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Bioengineered '*golden*' indica rice cultivars with β-carotene metabolism in the endosperm with hygromycin and mannose selection systems

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Summary

Vitamin-A deficiency (VAD) is a major malnutrition problem in South Asia, where indica rice is the staple food. Indica-type rice varieties feed more than 2 billion people. Hence, we introduced a combination of transgenes using the biolistic system of transformation enabling biosynthesis of provitamin A in the endosperm of several indica rice cultivars adapted to diverse ecosystems of different countries. The rice seed-specific glutelin promoter (Gt-1 P) was used to drive the expression of phytoene synthase (*psy*), while lycopene β-cyclase (*lcy*) and phytoene desaturase (*crtI*), fused to the transit peptide sequence of the pea-Rubisco small subunit, were driven by the constitutive cauliflower mosaic virus promoter (CaMV35S P). Transgenic plants were recovered through selection with either CaMV35S P driven *hph* (hygromycin phosphotransferase) gene or cestrum yellow leaf curling virus promoter (CMP) driven *pmi* (phophomannose isomerase) gene. Molecular and biochemical analyses demonstrated stable integration and expression of the transgenes. The yellow colour of the polished rice grain evidenced the carotenoid accumulation in the endosperm. The colour intensity correlated with the estimated carotenoid content by spectrophotometric and HPLC analysis. Carotenoid level in cooked polished seeds was comparable (with minor loss of xanthophylls) to that in non-cooked seeds of the same transgenic line. The variable segregation pattern in T_1 selfing generation indicated single to multiple loci insertion of the transgenes in the genome. This is the first report of using nonantibiotic *pmi* driven by a novel promoter in generating transgenic indica rice for possible future use in human nutrition.

Keywords: β-carotene, biolistic transformation, cestrum promoter, golden indica rice, phosphomannose isomerase, provitamin A.

Introduction

Vitamin A plays an important role in a wide variety of physiological functions of all mammals. Vitamin-A deficiency (VAD) affects the proper functioning of the immune system, the rod cells in the retina of the eye, and mucous membranes throughout the body. Night blindness is the first symptom of VAD. Corneal xerosis, keratomalacia and total blindness are severe VAD manifestations. VAD may cause increased morbidity and mortality in children by impairing the specific and nonspecific immune mechanism (Gerster, 1997). It is estimated that 124 million children world-wide are deficient in vitamin A. Since mammals cannot manufacture vitamin A, diet is the source of all human vitamin A and provitamin A. Most dietary vitamin A is derived from plant food in the form of provitamin A, the carotenoids, which are converted to vitamin A in the body (Sivakumar, 1998).

Carotenoids, which are present in all photosynthetic and many non-photosynthetic organisms, are a widely distributed class of natural pigments containing 40 carbon atoms. Most of the orange, yellow or red colours found in different organs of many higher plant species result from the accumulation of carotenoids in the cells. A characteristic of some carotenoids, such as β-carotene, α-carotene, γ-carotene and β-cryptoxanthin

is that they can be converted in mammals by central cleavage into one or two molecules of 20-carbon moiety vitamin A (retinol). Moreover, they have the ability to protect the tissues and cells as scavengers of reactive oxygen species (ROS). In plants, their predominant function is in the mechanism of photosynthesis as a constituent of light harvesting complexes and photosystems to collect light energy and to detoxify excited chlorophyll and oxygen species at high light intensities (for review see Sandmann, 2001).

Biosynthesis of carotenoids in plants takes place within the plastids, chloroplasts of photosynthetic tissue, and chromoplasts of fruits and flowers. Chlorophyll, tocopherols, plastoquinone, phylloquinone, gibberellins and carotenoids all share a common biosynthetic precursor, geranylgeranyldiphosphate (GGPP), which is derived from plastidic isoprenoid metabolism. It has been established that four enzymes in plants, i.e. phytoene synthase, phytoene desaturase, ζ-carotene desaturase, and lycopene cyclase catalyse to complete the pathway toward β-carotene (provitamin A) biosynthesis from GGPP (for review see Britton, 1988; Cunningham and Gantt, 1998; Sandmann, 1994, 2001). The first step in carotenoid biosynthesis is the condensation of two molecules of GGPP to produce phytoene by the enzyme phytoene synthase (PSY). PSY is firmly associated with the chromoplast membrane in its active form (Schledz *et al*., 1996). In contrast to plants, anoxygenic photosynthetic bacteria, non-photosynthetic bacteria and carotenoid-synthesizing fungi do not possess a distinct phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) to catalyse the conversion of phytoene to lycopene. In non-photosynthetic bacteria, phytoene is converted to all-*trans* lycopene by a single enzyme phytoene desaturase (CRTI). The cyclization of lycopene by two different lycopene cyclases specific for α- and ε-ionone end-groups of LCY marks a branching point in the pathway where one branch leads to α-carotene and its oxygenated derivative lutein, while the other forms β-carotene and the derived xanthophylls, such as zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (for review see Hirschberg, 2001).

The genes necessary for these enzymes have been isolated and their function elucidated from a variety of bacteria, fungi and plants (Al-Babili *et al*., 1996; Armstrong *et al*., 1990; Bartley *et al*., 1991; Buckner *et al*., 1993; Hundle *et al*., 1991; Misawa *et al*., 1990; Scolnik and Bartley, 1994, 1996; To *et al*., 1994).

