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ORIGINAL RESEARCH ARTICLE



Engineering provitamin A synthesis pathway with β -carotene metabolism in rice endosperm of a restorer line BR827R

S. Rehana^{1*} , N. Baishakh², K. Datta³, N. Oliva⁴, E. Abrigo⁴, M.A. Mazid⁵, Shah-E-Alam⁶, M.R. Uddin⁷ and Swapan K. Datta⁸

¹Bio-Technology and Genetic Engineering Discipline, Khulna University, Khulna 9208, BANGLADESH

²School of Plant Environmental and Soil Sciences, Louisiana State University, 214 M.B. Sturgis Hall Baton Rouge, LA 70803, USA

³Laboratory of Translational Research on Transgenic Crops, Department of Botany, University of Calcutta, Kolkata-700073 (West Bengal), INDIA

⁴Plant Tissue Culture Laboratory, International Rice Research Institute, DAPO 7777, Metro Manila, PHILIPPINES

⁵525/A, Senpara Parbata, Mirpur 14, Dhaka 1216, BANGLADESH

⁶Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh 2202, BANGLADESH

⁷Department of Agronomy, Bangladesh Agricultural University, Mymensingh 2202, BANGLADESH

⁸Department of Crop Science, Institute of Agriculture, Visva Bharati University, Santiniketan- 731235 (West Bengal), INDIA

*Corresponding author's E-mail: saydarehana@gmail.com

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ABSTRACT

Vitamin A deficiency (VAD) is a serious public health problem in South Asia particularly in Bangladesh. *Indica* rice as a major staple in the country completely lacks vitamin A or compounds with provitamin A activity after milling. A combination of transgenes has been introduced enabling biosynthesis of provitamin A in the endosperm of a restorer line using biolistic system of transformation. The rice seed-specific glutelin promoter (*Gt-1 P*) was used to drive the expression of phytyl synthase (*psy*), while lycopene b-cyclase (*lcy*) and phytoenenedesaturase (*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 *CaMV35sP* driven *hph* (hygromycinphosphotransferase) gene. Molecular analysis demonstrated stable integration and expression of the transgenes. The variable segregation pattern in T_1 generation indicated single to multiple insertions of the transgenes in the genome. This is the first report of the development of a transgenic restorer line with carotenogenic pathway into the endosperm for use of hybrid rice improvement.

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INTRODUCTION

Vitamin A is an essential vitamin for mammals. Mammals are not able to produce carotenoids because of the complete absence of the biosynthetic pathway beginning with the synthesis of phytoene from GGPP. Dietary β -carotene therefore serves as an important precursor for the synthesis of vitamin 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). Dietary β -carotene is converted into vitamin A, also known as retinol, by oxidative cleavage of

the central double bond followed by a reduction of the terminal aldehyde.

Vitamin A plays role in the normal development of humans (Bendich, 1993, 1994; West *et al.*, 1989) and in other mammals. Furthermore, recent investigations have reported that vitamin A quenches free radicals and prevents cellular oxidative damage, as well as supporting the human immune system (Bendich, 1989, 1993; Ross, 1992). In mammals β -carotene and some structurally related compounds have provitamin A character. Carotenoids, present in all photosynthetic and many non-photosynthetic organisms are a widely distributed class of

natural pigments containing 40 carbon arms. Carotenoid biosynthesis represents one intracellularly specialized branch of general isoprenoid metabolism in plants. Whereas sesqui- and triterpenoids are produced in the cytoplasm, mono-, di- and tetraterpenoids are synthesized in plastids (Kleinig, 1989). These pigments are well known as essential components of the photosynthetic apparatus. Carotenoids are also produced in the chloroplast of flowers and fruits, where they serve as visual attractants of insects and animals to facilitate pollination and seed dispersal. In some cases, such as maize and potato, carotenoids also can be formed in the amyloplasts of plant storage tissues.

