68-14	41	39	2	0.0016	2/	31	0	0.00813	1
VPIR68-16	41	35	6	1.8292	1	41	5	1.7707	≤ 2
VPIR68-43	56	51	5	0.304	2/<	55		0.3619	≤ 2

[a] Presence of *crt*I and *hph* genes was confirmed by PCR analysis [b] Chi squared values calculated for segregation ratios of 3:1 and 15:1. A value of 3.84 at $df = 1$ is equivalent to the 95% confidence level

tained less than 1 μg of total carotenoids per gram of polished rice seeds, while a few lines contained 2–3 μg/g. One transgenic plant, VPBR29-16, contained 3.128 μg of total carotenoid per gram of T_0 polished rice seeds, the highest value obtained (Table 5). Based on a β-carotene calibration curve, the highest β-carotene content in polished T_0 seeds (1.808 μg/g) was found in plant VPBR29-35. The lutein content ranged between 0.085 and 0.284 μg/g in the three cultivars transformed (see Table 6 and Fig. 7). Other carotenoids found in the transgenic rice seeds were cryptoxanthin and α-carotene. It was also noted that the tocopherol content (detected by its fluorescence emission at 330 nm upon excitation at 290 nm) was 2- to 3-fold higher in transgenic as compared to non-transgenic seeds.

Gene integration patterns and differences in carotenoid expression levels

All 24 transgenic founders examined for marker-free status were also studied for gene-integration pattern, as

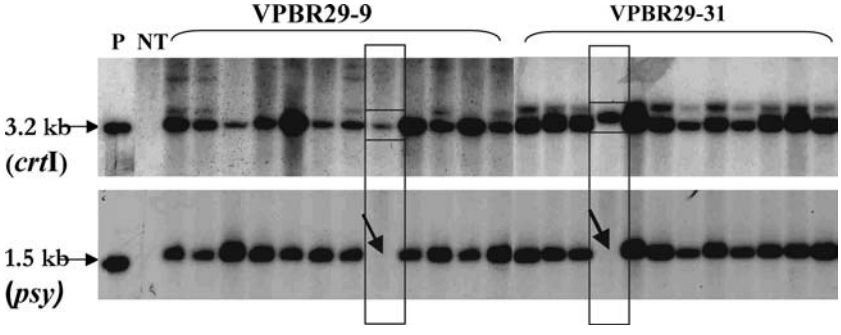

Fig. 4 Southern analysis of truncated T-DNA integration in T_1 progenies of transgenic founders VPBR29-9 and VPBR29-31. In the two *boxed lines*, the *crtI* gene is present but the *psy* gene is absent (*arrow*), although both genes were originally present on the same T-DNA. P, plasmid-positive control; NT, non-transgenic control

Table 4 Analysis of truncated T-DNA integration at T_1

Transgenic founder	Number of plants with truncated T-DNA	pCacar-IRRI T-DNA		pTok233 T-DNA	
		psy	*crt*	*hpt*	*gus*
VPBR29-9	1	–	+		
VPBR29-31	2	–	+		
VPBR29-33	14			–	+
VPIR68144-6	5			–	+

