Tropical Agricultural Research Vol. 14: 22-30 (2002)

Agrobacterium Mediated Transfer of Synthetic *cry* 1 Gene to Rice Calli of BG 380

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ABSTRACT. *Rice (Oryza sativa L. ssp. indica) is the primary source of food for 50% of the world population, and therefore is among the most important crop plants on earth. Human consumption accounts for 85% of the total rice production, thus making rice the major staple food for more than 2 billion people in Southeast Asia. Lepidopteran pest attack is a considerable constraint on rice growing. Chemical control of these pests is neither ecologically acceptable nor cost effective. Therefore, the main objective of this research was to transfer synthetic cry 1 gene into rice plants and develop a pest resistant rice variety. Seeds of rice variety BG 380 were cultured on MS media for callus induction. Rice calli were co-cultivated with Agrobacterium tumefaciens recombinant strain C58 (containing $p^{CAMBIA1301}$ vector). Transformants were screened on 200 mg l^{-1} hygromycin. Southern blot analysis and histochemical Gus assay were used for confirmation of gene transfer. Positive transformants were transferred to N6 medium for regeneration. Transfer of the cry gene in to calli was confirmed by the positive results of the Southern blot. Plant regeneration was observed after three weeks on N6 medium.*

INTRODUCTION

Rice (*Oryza sativa*) is the major staple for more than 2 billion people in Southeast Asia, where over 90% of the world rice production is consumed. During the last decade, rice acreage has increased by 10% where as rice consumption has increased by 30%. In the year 2000, Sri Lankan rice production was 2.86 million mt (Central Bank, 2000). In 2005, Sri Lanka's population will exceed 20 million. It requires production of 3.1 million mt per year and 4.5 t ha^{-1} of average yield. However, the current average yield of rice is 3.56 t ha-1 (Department of Census and Statistics, 1998). Therefore, it is important to increase the local productivity of rice. About 30% of the potential production of rice is destroyed due to the pest and disease incidences. Therefore it is essential to minimize the yield loss caused by pest and diseases. Transfer of "pest resistant genes" into rice plants is one of the methods to achieve the above objective. Many rice varieties with improved characters have been released in the developing countries since the releasing of IR8 variety. However, much of the rice is grown under adverse conditions such as high incidence of pests and diseases, drought and sub optimum temperatures. Most of the newly improved rice varieties are not adequately adapted to these stresses. As a consequence their cultivation has expanded only about 25–30% of the total rice area in the zones that are apparently better suited to these varieties. Even in those areas, pest and disease problems and fluctuating rainfall pattern

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cause considerable instability in yields. Lepidopteran pest attack is a considerable constraint for rice growing in many countries. Therefore, the main objective of this study is to transfer synthetic Bt *cry* gene to rice aiming for lepidopteron pest resistance.

MATERIALS AND METHODS

Explant and bacterial strain

Viable dehusked mature rice seeds of variety BG 380 were used as the explants.

Agrobacterium tumefaciens strain C58 supplied by Dr. N. Yakandawala (ETH, Zurich) was used as the host strain for vector $p^{CAMBIA1301}$. This strain was maintained on YEB medium (Luis and Simpson, 1988) with 50 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin at 28°C.

Vector

Agrobacterium binary vector $p^{CAMBIA1301}$ supplied by Dr. N. Yakandawala (ETH, Zurich) carrying the synthetic *cry* 1 gene was used for transformation. This vector has the hygromycin resistant gene and *Gus* genes (exon1 and exon2) driven by CaMV35S promoter (Fig. 1).

Culture of ex-plants

Mature seeds were de-husked and dipped in water for one minute into which a few drops of teepol had been added. The seeds were then washed with distilled water and transferred into 70% ethanol and kept for one minute. The seeds were washed with sterilised distilled water. Then 10% calcium hypoclorite solution was added and kept in the shaker for about 30 min. These seeds were rinsed in sterilised water for about five minutes and transferred to a sterilised filter paper to absorb excess water. Sterilised mature seeds were cultured on Murashige and Skoog (MS) medium supplemented with mg l^{-1} 2,4-D and kept in dark at 28°C and 80% RH.