It has been established that four enzymes in plants, i.e. phytone synthase, phytone desaturase, ζ -carotene desaturase, and lycopene cyclase to complete the pathway toward β -carotene (provitamin A) biosynthesis from GGPP (for review see Britton, 1988; Cunningham and Grantt, 1998; Sandmann, 1994, 2001). The first step in carotenoid biosynthesis is the condensation of two molecules of GGPP to produce phytone by the enzyme phytone synthase (PSY). PSY is firmly associated with the chloroplast membrane in its active form (Schledz et al., 1996). Comparing to plants, anoxygenic photosynthetic bacteria, non-photosynthetic bacteria and carotenoid-synthesizing fungi do not possess a distinct phytone desaturase (PDS) and ζ -carotene desaturase (ZDS) to catalyse the conversion of phytone to lycopene. In non-photosynthetic-bacteria, phytone is converted to all-trans lycopene by a single enzyme phytone desaturase (CRTI). In order to cyclase of lycopene two different lycopene cyclase specific 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 derived 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 fungi, bacteria and plants (Al-Babili et al., 1999; Armstrong et al., 1990; Misawa et al., 1993; Buckner et al., 1993; Hundle et al., 1991; Misawa et al., 1990; Scolnik and Bartley, 1994, 1996; To et al., 1994).

Conventional interventions (supplementation, fortification, food based diversification etc) have been helpful in defeating VAD but were not sufficiently effective. Plant breeding to alter, modify or introduce this biosynthetic machinery into the target tissues in rice has been impossible as of now, as no endosperm active carotenoid-biosynthetic genes have found thus far in the available rice gene pool (Tan et al., 2005). Therefore recombinant DNA technology and plant biotechnology, with above-mentioned molecular tools in hand, represents an alternative method to combat VAD. Moreover it may represent a sustainable strategy (Zimmerman and Hurrell, 2002).

Golden Rice as published (Ye et al., 2000) demonstrates the feasibility of the scientific approach but does not yet represent a product. The carotenogenic pathway introduced earlier in the endosperm of various *indica* rice cultivars well established in different developing countries (Datta et al., 2003) cannot be

used directly for developing provitamin A enriched hybrids. Therefore, this study was undertaken to develop a provitamin A enriched restorer (r) line which will directly help to produce carotenoid enriched hybrid rice. Through biolistic method of gene transformation Phytone synthase (*psy*), bacterial phytone desaturase (*crtl*), and lycopene cyclase (*lcy*) genes were introduced into the endosperm of a restorer line to derive the accumulation of β -carotene. This is the initial report to develop a transgenic β -carotenoid restorer line, a new tool for improving hybrid rice.

MATERIALS AND METHODS

Selection of genotype and plasmid for transformation experiments

An elite *indica* restorer line (BR827R) was selected for transformation on the basis of its superior grain quality. Altogether three different plasmids were used for the co-transformation experiments. The vector pBall3 (Figure 2) contained the daffodil phytone synthase (*psy*) gene (Burkhardt et al., 1997) under control of an endosperm-specific *Gt1* promoter and a bacterial phytone desaturase (*crtl*) gene fused to a transit peptide sequence of a pea-rubisco small subunit (Misawa et al., 1993) to direct the expression of this bacterial gene into the plastids by constitutive 35S promoter. In order to yield the plasmid pTCL6 (Figure 2) under control of the 35S promoter and nopaline synthase terminator, lycopene β -cyclase (*lcy*) cDNA (Al-Babili et al., 1999) was subcloned from pCyBlue with the *KpnI*-*Bam*HI site of pGL2 (Gritz and Davies, 1983); to the selectable marker gene, plasmid pGL2 (Figure 2) containing the selectable marker gene *hph* for hygromycin phosphotransferase under CaMV 35S promoter (Datta et al., 1990).

Experimental design for transformation experiments

Rice immature embryos were used as target explants for co-transformation (Figure 3) of the above-mentioned vectors using the PDS-1000He particle gun. Selection started 16-20 hours after bombardment on fresh callus induction medium containing 40-mg/L hygromycin as described earlier (Figure 3) (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 \pm 2 °C and 70-85% relative humidity. A stepwise methodology of rice transformation experiment has been given in figure 1.

Polymerase chain reaction (PCR) and southern blot analysis

Genomic DNA was isolated from 1-month-old plants using the micro prep method and 50-100 ng of template DNA was used for PCR analysis with gene-specific primers (Table 1) as described earlier (Baisakh et al., 2001). 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 Thomson, 1980). Ten micrograms of DNA were digested overnight with *Eco*RI-*Hind*III

for *psy* and *lcy*, *Bam*HI for *crtI* 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.