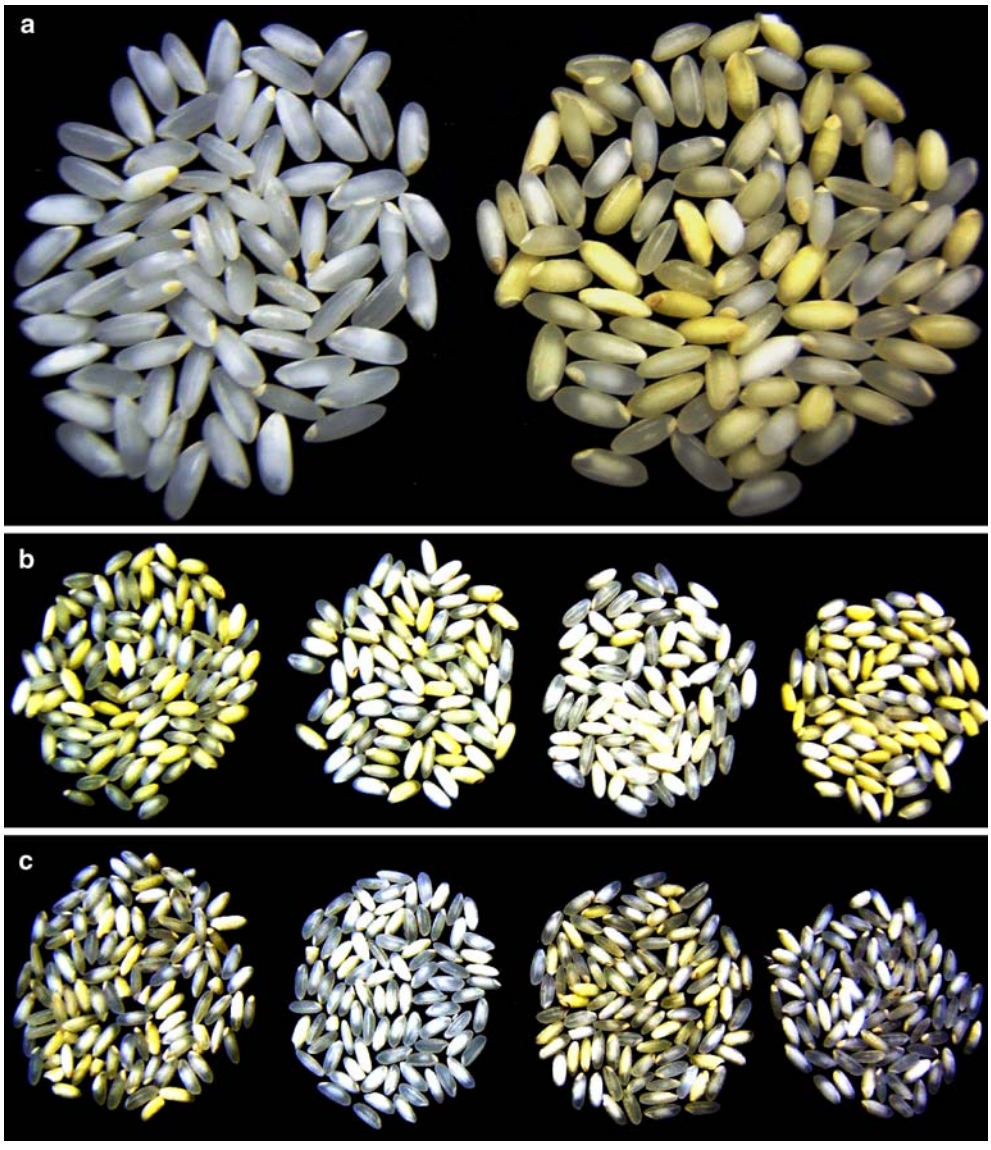

Fig. 5 a–c Accumulation of carotenoids in polished rice seeds of transgenic rice lines. **a** Transgenic BR29-17-37 (*right*) in comparison with the non-transgenic BR29 (*left*). **b, c** Variation in carotenoid accumulation among the T1 progenies of transgenic founder VPBR29-17 with a rearranged gene-integration pattern (**b**) and VPBR29-16 with a simple gene integration pattern (**c**)

well as for the expression of carotenoid genes in the T_0 and T_1 generations. Both simple and rearranged types of gene-integration patterns were observed in the T_0 generation (with a few exceptions representing truncated gene integrations). Simple gene-integration patterns (single band) detected in the T_0 generation were maintained in T_1, while rearranged patterns in T_0 (2–3 bands) subsequently became either more complex, or were resolved into a mixture of simple and rearranged gene-integration patterns. Wide variation was observed in the level of carotenoid expression in polished rice seeds of T_1 progenies of transgenic founders that exhibited rearranged gene-integration patterns in T_0. Surprisingly, in all T_0 transgenic plants with a simple gene-integration pattern, the level of carotenoid gene expression was not retained in the T_1 generation; in most such cases, carotenoid accumulation was lower than in the T_0 plant. However, enhanced carotenoid levels were observed in most of the T_1 progenies of plants showing rearranged gene integration at T_0 (Table 5).