Callus multiplication and transformation

After formation of the callus the scutellum-derived calli were transferred into fresh MS medium. Subculture was done once a week for five weeks and only the embryogenic callus was transferred to the fresh medium. Transformation was carried out according to the protocol used at ICGEB (ICGEB, 1996).

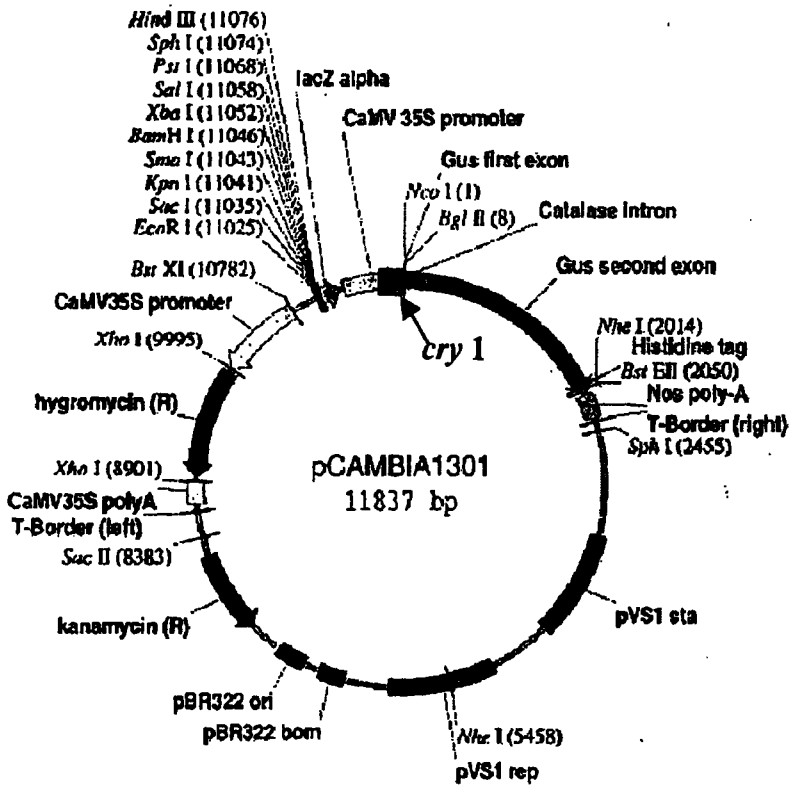

Fig. 1. Map of recombinant $p^{CAMBIA1301}$ vector.

Preparation of bacterial cultures

A. tumefaciens strain C58 carrying synthetic *cry* 1 cloned $p^{CAMBIA1301}$, was inoculated to 25 ml of YEB and kept overnight in a shaking water bath at 28°C. This culture was centrifuged at 5000 rpm for 10 min after 16 h. Supernatant was discarded and the pellet was re-suspended in plant inoculation medium (1/2 MS with 3% sucrose, 2 mg l^{-1} 2,4-D and 0.4% agar).

Preparation of ex-plants and *Agrobacterium* inoculation

Calli obtained after fourth subculture were plasmolyzed one hour prior to inoculation by introducing them into plasmolysis medium (MS basal medium with 10% sugar and 0.4% agar at pH 7.2). Plasmolyzed calli were directly transferred to bacterial suspension in the inoculation medium. These were incubated for a total of 30 min in two phases; 10 min shaking and 20 min stationary.

Co-cultivation

Inoculated calli were transferred to co-cultivation medium (MS basal medium with 3% sucrose, 200 µM acetosyringone, 4% glucose and 2% $MgCl_2$ at pH 5.8). After 48 h co-cultivated rice calli were transferred to MS with 500 mg l^{-1} cefotaxime to kill the bacteria. These were then transferred to MS with 100 mg l^{-1} hygromycin to select the transformants.

Confirmation of gene transfer: *Gus* analysis

Hygromycin positive calli were incubated for 48 h with 100 µg ml^{-1} 5-bromo-6-chloro-3-indolyl-beta-D-glc-A (Biosynth International, USA) under total dark conditions at 37°C.