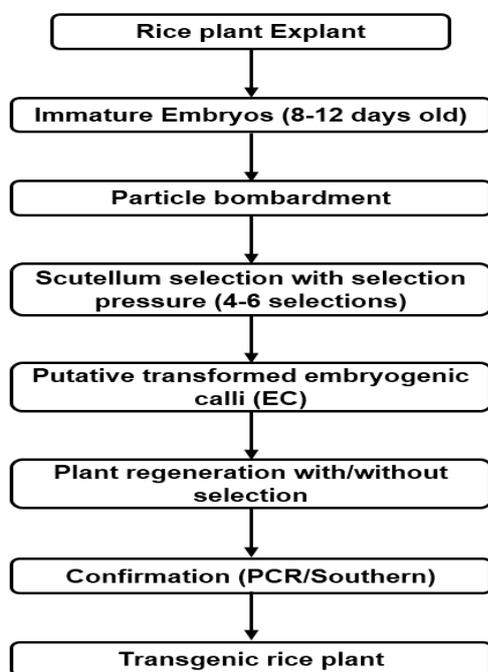


Figure 1. Stepwise rice transformation experiment.

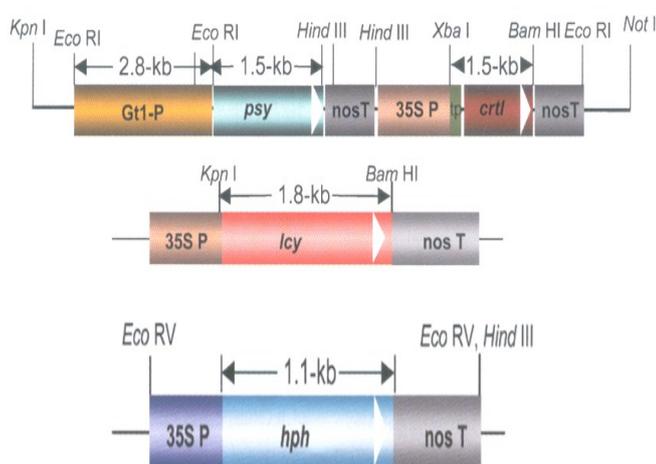


Figure 2. Partial map of the plasmids: a. pBaal3 containing *psy* and *crtI*, b. pTCL6 containing *lcy* and c. pGL2 with *hph*.

Table 1. General features of selected plasmids for transformation experiments.

Plasmid	Target gene (s)/ sequence (s)	Primer sequences	Specific features	Source
pBaal3	<i>Psy</i> and <i>Crt 1</i>	<i>Psy</i> F: TGGTGGTTGCGATATTACGA, <i>psy</i> R: ACCTTCCCAGTGAACACGTC <i>CrtI</i> F: GGTCGGGCTTATGTCTACGA, <i>crtI</i> R: ATACGGTCGCGGTAGTTTTGG	Phytoene synthase and Carotene desaturase	Datta et al. (2003)
pTCL6	<i>Lcy</i>	<i>Lcy</i> F: CCAATCCCCAGAACCCCTAAT, <i>lcy</i> R: CTCGCTACCATGTAACCCGT	Lycopene cyclase	Datta et al. (1990)
pGL2	<i>hph</i>		Selectable marker gene	Datta et al. (1990)

RESULTS AND DISCUSSION

Assessment of T₀ transformants

Out of the eight independent bombardments (600 IE each) of BR827R, a small number of T₀ transgenic plants (46) produced, three independently transformed lines SBR827R7, SBR827R11 and SB827R12 were recovered and presented in the study. The insertion of the genes in the genome was primarily checked by PCR analysis (Figure 4) and then confirmed by southern blot analysis (Figure 5). The 1.5 kb and 1.6 kb size bands confirmed the integration of *psy* and *crtI* gene respectively (Figure 5). When the blot was hybridized with 1.5 kb *psy* probe the three lines showed expected size band (Figure 5). In case of *crtI* gene, hybridization occurred in the high molecular weight regions in lane 1 (Figure 5) containing not expected size DNA of *crtI* gene, suggesting rearrangement of the transgene. Two of them contained a fragment of the expected size that suggests a correct and intact integration of the *crtI* cDNA (Figure 5). Peter Burkhardt (1996) also reported while the plants were co transformed with plasmids pCPsyH and pCPdsH, both higher and expected size was observed. However, two transformants were fertile and one line SBR827R12 was sterile. Between the two fertile lines SB827R7 produced sufficient seeds and the other line SB827R11 produced very few seeds.