Discussion

Co-transformation with two binary plasmids carried by one *Agrobacterium* strain was successfully used to develop co-transformants of three elite *indica* rice cultivars, BR28 and BR29 (popular Bangladeshi high-yield varieties) and IR68144 (high iron rice). The efficiency of co-transformation was 53.90%, which is similar to the previously reported values for this system (Daley et al. 1998), as well as the 'two T-DNAs in one binary plasmid system' of co-transformation (Komari et al. 1996). The present system represents an improvement over the two *Agrobacterium* strain system of co-transformation described by De Block and Debrouwer (1991) and Poirier

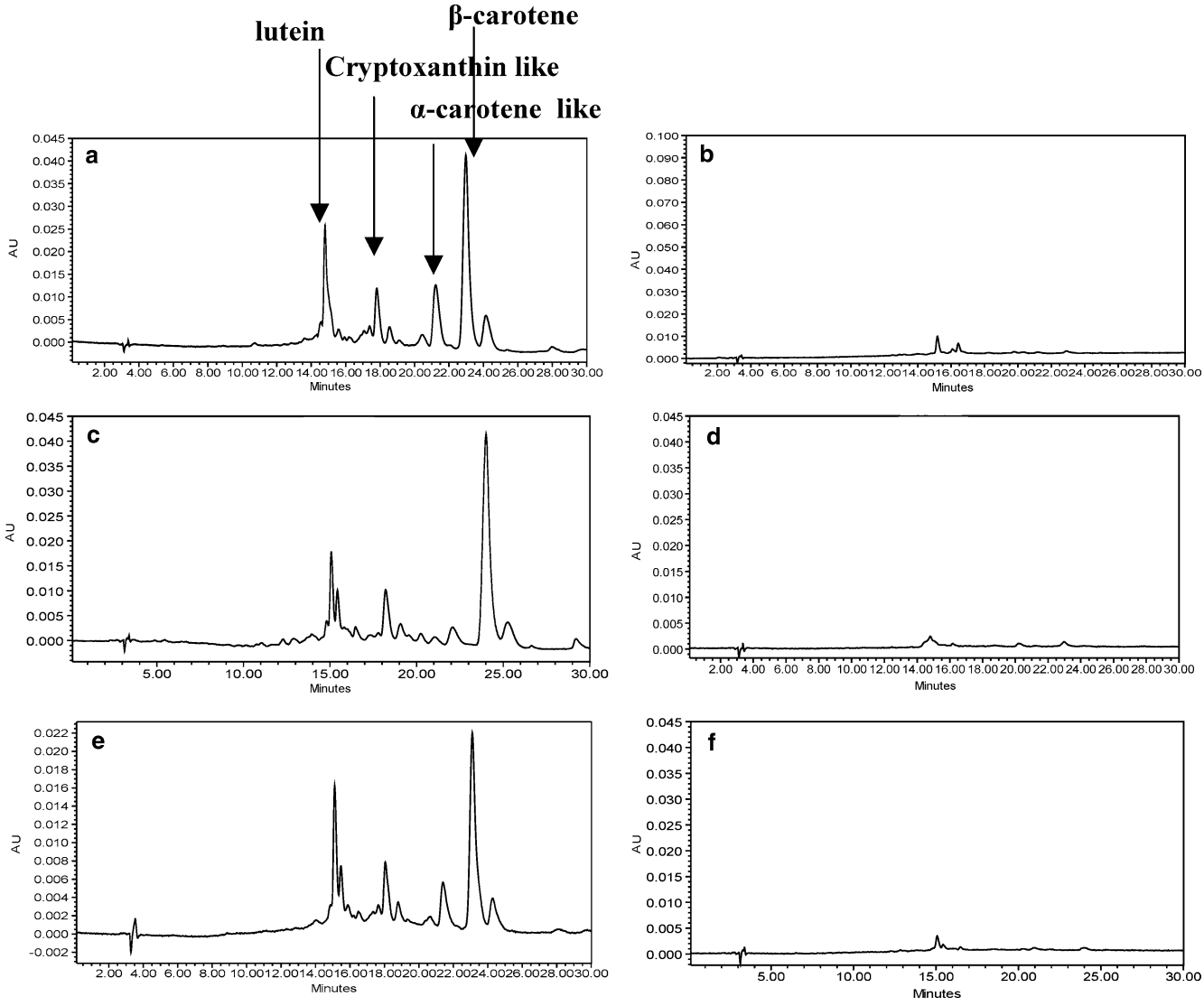

Fig. 6 a–f HPLC chromatograms showing the carotenoid profiles of polished rice seeds (T_1) from the transgenic lines VPBR28-5 (**a**), VPBR29-16 (**c**), and VPIR68-43 (**e**) compared with seeds of the respective control cultivars BR28 (**b**), BR29 (**d**), and IR68144 (**f**)

et al. (2000). Using advanced vector systems, recent studies on the development of marker-free transgenic crops have achieved co-transformation efficiencies of 70–90% (Miller et al. 2002; Breitler et al. 2004), but these systems have the disadvantage that they require specialized binary vectors.