Southern blot analysis: Isolation of genomic DNA from co-cultivated rice calli

Putatively transformed calli were used to isolate chromosomal DNA according to the method described by Walbot, 1989. Co-cultivated calli (0.1 g) were homogenized in cold mortar by rapid grinding, 0.5 ml of solution I {15% sucrose, 50 mM Tris. HCl (pH 8.0), 50 mM Na_2EDTA and 250 mM NaCl} was added and ground again. Fluid was transferred into ice-cold centrifuge tubes. After centrifugation pellets were re-suspended in solution II {20 mM Tris. HCl (pH 8.0) and 10 mM Na_2EDTA}. Twenty micro litres of 20% SDS was added and mixed. This was incubated at 70°C for 15 min and placed on ice. Hundred and fifty micro litres of 7.5 M ammonium acetate was added and kept on ice again. After centrifugation at 12000 rpm for 45 min at 4°C, supernatant was treated with 0.7 ml of isopropanol and incubated at room temperature. After centrifugation, 70% ethanol was added to the pellet. Finally precipitated DNA was re-suspended in 50 µl of TE (pH 8.0) and stored at –20°C.

Vector DNA extraction and gel electrophoresis

DNA was extracted from *Agrobacterium tumefaciens* strain C58 using JET prep plasmid DNA isolation and purification kit (Genomed, Germany). DNA isoladed from *Gus* positive rice calli, *Gus* negative rice calli, untransformed rice calli and vector DNA were subjected to gel electrophoresis on a 0.8 agarose gel according to Sambrook *et al.* (1989).

Alkaline transfer

Gel was soaked for 15 min in 0.4 M NaOH to denature the DNA. Denatured DNA was transferred to the positively charged nylon membrane according to the manufacturer's instructions (Boehringer Manheim Biochemica, Germany).

Hybridization

Nylon membrane was baked at 120°C for 15 min. This membrane was probed with Dig labelled synthetic *cry* I probe using Dig DNA labelling and detection kit according to the instructions of the manufacturer. Fixed membrane was pre-hybridised with 20 ml of hybridisation buffer (5 x SSC, 1% blocking reagent, 0.1% N-luroylsarcosine and 0.02% SDS) per 100 cm^2 of filter at 68°C for 1 h. Membrane was soaked in 2.5 ml of hybridisation buffer containing 5 µl of freshly denatured labelled probe and incubated for 6 h with occasional redistribution of the solution. Hybridised membrane was washed at room temperature with 50 ml of 2 x SSC supplemented with 0.1% SDS for 10 min. Secondary washing step was carried out at 68°C with 50 ml of 0.1% x SSC supplemented with 0.1% SDS. Properly washed membrane was subjected to immunological detection according to the manufacturer's instructions.

Callus regeneration

Gus positive calli were transferred to MS and N6 media with 2 mg l^{-1} BAP, 1 mg l^{-1} kinetin, 1 mg l^{-1} NAA and 500 mg l^{-1} casein hydrolysate. The cultures were incubated with a 16 h photoperiod at 3500 lux, 28°C and 85% RH.

RESULTS AND DISCUSSION

Confirmation of gene transformation

Gus analysis

Histochemical *Gus* assay was used to confirm the gene transformation. *Gus* gene is the most utilised reporter gene in plant genomic transformation. Calli infected with *A. tumefaciens* strain C58 took 30 h for colour development (Plate 1).

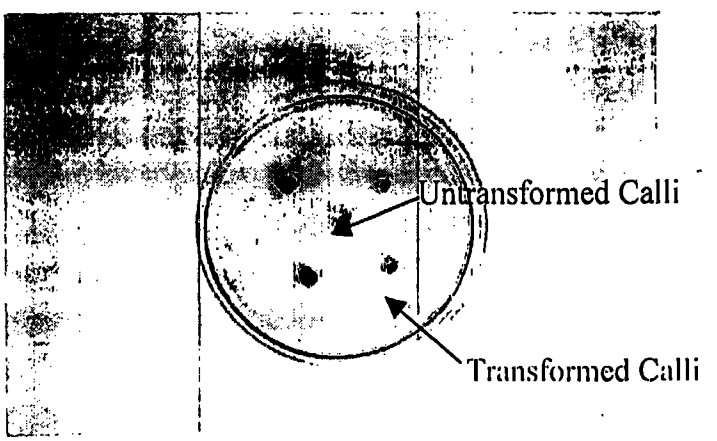

Plate 1. Transformed calli with an overnight dip in X-glcA at 37°C.