Evaluation of T₁ progeny

B-carotenoid positive T₁ progeny from both transformants were identified by southern blot analysis. Results of southern blot analysis of the progeny from both transformants are shown in Figure 5. In the progeny lines of SBR827R-7 showed (Figure 6) same integration pattern of 1.5-kb size *psy* gene like T₀ line and genomic DNA was digested with *Eco*R1/*Hin*DIII restriction enzyme. Out of 14 progeny lines of SBR827R-7, 9 lines were positive with expected size *psy* DNA. When the same blot was reprobated with *crtI* gene (PCR originated) all the *psy* positive lines showed integration of *crtI* gene in the genome (Figure 4). The banding pattern in T₁ was same as previously shown in T₀ (Figure 5) not expected size in all SBR827R-7 progeny lines. In case of SBR827R-11, two lines were positive but one showed rearranged banding pattern (Figure 4). In T₀, SBR827R-11 gave expected size 1.6 kb sized *crtI* (Figure 5), but in case of T₁ some rearrangement may be occurred. Between two positive lines one showed expected size SBR827R11-4, 1.6-kb *crtI* but

SBR827R11-2 showed comparatively upper band means high molecular weight of the transgene (Figure 6). The different banding pattern in SB827R11 indicates that a rearrangement of the transgene may occur in successive generations. This may be due to deletion, addition or translocation of the transgene. However, further study on this aspect is needed. Multiple

generations and additional progeny analysis may provide useful information regarding such rearrangements. Goto et al. (1993) showed differences in banding patterns among T₂ progenies of transgenic rice plants. Nayak et al. (1997) also reported different southern patterns among progenies of specific transformation event they analyzed.

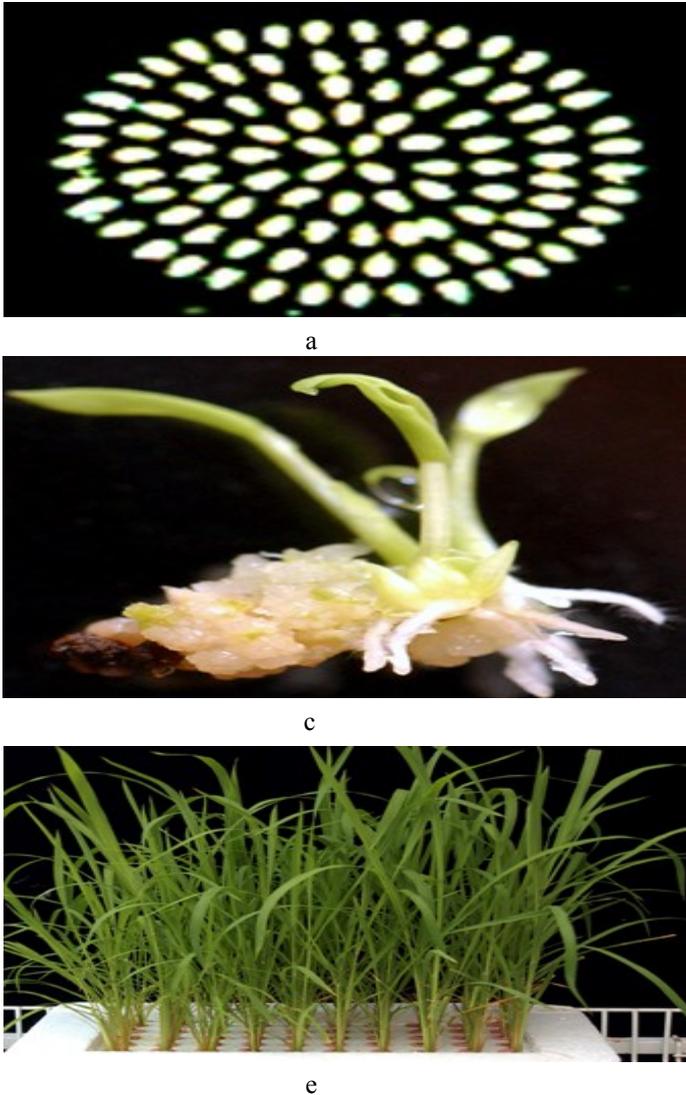


Figure 3. Generation of transgenic rice plant by particle bombardment mediated transformation; a. circular arrangement of IE; b. Resistant calli in selection medium; c. Regenerated plantlet; d. Plantlet in rooting medium; e. In yoshida's solution.