Over 50% of the co-transformants obtained with both systems showed segregation of the marker gene (Komari et al. 1996; Daley et al. 1998). These values were based on the phenotypic expression of both the selectable marker gene and a reporter gene, but in such cases a gene may be present but not be expressed. Here, we studied the segregation of the selectable marker based on the presence of the gene, and found that the selectable marker segregated away from the genes of interest in 58.33% of co-transformants. In 13 out of 24 transgenic founders studied in the T_1 generation, the *gus* reporter gene, though present, was not expressed. This could be due to gene silencing, gene rearrangement, or integration of a truncated gene in another locus. Integration of truncated T-DNA is a common phenomenon in *Agrobacterium*-mediated transformation (Vain et al. 2003); indeed, in the T_1 generation, we observed four cases in which a truncated T-DNA had been integrated at one of the loci (Table 7). In two of these, the *hph* gene was missing from the T-DNA carrying the marker genes, while in another the *psy* gene had been deleted from the T-DNA containing the carotenoid genes (Fig. 4).

The type of *Agrobacterium* strain used for insertion of the T-DNA can influence transgene integration into the plant genome (De Block and Debrouwer 1991). Nopaline-derived *Agrobacterium* strains favour insertion of multiple T-DNAs at genetically linked loci; while octopine-derived strains favour integration at unlinked loci (Breitler et al. 2004). Here, we used the octopine-derived strain LBA4404, and this could ex-