In cross sections of transformed calli, some cells showed colour change while some did not (Plate.2). This could be due to either somaclonal variation or different levels of exposure to *Gus* chemical. Untransformed calli did not develop blue colour (Plate 3) confirming the absence of endogenous *Gus* activity in rice. Slight tissue browning was observed in calli used for this experiment. Because of this, blue colour intensity of calli was reduced to some extent. Positive *Gus* analysis indicates that the *Gus* gene in T-DNA region has expressed in rice genome. This proves that the T-DNA region has integrated with the rice genome.

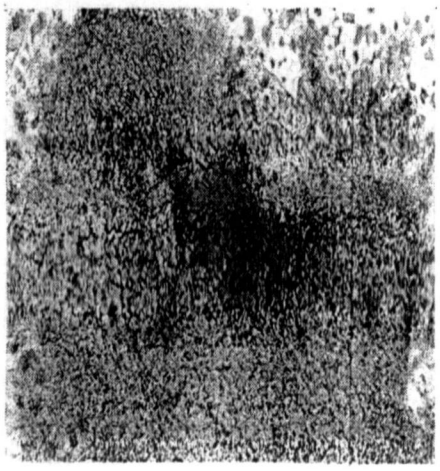

Plate 2. *Gus* expression at cellular level of co-cultivated rice calli.

Plate 3. Cross section of an untransformed calli.

Southern blot

All the *Gus* negative calli subjected to Southern hybridisation gave negative results giving proof to their un-transformed nature (Plate 4). Lane 6 gave two distinct hybridisations. These could be due to multiple copies of the transgene. This could be further clarified by southern hybridisation of digested DNA from transformed calli. Lane 1 carried DNA of the recombinant vector used as the positive control.

Regeneration of rice calli on N6 and MS

Between the two media (MS and N6) used for the regeneration, calli of the N6 medium showed better results. Almost all the replicates of BG 380 showed green shoot primordia (Plate 5) on N6 medium after three weeks. The calli on MS medium showed no regeneration and most of the calli turned to dark brown or black colour with time.

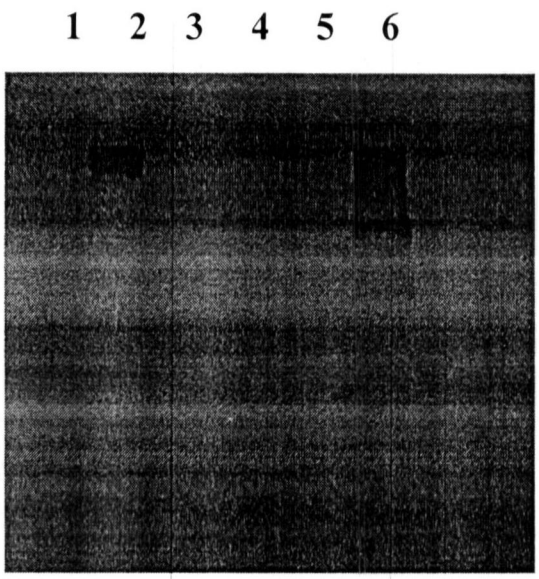

Plate 4. Southern blot of transformed calli with Dig labeled *cry* probe.
[Note: Lane 1 - Vector DNA, Lanes 2-4 - DNA from *Gus* negative calli, Lane 5 - DNA from non-transformed calli, Lane 6 - DNA from *Gus* positive calli].

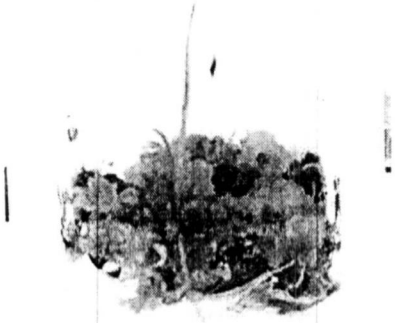

Plate 5. Regenerating calli of BG 380 on N6 medium after 4 weeks.