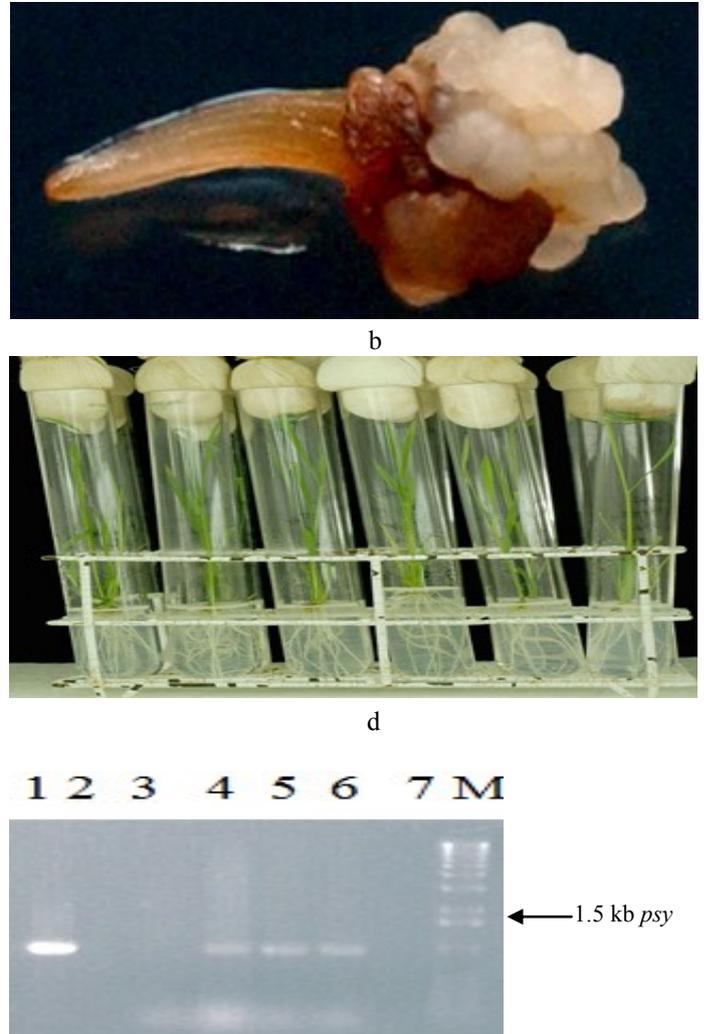


Figure 4. PCR analysis showing amplification of 1.5 kb size band of psy gene in the primary transgenics. Lane 1, plasmid control; lane 2, blank; lane 3, non-transformed control plant; lane 4, 5, 6 three individual transformants; lane 7, blank; and M, 1 kb DNA molecular marker.