Conventional interventions (distribution, fortification, diet diversification, etc.) have been helpful in defeating VAD but were not sufficiently effective. Plant breeding to alter, modify or introduce this biosynthetic machinery *in toto* into the target tissues in rice has been impossible as of now, as no endosperm-active carotenoid-biosynthetic genes have been found thus far in the available rice gene pool (Tan *et al*., manuscript in preparation). Therefore recombinant DNA technology and plant biotechnology, with the above-mentioned molecular tools in hand, represents an alternative method to combat VAD. Moreover, it may represent a more sustainable strategy (Zimmerman and Hurrel, 2002). Transgenic approaches have been used effectively to modify the carotenoid content in plants to enhance their nutritional value, which includes modification of the carotenoid pathway by shifting to another carotenoid product in tomato (Römer *et al*., 2000), increasing the amount of existing carotenoids by over-expression of phytoene synthase in *Brassica napus* (Shewmaker *et al*., 1999), and engineering a carotenogenic pathway in rice endosperm, which is completely devoid of carotenoids (Ye *et al*., 2000). The functional expression of phytoene synthase in transformed rice endosperm has been demonstrated (Burkhardt *et al*., 1997). The functional expression of the entire pathway, namely of phytoene synthase (from *Narcissus pseudonarcissus*; Schledz *et al*., 1996), *crtI* (from *Erwinia uredovora*; Misawa *et al*., 1993), and lycopene cyclase (from *N. pseudonarcissus*; Al-Babili *et al*., 1996) led to carotenoid production in the japonica rice cultivar Taipei 309, the transgenes being introduced by *Agrobacterium*-mediated transformation using the *hph* gene as a selectable marker (Ye *et al*., 2000). This was a landmark concept in establishing an entire metabolic pathway functional in an alien background through transgenesis. This concept was extended to indica rice cultivars consumed by 90% of the Asian population that are adapted to different agro-ecological zones of several tropical Asian countries (Khush, 2001). This would be advantageous, as the transgenic indica lines would directly serve the needs of the farmers in a specific ecosystem and save time, labour, and avoid the sterilty problems of conventional breeding involving indica × japonica crosses.

We report here the introduction of a carotenogenic pathway in the endosperm of various indica rice cultivars well established in different developing countries such as BR29 in Bangladesh, Immyeobaw in Myanmar, IR64 in several Asian countries, and Nang Hong Cho Dao and Mot Bui in Vietnam. Phytoene synthase (*psy*), bacterial phytoene desaturase (*crtI*), and lycopene cyclase (*lcy*) were used to drive the accumulation of β-carotene into the endosperm of rice seeds. A further significant difference of this report was that we used a novel cestrum yellow leaf curling virus promoter driven *pmi* as a selectable marker gene (Positech™ selection system, Syngenta International Patent Application no. WO 01/73087 A1) in addition to the antibiotic hygromycin resistance *hph* gene (Datta *et al*., 1990) used earlier (Ye *et al*., 2000) in the biolistic transformation method.

Table 1 Transgenic rice obtained from eco-geographically diverse indica genotypes with β-carotene biosynthesis genes

Cultivars/ genotypes	Genes of interest	Selectable marker gene	Number of plants regenerated*	Transgenics (PCR^+/S^+)
IR64	*psy, crtI, lcy*	*pmi*	60	54
	psy, crtI	*hph*	36	1
	crtI	*hph*	106	34
IR68144	*psy, crtI, lcy*	*pmi*	300	61
BR29	*Psy, crtI, lcy*	*pmi*	155	48
	psy, crtI, lcy	*hph*	759	396
	psy, crtI	*hph*		12
	lcy	*hph*		20
Nang Hong Cho Dao	*psy, crtI, lcy*	*hph*	15	3
Mot Bui	*psy, crtI, lcy*	*hph*	13	2
Immyeobaw	*psy, crtI, lcy*	*hph*	30	1
IR68899B	*Psy, crtI, lcy*	*hph*	15	7

*All regenerated plants are not analysed; PCR^+/S^+ = positive by PCR and/or Southern analyses; *pmi* = phosphomannose isomerase; *hph* = hygromycin phosphotransferase.

Results

Transformation

More than 600 primary transgenic rice plants (T_0) of different cultivars were obtained from mannose-resistant and antibiotic-resistant cells (Table 1). The insertion of the genes in the genome was primarily checked by PCR analysis (data not shown) and then confirmed by Southern blot analysis (Figure 2a–c). The 1.5 kb and 2.1 kb size bands confirmed the integration of *psy* gene and the expression cassette of the *crtI*, respectively, and the 1.8 kb band corresponded to the expected size of the *lcy* cDNA. Apart from the expected size bands, many transgenics carried rearranged transgene copies of the three genes (Figure 2a–c). Independent transformants contained one to several copies of introduced genes, as observed from the Southern analysis with the use of a restriction enzyme (*Kpn*I), which cuts once in the plasmid vectors for the genes (data not shown). In most cases, transgenes (at least one copy from the three) were clustered at a single site, which was evident from the co-segregation of the transgenes in the subsequent selfing generation.

Expression

Reverse transcription polymerase chain reaction (RT-PCR) indicated the *mRNA* transcription of the transgenes in the seeds by the presence of the 0.93 kb and 1.03 kb amplicons (Figure 3) expected for the *psy* and *crtI* cDNAs, respectively, and the absence in the non-transgenic control.

Most transgenics (more than 90% of the plants) exhibited a normal morpho-agronomic phenotype with normal seed setting like the wild-type plants (Figure 4). However, less than 10% of the transgenic plants showed a phenotypic difference from their respective non-transformed control plants such as short stature, dark and stay-green nature, and late flowering, and some of them had a much smaller number of seeds.

Mature seeds from individual transgenic lines were polished, and the variation in the yellow colour intensity of the endosperm seemed to indicate the variation of the level of carotenoid formation and accumulation among individual lines of different cultivars (Figure 5a–d).

Quantification

Polished seeds or the endosperm from individual lines were analysed quantitatively by spectrophotometry and qualitatively for β-carotene and other xanthophylls by high-performance liquid chromatography (HPLC). Some of the lines even having all three genes integrated did not accumulate a detectable amount of carotenoid.