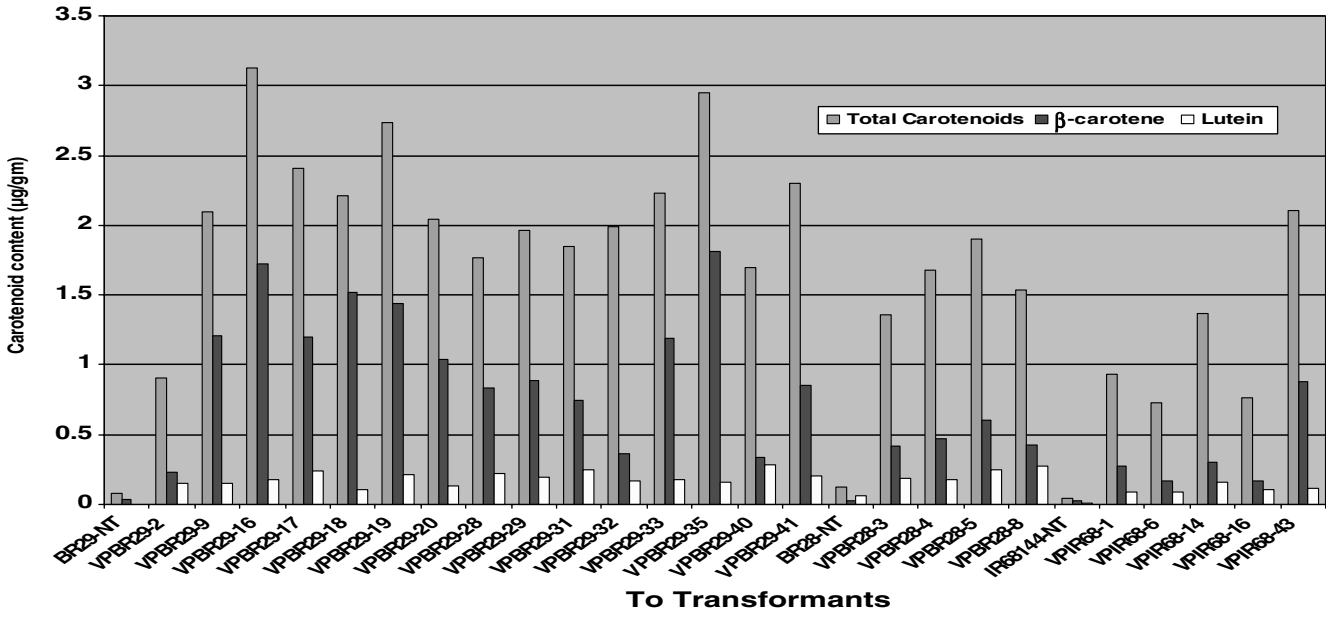

Fig. 7 Summary of the carotenoid profiles of the 24 T_0 transgenic founders selected for segregation analysis in T_1

plain the high frequency of unlinked markers obtained in the T_1 generation. Based on chi square values, it was found that most of the T_1 transgenic events studied showed single-locus integration of T-DNA carrying carotenoid genes, while the marker gene T-DNA integrated at multiple loci. However, the sample size used for T_1 analysis was too small to allow any general conclusions to be drawn.

Earlier studies have reported the accumulation of 1–1.6 µg/g of total carotenoids in polished rice seeds of *indica* as well as *japonica* rice cultivars (Ye et al. 2000; Datta K et al. 2003a; Hoa et al. 2003). Based on the

Table 5 Comparative analysis of T_0 and T_1 generations of transgenic *indica* rice lines for gene integration pattern and differential accumulation carotenoids in seed endosperm

T_0 transgenic plant	Gene integration pattern[a]		Total carotenoid content in polished rice seeds (µg/g)	
	T_0	T_1	T_0	T_1
VPBR29-2	S	S	0.908	1.049–1.212
VPBR29-9	R	S/R	2.096	0.554–2.123
VPBR29-16	S	S	3.128	0.501–1.503
VPBR29-17	R	S/R	2.404	0.830–3.011
VPBR29-18	S	S	2.216	1.010–1.650
VPBR29-19	S	S	2.736	0.522–2.250
VPBR29-20	R	S/R	2.044	1.502–2.112
VPBR29-28	R	R	1.764	0.332–2.105
VPBR29-29	R	R	1.960	0.423–1.911
VPBR29-31	R	S/R	1.844	1.118–2.309
VPBR29-32	R	S/R	1.992	0.663–2.002
VPBR29-33	R	R	2.232	2.012–2.407
VPBR29-35	S	S	2.948	1.590–1.701
VPBR29-40	R	S/R	1.700	1.227–1.750
VPBR29-41	R	S/R	2.304	1.551–2.501
VPBR28-3	R	S/R	1.356	0.225–1.542
VPBR28-4	R	R	1.680	0.890–1.819
VPBR28-5	R	R	1.900	0.346–2.103
VPBR28-8	R	R	1.536	1.004–1.700
VPIR68-1	S	S	0.932	0.890–1.121
VPIR68-6	R	R	0.724	0.991–1.340
VPIR68-14	S	S	1.368	0.702–1.244
VPIR68-16	R	R	0.764	0.456–2.122
VPIR68-43	R	R	2.102	0.223–1.890

[a] S, simple integration pattern; R, rearranged

Table 6 Accumulation of total carotenoids (TC), β-carotene (β), and lutein (L) in polished seeds of T_0 transgenic rice lines

Cultivar	Content of total carotenoids, β-carotene and lutein											
	< 1 μg/g			1–2 μg/g			2–3 μg/g			> 3 μg/g		
	TC	β	L	TC	β	L	TC	β	L	TC	β	L
BR28	2	6	6	4								
BR29	20	26	34	11	8		2			1		
IR68144	26	28	28	1			1					

Table 7 Summary of the generation of marker-free transgenic rice lines showing accumulation of β-carotene in seed endosperm