Although callus induction and plant regeneration are controlled by many factors, media components are very important in *in vitro* culture of rice. When MS and N6 media were compared MS medium has a higher concentrations of mineral and salt than N6 medium. Suitable levels of minerals and salts are very important for the

regeneration. For some plants, however the levels of salts in MS medium is either toxic or unnecessarily high (Anderson, 1975; Adams *et al.*, 1979). Higher concentration of minerals and salts in MS medium may be toxic for most of the varieties used. One of the major differences among N6 and MS media was the content of minerals and salts, particularly nitrogen salts (ammonium and nitrate ions). The difference in the total nitrogen ratio of these two media suggests that it plays an important role in obtaining plant regeneration. So the amount of minerals and salts found in N6 medium may be adequate for the shoot primordia initiation in the varieties used, compared to MS medium. It is evident that MS medium contains a high concentration of nitrogen as ammonium ion while N6 has low concentration. It is also may be a reason for very low regeneration ability of calli on MS medium, as high ammonium concentrations reduce cell growth in some plant systems. Development of the embryo into a plant is enhanced by the presence of amino acids in the culture medium (Paris *et al.*, 1953). As N6 medium contain very high amount of glycine compared to MS medium, it maybe also one of the reasons to get higher regeneration of calli on N6 medium. Along with that casein hydrolysate is also used as a source of amino acid in the medium, this complex also may have an effect on the growth of the tissues.

After successfully establishing an efficient transformation system for BG 380 including a complete regeneration protocol, the future lies in the direction of analysis of the regenerated transfomants for the efficient production of the synthetic *cry* 1 protein *via* a Western blot (under study). Afterwards successful transformants should be tested under restricted conditions for their efficacy against lepidopteran pests. Based on these, if necessary further improvements could be made at the molecular level to obtain a better product.

CONCLUSIONS

Based on the positive Southern hybridisation it is evident that the calli derived of BG 380 variety was successfully transformed with the synthetic *cry* 1 gene. The medium N6 is more suitable for callus regeneration of variety BG 380 than MS.

ACKNOWLEDGEMENTS

Authors thank Dr N. Yakandawala for the vector $p^{CAMBIA1301}$ and critical evaluation of the procedures and Dr.(Mrs) Ranamalee Amarasinghe for assessment.

REFERENCES

Adams. R.M., II, Koenigsberg. S.S. and Langhans. R.W. (1979). *In vitro* propagation of the butterwort *Pinguicula moranensis* H. B. K. Hort. Sci. 14: 701-702.

Anderson. W.C. (1975). Propagation of rhododendrons by tissue culture. Part development of a culture medium for multiplication of shoots. Proc. Int. Plant. Int. Plant Prop. Soc. 25: 129-135.

Central Bank. (2000). Annual Report of the Central Bank of Sri Lanka. pp. 54-56.

Department of Census and Statistic. (1998). Statistical Abstract of the Democratic Socialist Republic of Sri Lanka 1998. pp. 80-111.

ICGEB. (1996). Plant Transformation Workshop Handbook. International Centre for Genetic Engineering and Biotechnology, New Delhi, Nov. 4-15,1996.

Luis, H.E. and Simpson, J. (1988). Foreign gene expression in plants. pp. 131-160. *In*: Shaw, C.H. (Ed). Plant Molecular Biology: A Practical Approach, IRL Press, Oxford, Washington DC.

Paris, D., Rietsema, J., Satina, S. and Blakeslee, A.F. (1953). Effect of amino acids, especially aspartic and glutamic acid and their amides, on growth of *Datura stramonium* embryos *in vitro*. Proc. Natl. Acad. Sci. USA. 39: 1205-1212.

Sambrook, J., Fritch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Edition Vol. 3, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Walbot, V. (1989). Preparations of DNA from single rice seedling. pp. 149-151. *In*: ICGEB. Plant Transformation, Rice Genetics Newsletters 1996, Int. Ctr. Genetic Eng. and Biotech. Workshop, Newdelhi.