Estimation of carotenoids from yellow seeds showed total carotenoid levels ranging from 0.297 μg/g (as in one line of BR29, KDGR29-104) to 1.05 μg/g (in one line of Nang Hong Cho Dao, NHCD3) in the T_1 seeds of the individuals of transgenic lines of different cultivars (Figure 6a–c). To find the effect of cooking on carotenoid level, we cooked polished homozygous seeds of transgenic IR64 (64E26) 'in laboratory conditions with covers on the top of the container' for 10–15 min with water just sufficient to submerge the seeds. HPLC analysis showed a reduction in the total carotenoid content by ~10% in the cooked grains (Figure 6d) compared with the non-cooked rice (Figure 6c). However, the β-carotene (provitamin A; BC) level was not much affected by

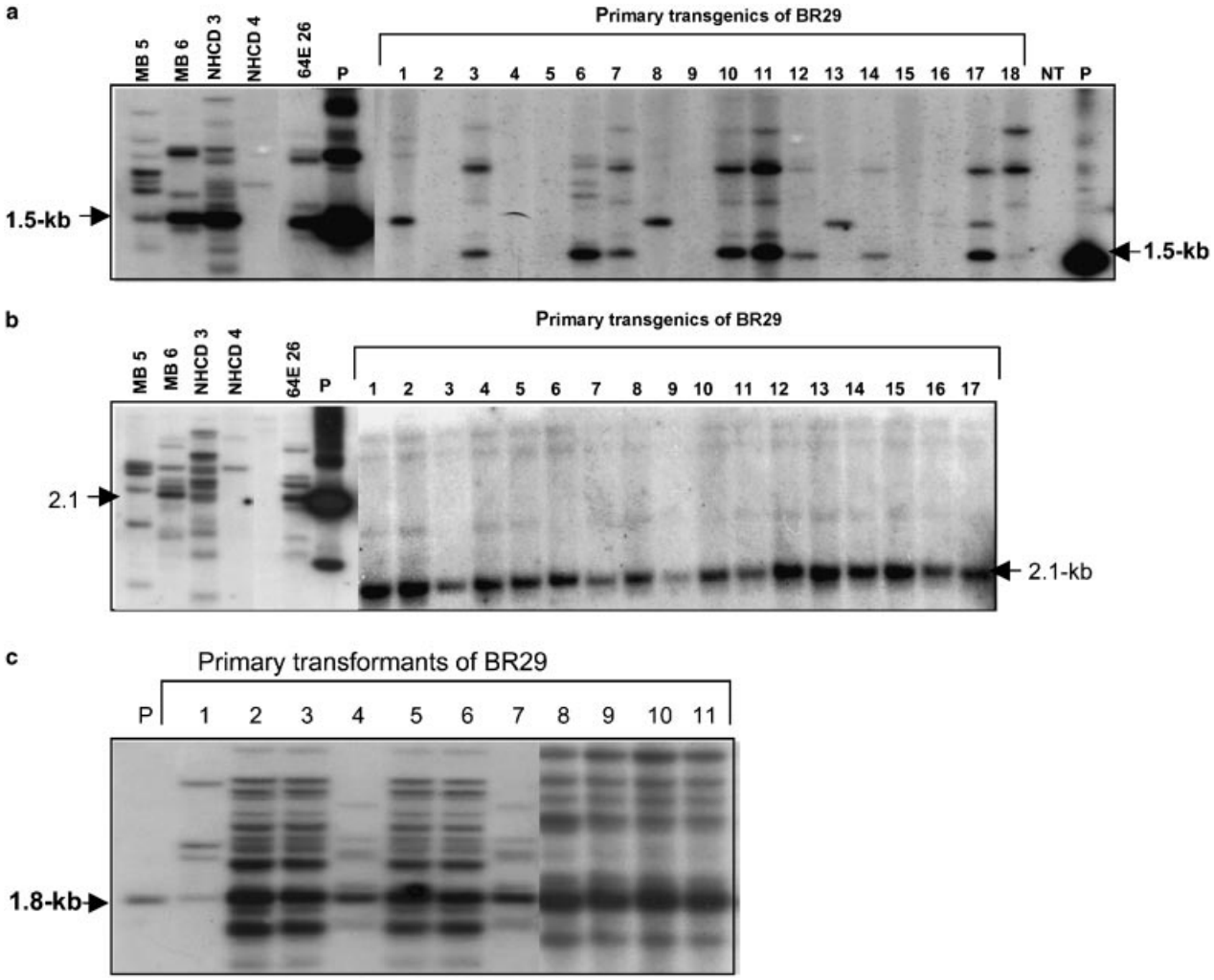

Figure 2 Southern blots showing the integration of (a) *psy*, (b) *crtI* and (c) *lcy* in the primary transgenics of indica rice. NT = non-transformed control, P = positive control (*Eco*RI/*Hin*dIII-digested pBaal3 for *psy* and *crtI* and *Kpn*I/*Bam*HI digested pTCL6 for *lcy*). Ten μg of genomic DNA were double digested overnight with *Eco*RI and *Hin*dIII for *psy* and *crtI* and with *Kpn*I and *Bam*HI for *lcy*, electrophoresed in 1% TAE-agarose gel, Southern blotted and hybridized with (α-^{32}P) dCTP-labelled probes of *psy*, *crtI* and *lcy* (PCR-generated).

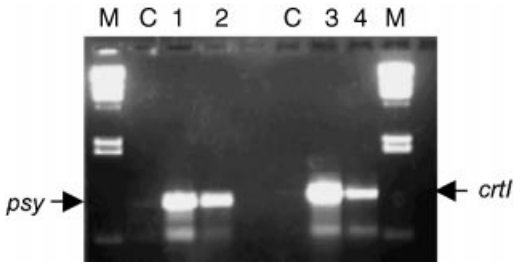

Figure 3 Expression of the transgenes in the primary transgenics. (a) RT-PCR showing *mRNA* transcription of *psy* (arrow mark at the left) and *crtI* (arrow mark at the right) in the polished seeds of NHCD3 (nos. 1 and 3) and 64E26 (nos. 2 and 4), whereas the non-transgenic control (C) did not show any amplification. Note that the expression in the NHCD3 was higher than in 64E26.

