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Transformation of elite rice cultivars ASD16 and IR64 with *cry2Ac* gene for resistance to rice lepidopteran pests

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With the view to imparting resistance to major lepidopteran pests of rice, attempts were made to transform elite *indica* genotype ASD16 and IR64 with *cry2Ac* gene. *Agrobacterium* and particle bombardment-mediated transformation were carried out using the vector pS2AcP2 harbouring *cry2Ac* gene, the selectable marker gene, *hph* and the scorable marker gene, *gusA*. In *Agrobacterium*-mediated transformation experiment with mature seed derived calli, two lines in ASD16 were regenerated with a regeneration frequency of 1.1 per cent. In particle bombardment-mediated transformation experiments with immature embryos, 15 lines in IR64 were regenerated with a regeneration frequency of six per cent. Presence of transgenes in these lines could not be demonstrated through PCR.

Key words : Rice, *Agrobacterium tumifaciens*, *Bacillus thuringiensis*, Stemborer, Leaffolder, *cry2Ac* gene, Biolistic gun

INTRODUCTION

Rice is one of the most important cereal crops, providing staple food for nearly one-half of the global population (FAO, 2004). Globally rice is cultivated in 154 m.ha with an annual production of around 645 MT and average productivity of 4.12 tonnes per hectare (USDA, 2007). At least 114 countries grow rice and more than 50 have an annual production of 100,000 tonnes or more.

Savary *et al*. (2000) reported that 24-41% of rice yield was lost every year because of rice stemborer and other insect pests, diseases and weeds. The estimated biotic stress causes annual rice yield loss upto 40% (Oerke and Dehne, 2004).

The most destructive insect pests of the rice crop are yellow stemborer (YSB; *Scirpophaga incertulas* Walk.) and rice leaffolder (RLF; *Cnaphalocrocus medinalis*). Globally, YSB alone causes yield losses of 10 MT and accounts for 50% of all insecticides used in rice field (Huesing and English, 2004). For a long time, the control of these pests has depended chiefly on the use of large amounts of poisonous chemical insecticides, mostly as sprays, which cause considerable environmental pollution and represent a health hazard to farmers as well as significantly increasing the costs of rice production (Tang *et al.,* 2006).

The different insecticidal gene used for the control of insect pests includes protease inhibitors, lectins, amylase inhibitors and ä-endotoxins (*Bt* gene) produced by the soil bacterium, *Bacillus thuringiensis.* Among them, *Bt* gene offers a great scope for controlling insect pests (Shelton *et al.,* 2000).

Keeping the above points in mind the present investigation has been envisaged to evolve transgenic indica rice cultivars expressing Bt toxin to provide protection against lepidopteran pests with the following objectives:

- Confirmation of *cry2Ac* gene construct through molecular analysis
- *Agrobacterium* and particle bombardment-mediated transformation of elite *indica* rice cultivars, IR64 and ASD16 using pS_2AcP_2 harbouring *cry2Ac* gene

MATERIALS AND METHODS

Gene construct :

The binary vector, pS_2AcP_2 (based on pCAMBIA1301; Fig.1) containing *cry2Ac* gene (source: Dr. V. Udayasuryan, Department of Plant Molecular Biology and Biotechnology, CPMB) driven by *CaMV*35S promoter, *hph* and *gusA* gene. *Agrobacterium*- strain LBA4404 (pS_2AcP_2) was used for transformation experiments.

Back-transformation of E. coli DH5á :

Agrobacterium total DNA isolation:

Total DNA was isolated from *Agrobacterium* strain LBA4404 harbouring pS_2AcP_2 by following a modified protocol of Chen and Kuo (1993).