Cultivar	Total transformants generated	Number of transformants with both T-DNAs (%)	Number of lines screened at T_1	Number of marker-free transgenic lines in T_1 (%)	Number of lines showing truncated integration
BR28	11	6 (55.45%)	4	2 (50.00%)	0
BR29	61	34 (55.75%)	15	10 (66.66%)	3
IR68144	54	28 (51.85%)	5	2 (40.00%)	1
Total	126	68 (53.90)	24	14 (58.33)	4

recommended daily allowance (RDA), this may not be enough to meet the daily requirement for pro-vitamin A. The main aim of this study was to develop a marker-free transgenic rice with enhanced accumulation of carotenoids in the rice seeds. Here, we have achieved accumulation of more than 3.0 μg/g total carotenoids in T_2 polished rice seeds in transgenic rice cultivar BR29, with about 2 μg/g in BR28 and IR68144. The highest accumulation of β-carotene (1.812 μg/g in polished T_2 rice seeds) was found in plants derived from the transgenic founder VPBR29-17-37. The level of β-carotene did not always represent a constant proportion of the total carotenoid content (Fig. 7). This phenomenon has also been reported in potato (Ducreux et al. 2004). However, β-carotene always represented the major component of the total carotenoids, followed by lutein and other carotenoids. A possible reason for the high levels of β-carotene could be that the activity of β-carotene hydroxylases becomes rate limiting as the carotenogenic flux increases, resulting in a metabolic bottleneck. Accumulation of α-carotene in polished rice seeds was found to be very low when compared with β-carotene; enzyme activities on this branch of the carotenoid pathway are thus probably not rate limiting. Upon stimulation of carotenogenesis, the tocopherol content in potato increased (Romer et al. 2002; Ducreux et al. 2004). Similarly, expression of two genes of the carotenoid biosynthetic pathway, *psy* and *crtI*, in rice endosperm, results in a 2- to 3-fold increase in tocopherol levels in polished transgenic rice seeds as compared to the control seeds. Of the 68 co-transformants obtained, 24 showed considerable accumulation of carotenoids, while the remaining 44 showed poor to negligible accumulation of carotenoids in the polished seeds. Since accumulation of the final product is dependent on the proper coordination of all enzymes in the metabolic pathway, one cannot reasonably expect to generate valuable transgenic plants after a few transgenic events. Large numbers of transgenic events need to be developed to select the best phenotype based on product accumulation as well as agronomic performance.

Gene rearrangement is very commonly observed in *Agrobacterium*-mediated transformation and cannot be correlated with gene expression. Stable and heritable expression of transgenic *Bt* rice with a rearranged gene integration pattern has been observed over eight generations (Datta et al. 2003a, 2003b). Due to the complex character of the carotenoid biosynthetic pathway, four to five generations will be required stabilize gene expression. The performance of transgenic plants in different agronomic and climatic conditions also needs to be studied. T_1 progenies of transgenic events with simple gene-integration patterns did not maintain the level of carotenoid gene expression found in the T_0 generation; in most cases the amount of product declined, with a marked variation in carotenoid expression among T_1 progenies. In cases where the genes were re-arranged during integration, wide variation in carotenoid expression was observed among T_1 progenies and, in some progenies, the carotenoid content was even found to be higher than that in the parents. By using the skills of rice breeders together with carotenoid analysts, the plants that combine best stable high carotenoid accumulation (in polished rice seeds) and superior agronomic performance could be selected prior to releasing the genetically engineered products to the public.

Reasonable levels of β-carotene and lutein accumulation have been reported in transgenic tomato and potato (Romer et al. 2000; Ducreux et al. 2004). However, considering the importance of rice as the staple food of more than half the world's population (mainly in developing countries), any improvement in the content of β-carotene and other carotenoids in

polished rice seeds will directly benefit developing countries in helping to prevent VAD. The carotenoids in polished rice seeds should not be the sole source of vitamin A, but increased levels could prove a useful supplement. Furthermore, enhanced accumulation of carotenoids in the high-iron rice cultivar IR68144 could help simultaneously to combat both VAD and iron-deficiency anaemia.

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