Figure 4 Plants (IR64) in the transgenic greenhouse showing a normal phenotype of the transgenic plants with good seed set.

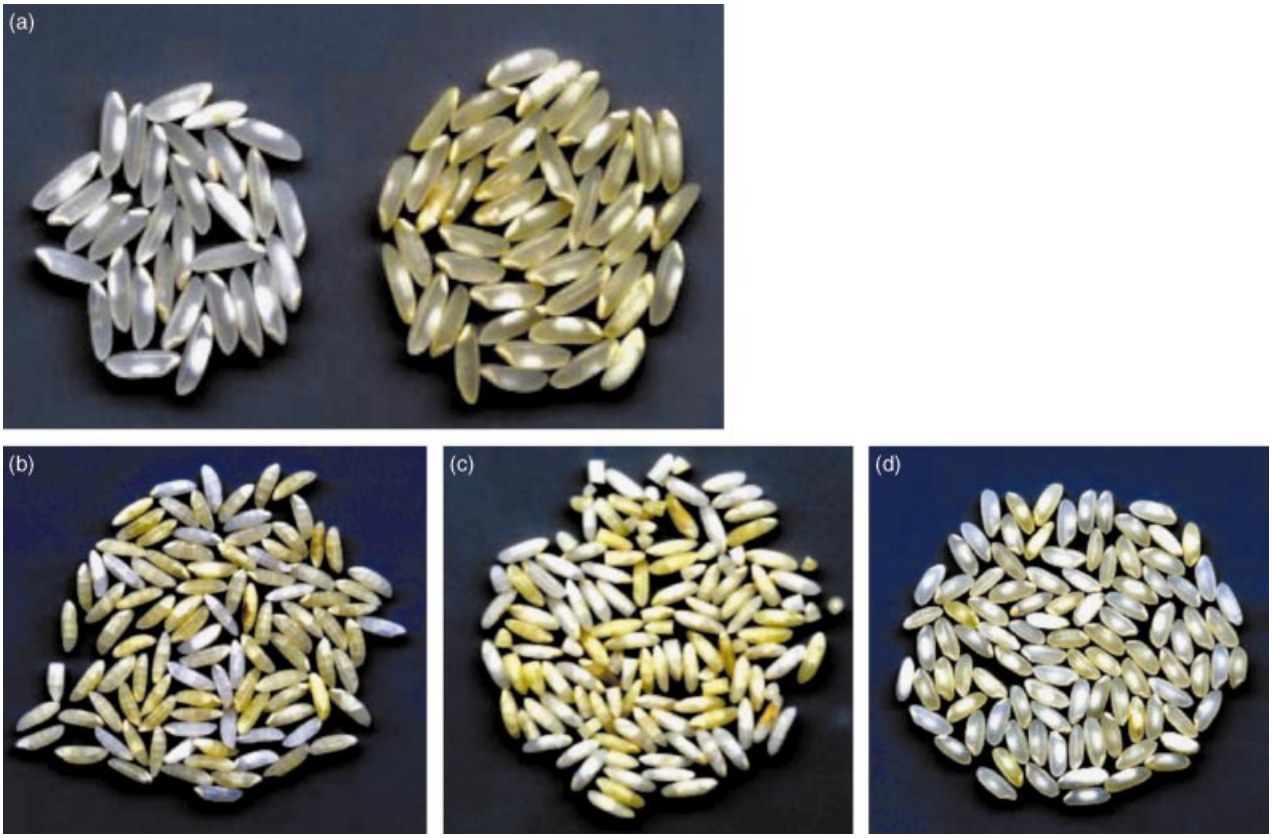

Figure 5 Yellow endosperm of the polished grains from different transgenic indica rice cultivars: (a) homozygous IR64 transgenic seeds (64E26, right side) *vis-á-vis* the white endosperm (at the left); (b) segregating yellow and white seeds of Nang Hong Cho Dao (NHCD3) (c) Mot Bui (MB5) and (d) BR29 (KDGR29-104).

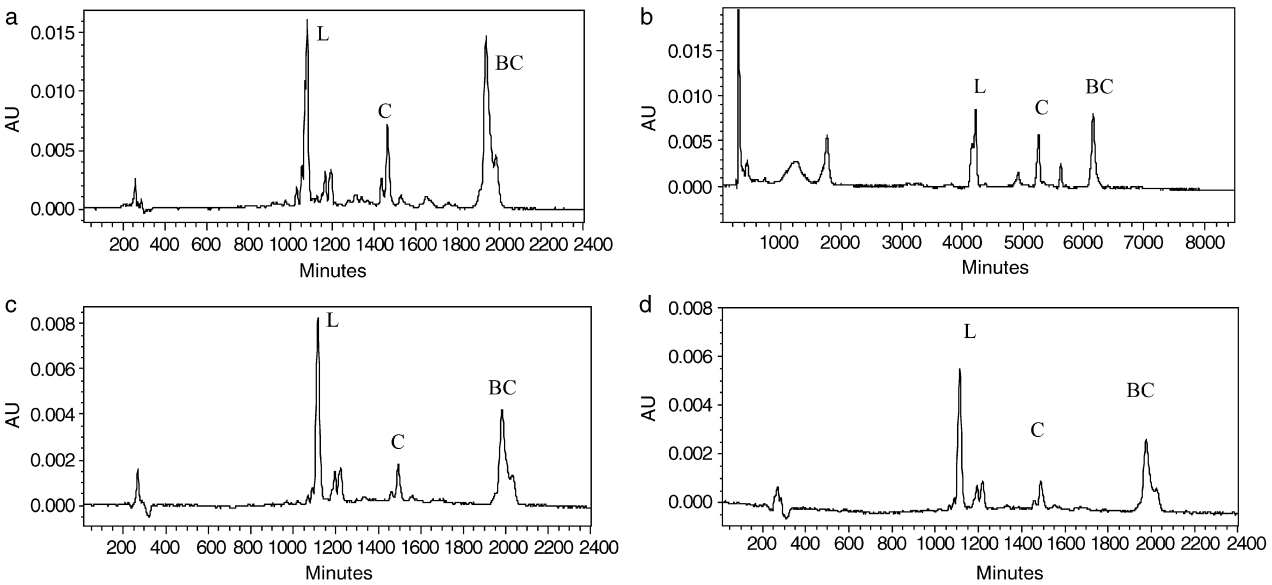

Figure 6 HPLC chromatograms showing the β-carotene peaks (BC) in the carotenoid extracts from polished yellow seeds of (a) NHCD3, (b) KDGR29-104, (c) 64E26 and (d) cooked 64E26. Other peaks correspond to other carotenoid compounds such as lutein (L) and cryptoxanthin (C).