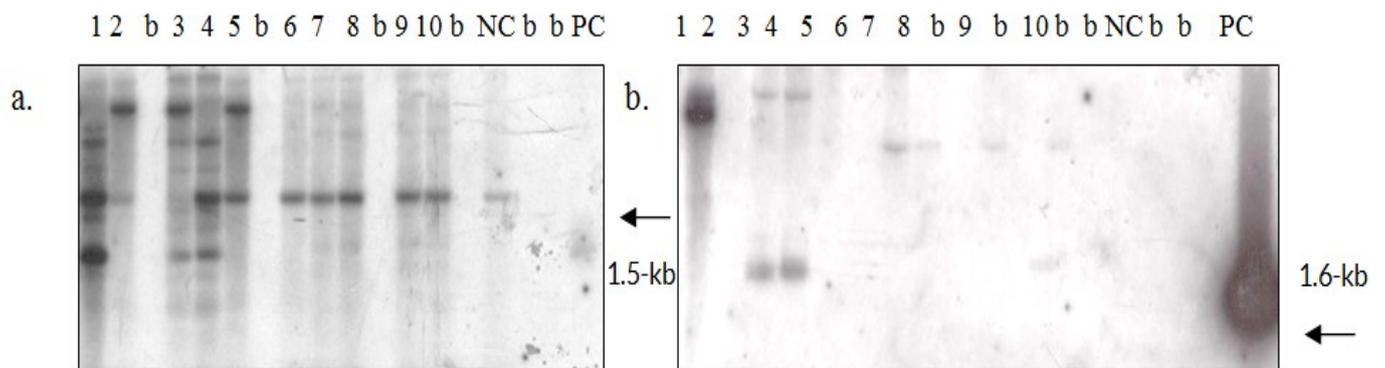


Figure 5. Southern blots showing the integration of a) psy, b) crtI in the primary transgenics of restorer line BR827R(EcoRI/HinDIII-digested Pbaa13 forpsyand BamHI forcrtI). Ten µg of genomic DNA were digested overnight, electrophoresed in 1% TAE-agarose gel, southern blotted and hybridized with (32p) dCTP-labelled probes ofpsy and crtI (PCR-generated). Lanes 1.SBRR7, 2.SBRR10, 3.SBRR11, 4. SBRR12, 5.SBRR13, 6.SBRR14, 7.SBRR15, 8. SBRR16, 9.SBRR17 and 10.SBRR18. b. blank, NC-negative control, PC-positive control.

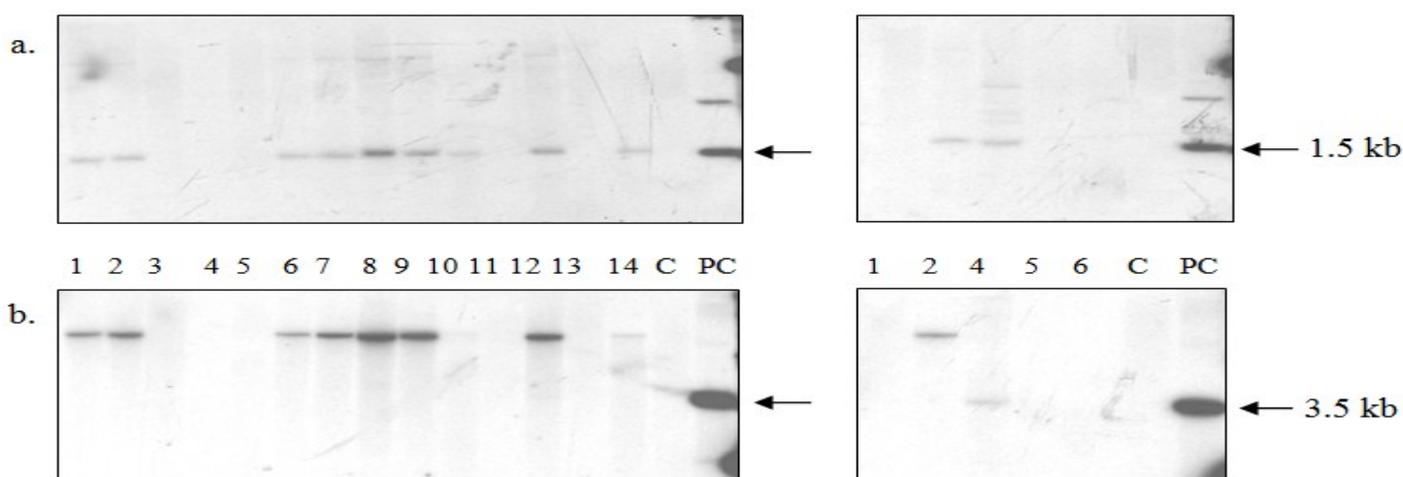


Figure 6. Southern blots showing the integration of a) *psy*, b) *crtI* in the T_1 progenies of SBRR7 and SBRR11 line. Ten μ g of genomic DNA was digested with *EcoRI*/*HindIII* for overnight, electrophoresed in 1% TAE-agarose gel, southern blotted and hybridized with (32p) dCTP-labeled probes of *psy* and *crtI* (PCR generated).

Conclusion

This investigation concluded that a restorer (r) line BR827R for hybrid rice production was used to explore the potential for transformation of *Indica* rice adapted in Bangladesh. Rice immature embryos were transformed with pBaal3, pTCL6 and pGL2 using the particle gun transformation system. The transgenic plants were confirmed by PCR and Southern Blot analysis. Hybridization with *psy*, *crtI*, *lcy* and *hph* probes suggested the integration of the respective genes in the genome of the transgenic BR827R plants.

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