the cooking process, but a major fraction of lutein (L)/zeaxanthin not possessing provitamin A-acticity as well as some cryptoxanthin (C) was lost.

Progeny

Transgenic lines showing an appreciable carotenoid expression and accumulation based on the yellow colour of the endosperm and HPLC analysis data were advanced to the next generations. From each desirable line, we grew 60 T_1 progenies to study the inheritance of integrated transgenes and the identification of putative homozygous lines. PCR and Southern blotting analyses showed single-locus mendelian segregation (3 : 1) to a variable segregation pattern representing the insertion of transgenes in one or more than one locus (data not shown). After maturity of the seeds of progeny lines, the estimation of carotenoid of the polished seed by spectrophotometry and HPLC analysis showed a varied amount of β-carotene in different lines (e.g. 0.670 μg/g in 64E26). Figure 6c shows an HPLC chromatogram of polished seeds of a homozygous progeny of IR64E26 and Figure 6d shows the carotenoid composition in cooked rice endosperm of the same line.

Discussion

Rice plants possess carotenoids in photosynthetic tissues but not in the endosperm, the staple food for half of the world population. To direct the accumulation of carotenoid compounds in the endosperm we used phytoene synthase driven by endosperm-specific glutelin promoter and phytoene desaturase with a transit peptide sequence driven by the CaMV constitutive promoter. Several improved cultivated varieties of rice were used for the direct introduction of the carotenogenic pathway so that the homozygous transgenics of choice cultivars could fit directly into the target area, thereby saving time and cost in further breeding. The phosphomannose isomerase selectable marker was used as an alternative to the antibiotic-resistant hygromycin phosphotransferase selection system.

The phenotypic variations observed in some transgenics were stably inherited in the subsequent T_1 generation. Because of competition for the common precursor (GGPP) shared between carotenoid and the gibberellin biosynthesis pathway, over-expression of phytoene synthase may cause the lack or deficiency of gibberellin, which may result in dwarfism (Fray *et al*., 1995). The pleiotropic effect observed in less than 10% of regenerated transgenic plants could also be attributed to somaclonal variation (a phenomenon often observed in *in vitro* culture), and not necessarily to alien gene integration or expression. Such variation cannot be ruled out in any breeding programme aiming at the genetic improvement of crops. Hence, the selection of the correct transgenic line is important based on agronomic performance without any phenotypic cost, which requires the production of a large number of independent transgenics.

The transgenes showed a varied inheritance pattern, either as a single mendelian locus or two independent functional loci, which is not uncommon in biolistic transformation (Baisakh *et al*., 1999; Christou *et al*., 1991; Kohli *et al*., 1998). Moreover, the *Agrobacterium* method has been shown to result in a similar pattern of transgene(s) segregation in the progenies of glyphosate resistant transgenic soybean (Clemente *et al*., 2000). The transgenes showed a stability of expression over two (up to T_2) generations, evidenced by the yellow endosperm of seed progenies. We found that β-carotene was synthesized in transgenics (64E26) with only *psy* and *crtI* genes, which has also been reported earlier in a japonica variety, T-309 (Ye *et al*., 2000). This could be due to either feedback regulation originating from carotene intermediates and activation of endogenous carotenoid biosynthesis genes, or to the constitutive expression of downstream carotenoid biosynthetic enzymes in rice endosperm. However, the accumulation of carotenoids in transformants lacking the cyclase (64E26) was less as compared to those containing all the three genes (NHCD3) as evidenced from the colour of the endosperm. As was desired, the levels of carotenoid were maintained even after cooking, with a minimal loss in some xanthophylls. However, other factors such as time and conditions of storage, time of milling, etc. may also lead to losses.

Selection with the *hph* gene (Datta *et al*., 1990) is routinely used in cereals, particularly in rice transformation. This is the first report of Positech™ selection with *pmi* under a novel promoter showing successful and efficient in generation of a large number of transgenic indica rice with genes for β-carotene biosynthesis. This system has also been proven in other crops (Wright *et al*., 2001; Hansen and Wright, 1999). The present nonantibiotic selection system could be an advantage for overcoming public concern and obtaining acceptance of transgenic nutritional rice (Datta, 2000). Transgenic plants selected through this method appeared to be normal and healthy.

A minimal vector approach (expression cassette without backbone) used in transformation did not affect the transformation events or expression level (data not shown). Interestingly, in our study we found that the transgenics having multiple copies and rearranged fragments of the transgenes showed a higher expression of carotenoids. This is clear from

the yellower colour of the endosperm of NHCD3 (with more than 10 copies) compared with 64E26 (with 6 copies) and KDGR29-104 (1–2 copies). This was also evident from the higher *mRNA* expression (from an equal amount i.e. ~2 μg of total RNA loaded) in the seeds of NHCD3 *vis-à-vis* 64E26 (Figure 3). The dosage effect due to higher copy numbers has been reported to lead to high expression (Hobbs *et al*., 1993). However, a more detailed comprehensive study is required before a general statement could be made. Although a single or low copy number of the transgene is desirable, the possibility of silencing a single-copy gene has also been documented (Elmayan and Vaucheret, 1996), besides the frequent co-suppression and inactivation of multiple copies of transgenes (Vaucheret *et al*., 1998). However, the site of integration of the transgene(s) in the genome (position effect) could play for differential expression as observed from the Southern analysis with the use of a single cutter and a non-cutter for the transgene(s) (data not shown).

In view of daily dietary requirements, an increase in the amount of carotenoids, especially β-carotene (provitamin A) and others, such as cryptoxanthin, that are converted to vitamin A would be desirable, although the current levels of carotenoids in our transgenic seeds might already be sufficient to prevent vitamin A malnutrition on the basis of a daily diet of 300 g (R. Russel, personal communication). Efforts are currently underway with modified constructs to enhance the expression of the transgenes driven by different endosperm-specific promoters (globulin, glutelin and prolamin) in collaboration with Dr F. Takaiwa, NIAS, Japan. Care would need be taken to identify lines with clean transgene integration with Positech™ selection system without vector backbone, however, with high expression but no phenotypic agronomic trade-off that would go to farmers' fields and ultimately to end-users. These second-generation transgenic products with improved micronutrients, protein and vitamins would be perceived by the consumer as worthwhile (Philips, 2000). Moreover, plant systems also minimize safety risks due to contamination with human pathogens, in contrast to expression systems relying on cultured human or animal cells for the production of pharmaceuticals (Daniell, 1999).

Experimental procedures

Plasmid constructs

Altogether four different plasmids were used for the co-transformation experiments. The vector pBaal3 (Figure 1a) contained the daffodil phytoene synthase (*psy*) gene (Burkhardt *et al*., 1997) under control of an endosperm-specific Gt1 promoter and a bacterial phytoene desaturase (*crt* I) gene fused to a transit peptide sequence of pea-Rubisco small subunit (Misawa *et al*., 1993) to direct the expression of this bacterial gene into the plastids driven by the constitutive 35S promoter. The lycopene β-cyclase (*lcy*) cDNA (Al-Babili *et al*., 1996) was subcloned from pCyBlue to the *Kpn*I–*Bam*HI site of pGL2 (Gritz and Davies, 1983) under the control of the 35S

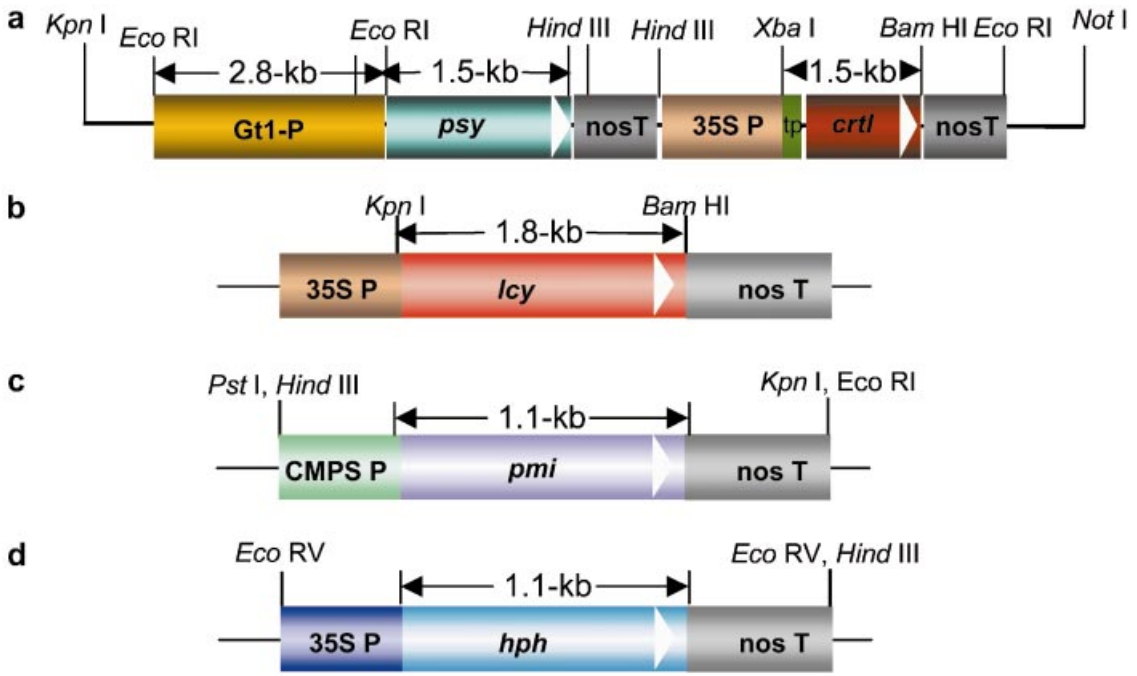

Figure 1 Partial maps of the plasmids: (a) pBaal3 containing *psy* and *crtI*, (b) pTCL6 containing *lcy*, (c) pNOV2820 containing *pmi*, and (d) pGL2 with *hph*.

promoter and nopaline synthase terminator to yield the plasmid pTCL6 (Figure 1b). For the selectable marker gene, either plasmid pNOV2820 (Figure 1c) that carried the phosphomannose isomerase gene driven by a constitutive cestrum promoter of yellow leaf curling virus (Syngenta International Patent Application no. WO 01/73087 A1) or plasmid pGL2 (Figure 1d) containing the selectable marker gene *hph* for hygromycin phosphotransferase under CaMV 35S promoter (Datta *et al*., 1990).

Cultivars and plant transformation

Seven popular indica rice cultivars suited to the diverse ecosystems of different countries – BR29 (from Bangladesh), Nang Hong Cho Dao and Mot Bui (from Vietnam), Immyeobaw (from Myanmar), IR64 (IRRI-bred elite cultivar), IR68899B (IRRI-bred maintainer line), and IR68144 (an IRRI-bred high iron and zinc line) were used for transformation. Immature embryos were used as target explants for co-transformation of the aforementioned vectors using the PDS-1000He particle gun.

For the Positech™ selection system involving the phosphomannose isomerase (*pmi*) gene, the immature embryos after bombardment were incubated in MS medium with 2.0 mg/L 2,4-D and 3% (w/v) sucrose/maltose, but without any selection for the first week. Then the embryogenic calli were transferred to MS medium containing 1% (w/v) D(+)-mannose as a selection agent, together with 2% (w/v) sucrose/maltose for 4–5 cycles at 2-week intervals. The mannose-resistant calli were transferred to the regeneration medium, MS with 1.0 mg/L NAA + 2.0 mg/L Kn + 3% sucrose with/without mannose.

The selection of hygromycin-resistant calli with *hph* as a selectable marker gene, and the regeneration and rooting were done as previously described (Datta *et al*., 1998). The putative primary transgenics and the subsequent seed progenies were grown in the containment greenhouse of IRRI, following a day/night temperature regime of 29/22 ± 2 °C and 70–85% relative humidity.

Polymerase chain reaction (PCR) and Southern blot analysis

Genomic DNA was isolated from 1-month-old plants using the microprep method and 50–100 ng of template DNA was used for PCR analysis with gene-specific primers as described earlier (Baisakh *et al*., 2001). The primer sequences used were as follows:

psy F: tggtggttgcgatattacga, *psy* R: accttcccagtgaacacgtc
crtI F: ggtcgggcttatgtctacga, *crtI* R: atacggtcgcgtagttttgg
lcy F: ccaatccccagaaccctaat, *lcy* R: ctcgctaccatgtaacccgt

Plant genomic DNA was extracted from the freshly harvested leaves of transgenic and non-transgenic control plants for Southern analysis, following the modified CTAB method (Murray and Thompson, 1980). Ten micrograms of DNA were double-digested overnight with *Eco*RI–*Hin*dIII for *psy* and *crtI*, and with *Kpn*I–*Bam*HI for *lcy* and run in 1% TAE-agarose gel. Southern membrane transfer, hybridization and exposure were done as previously described (Datta *et al*., 1998). PCR-amplified fragments of the three genes were radiolabelled with (α-^{32}P)-dCTP and used as hybridization probes.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the polished seeds of the transgenic and non-transgenic plants using the RNAeasy extraction kit (Qiagen, Germany). RT-PCR was performed on 2 μg of total RNA using the specific primers (as above) for *psy and crtI* following the previously described method (Datta *et al*., 2002). The RT-PCR products were resolved on 1.2% TAE-agarose gel.

Carotenoid extraction and HPLC

Polished seeds were milled to powder with a cyclone sample mill (Udy Co., USA). The carotenoid was twice extracted from 1 g of seed powder with acetone (4 mL the first time and 2 mL the second time). Half the proportion of petroleum ether : diethyl ether mix (1 : 1) was added to the combined extract and the clear phase was eluted after adding the water. The elution was evaporated under vacuum and dissolved in acetone. Spectrophotometer absorbance was measured at 470 nm. The acetone solvent was re-evaporated and carotenoid was redissolved in chloroform before injecting to the HPLC. Twenty μL of chloroform extract of carotenoid were applied to the HPLC (model 2690, Waters, USA) with a photodiode array detector (PDA 996, Waters, USA) using a C30 reverse-phase column (Waters, USA) with the following solvent system: solvent A = acetonitrile (ACN):tetrahydrofuran (THF):H_2O, 1.0 : 0.4 : 0.6, with 1% ammonium acetate; solvent B = ACN:THF:H_2O, 1.0 : 0.88 : 0.12 with a linear gradient of 100% A for first 3 min and slowly to 100% B for 10 min, up to a total run time of 24 min.

The total carotenoid (μg/g) was calculated based on the spectrophotometer reading, taking the dilution factor and extinction coefficient (134.5) into account. The amount of β-carotene was estimated based on the percentage coverage of its peak area with respect to the total area of carotenoids.

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References

Al-Babili, S., Hobeika, E. and Beyer, P. (1996) A cDNA encoding lycopene cyclase (Accession, X98796) from *Narcissus pseudonarcissus* L. (PGR 96–107). *Plant Physiol*. **112**, 1398.

Armstrong, G.A., Schmidt, A., Sandmann, G. and Hearst, J.E. (1990) Genetic and biochemical characterization of carotenoid biosynthesis mutants of *Rhodobacter capsulatus*. *J. Biol. Chem*. **265**, 8329–8338.

Baisakh, N., Datta, K., Oliva, N. and Datta, S.K. (1999) Comparative molecular and phenotypic characterization of transgenic rice with chitinase gene developed through biolistic and *Agrobacterium*-mediated transformation. *Rice Genet. Newsl*. **16**, 149–152.

Baisakh, N., Datta, K., Rai, M., Rehana, S., Beyer, P., Potrykus, I. and Datta, S.K. (2001) Development of dihaploid transgenic golden rice homozygous for genes involved in the metabolic pathway for β-carotene biosynthesis. *Rice Genet. Newsl*. **18**, 91–94.

Bartley, G.E., Vitanen, P.V., Pecker, I., Chamovitz, D., Hirschberg, J. and Scolnik, P.A. (1991) Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. *Proc. Natl Acad. Sci. USA*, **88**, 6532–6536.

Britton, G. (1988) Biosynthesis of carotenoids. In: *Plant Pigments* (Godwin, T.W., ed.), pp. 133–180. London: Academic Press.

Buckner, B., San Miguel, P. and Bennetzen, J.L. (1993) The *Y1* gene codes for phytoene synthase. *Maize Genet. Coop. Newsl*. **67**, 65.

Burkhardt, P., Beyer, P., Wünn, J., Klöti, A., Armstrong, G.A., Schledz, M., von Lintig, J. and Potrykus, I. (1997) Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J*. **11**, 1071–1078.

Christou, P., Ford, T.F. and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into zygotic embryos. *Bio/Technology*, **9**, 957–962.

Clemente, T.E., LaVallee, B.J., Howe, A.R., Conner-Ward, D., Rozman, R.J., Hunter, P.E., Broyles, D.L., Kasten, D.S. and Hinchee, M.A. (2000) Progeny analysis of glyphosate selected transgenic soybeans derived from *Agrobacterium*-mediated transformation. *Crop Sci*. **40**, 797–803.

Cunningham, F.X. and Gantt, E. (1998) Genes and enzymes in carotenoid biosynthsis. *Annu. Rev. Physiol. Plant Mol. Biol*. **49**, 557–583.

Daniell, H. (1999) GM crops: public perception and scientific solutions. *Trends Plant Sci*. **4**, 467–469.

Datta, S.K. (2000) Potential benefit of genetic engineering in plant breeding: rice, a case study. *Agric. Chem. Biotechnol*. **43**, 197–206.

Datta, K., Baisakh, N., Thet, K.M., Tu, J. and Datta, S.K. (2002) Pyramiding transgenes for multiple protection in rice against bacterial blight, stem borer and sheath blight. *Theor. Appl. Genet*. DOI 10.1007/s00122-022-1014-1.

Datta, S.K., Peterhans, A., Datta, K. and Potrykus, I. (1990) Genetically engineered fertile indica-rice plants recovered from protoplasts. *Bio/Technology*, **8**, 736–740.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M.F., Oliva, N., Abrigo, E., Khush, G.S. and Datta, S.K. (1998) Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring enhanced resistance to insect pests. *Theor. Appl. Genet*. **97**, 20–30.

Elmayan, T. and Vaucheret, H. (1996) Expression of single copies of a strongly expressed 35S transgene can be silenced posttranscriptionally. *Plant J*. **9**, 787–797.

Fray, R.G., Wallace, A., Fraser, P.D., Valero, D., Hedden, P., Bramley, P.M. and 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.

Gerster, H. (1997) Vitamin A functions, dietary requirements and safety in humans. *Int. Vit. Nutr. Res*. **67**, 71–90.

Gritz, L. and Davies, J. (1983) Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli and Saccharomyces cerevisiae*. *Gene*, **25**, 179–188.

Hansen, G. and Wright, M.S. (1999) Recent advances in transformation of agricultural plants. *Trends Plant Sci*. **4**, 226–231.

Hirschberg, J. (2001) Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol*. **4**, 210–218.

Hobbs, S.L.A., Warkentin, T.D. and DeLong, C.M.O. (1993) Transgene copy number can be positively or negatively associated with transgene tobacco transformants. *Plant Mol. Biol*. **15**, 851–864.

Hundle, B., Beyer, P., Kleinig, H., Englert, G. and Hearst, J.E. (1991) Carotenoids of *Erwinia herbicola* and an *Escherichia coli* HB101 strain carrying the *Erwinia herbicola* carotenoid gene cluster. *Photochem. Photobiol*. **54**, 89–93.

Khush, G.S. (2001) Green revolution: the way forward. *Nature Rev. Genet*. **2**, 815–821.

Kohli, A., Leech, M.J., Vain, P., Laurie, D.A. and Christou, P. (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot-spots. *Proc. Natl Acad. Sci. USA*, **95**, 7203–7208.

Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and 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., deFelipe, M.R., Lucas, M., Ikenaga, H. and 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.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight DNA. *Nucl. Acids Res*. **8**, 4321–4235.

Philips, R.L. (2000) Biotechnology and agriculture in today's world. *Food Nutr. Bull*. **21**, 457–459.

Römer, S., Fraser, P.D., Kiano, J.W., Shipton, C.A., Misawa, N., Schuch, W. and Bramley, P.M. (2000) Elevation of provitamin A content of transgenic tomato plants. *Nature Biotechnol*. **18**, 666–669.

Sandmann, G. (1994) Phytoene desaturase genes, enzymes and phylogenetic aspects. *J. Plant Physiol*. **143**, 444–447.

Sandmann, G. (2001) Carotenoid biosynthesis and biotechnological application. *Arch. Biochem. Biophys*. **385**, 4–12.

Schledz, M., Al-Babili, S., von Lintig, J., Haubruck, H., Rabbani, S., Kleining, H. and Beyer, P. (1996) Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. *Plant J*. **10**, 781–792.

Scolnik, P.A. and Bartley, G.E. (1994) Nucleotide sequence of *Arabidopsis* cDNA for phytoene synthase. *Plant Physiol*. **104**, 1471–1472.

Scolnik, P.A. and Bartley, G.E. (1996) A table of some cloned plant genes involved in isoprenoid biosynthesis. *Plant Mol. Biol. Rep.* **14**, 305–319.

Shewmaker, C.K., Sheehy, J.A., Daley, M., Colburn, S. and Ke, D.J. (1999) Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J*. **20**, 401–412.

Sivakumar, B. (1998) Current controversies in carotene nutrition. *Ind. J. Med. Res*. **108**, 157–166.

To, K.Y., Lai, E.M., Lee, L.Y., Lin, T.P., Hung, C.H., Chen, C.L., Chang, Y.S. and Liu, S.T. (1994) Analysis of the gene cluster encoding carotenoid biosynthesis in *Erwinia herbicola* EHO13. *Microbiology*, **140**, 331–339.

Vaucheret, H., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C. and Vernhetts, S. (1998) Transgene-induced gene silencing in plants. *Plant J*. **16**, 651–659.

Wright, M., Dawson, J., Dunder, E., Suttie, J., Reed, J., Kramer, C., Chang, Y. and Wang, H. (2001) Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase, *pmi*, gene as the selectable marker. *Plant Cell Rep.* **20**, 429–436.

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

Zimmerman, M. and Hurrel, R. (2002) Improving iron, zinc and vitamin A nutrition through plant biotechnology. *Curr. Opin. Biotechnol*. **13**, 142–145.