Preparation of DH5~ competent cells :

Single colony of DH5α was inoculated in 3 ml of LB (10 g/l tryptone, 5 g/l yeast extract, 10 mg/l NaCl, pH 7.2) broth and allowed to grow overnight. One millilitre

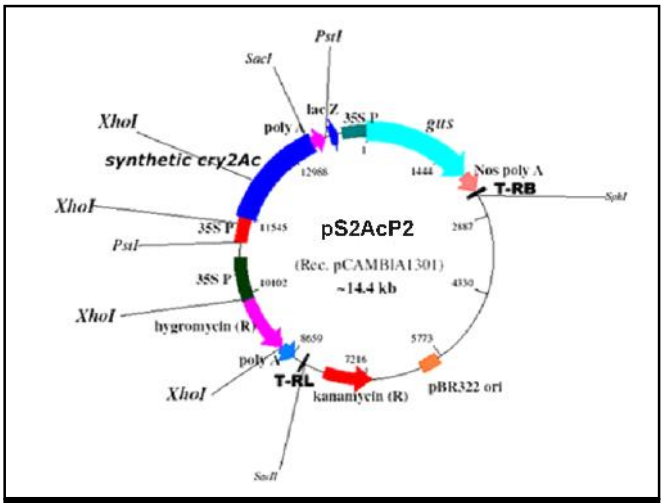

Fig. 1 : Physical map of pS2AcP2 harbouring *cry2Ac* gene

from this overnight grown culture was inoculated into 30 ml of LB broth and was grown at 37^{0}C with shaking at 200 rpm until 0.4-0.5 OD_{600} growth. The cell suspension was maintained at 0^{0}C for 20 min by keeping on ice. Then, the cells were harvested by centrifugation at 5000 rpm for 10 min at 4^{0}C followed by resuspension in 10 ml of sterile ice cold 50 mM $CaCl_2$ and incubated on ice for 20 min. The cells were again centrifuged at 5000 rpm for 10 min at 4^{0}C. The supernatant was discarded and the pellet resuspended in 2 ml of sterile ice cold 100 mM $CaCl_2$ and used immediately for transformation.

Bacterial transformation :

An aliquot of 100 ng of total DNA purified from *Agrobacterium* LBA4404 (pS_2AcP_2) was added to 100 µl of DH5α competent cell suspension. The mixture was incubated on ice for 30 min. It was then subjected to a heat shock at 42^{0}C for 90 sec and immediately returned to ice and kept as such for 30 min. This transformed bacterial cell suspension was then grown in 1 ml of LB broth for 1 h at 37^{0}C in a rotary shaker set at 200 rpm. After incubation, 100 µl of cell suspension was plated on LB medium with kanamycin (50 mg/l) and the plate incubated overnight at 37^{0}C for the colonies to develop.

Isolation of plasmid :

Alkali lysis method was used for isolation of plasmid from back transformed DH5α cells as was described by Sambrook *et al*. (1989). Single colonies were picked and grown in 3 ml of LB broth with kanamycin (50 mg/l) for 16 h at 37^{0}C and centrifuged at 10000 rpm for 2 min at 4^{0}C. The supernatant was discarded and the cells were resuspended in 100 µl TES (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.9% sucrose and kept on ice for 5 min. To the resuspended cells, 200 µl of lysis solution (0.1N NaOH + 1.0% SDS) was added, mixed well and kept on ice for 5 min. After lysis, 150 µl of neutralization solution (1.32 M sodium acetate pH 4.8-5.2) was added, mixed well by gentle inversion and kept on ice for 5 min. The supernatant was taken by centrifugation at 12000 rpm for 10 min and 300 µl of cold isopropanol added to precipitate the plasmid DNA. Centrifugation was done for 5 min at 12000 rpm and the supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol and air-dried. It was then dissolved in a minimal quantity of 0.1X TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and stored at -20^{0}C for further use.

Restriction digestion of DNA :

Restriction digestion of plasmid DNA was done as per the standard procedures (Sambrook *et al.*, 1989) using restriction endonuclease *Hin*dIII, *Sac*I, *Pst*I, *Xho*I (MBA, Fermentas) in an appropriate buffer at 37^{0}C for 1 h. The digested products were analyzed on a 0.8% agarose gel.

PCR analysis :

PCR was performed with plasmid DNA isolated from both DH5α and back-transformed DH5α cells using *gusA, hph, cry2Ac* specific primers as described by Sambrook *et al*. (1989).

hph gene :

PCR analysis was carried out using 1µl (100 ng) plasmid DNA in a 25 µl reaction mixture containing 2.5 µl of 10X PCR buffer (50 mM Tris-HCl pH 8.8, 50 mM KCl and 1.5 mM $MgCl_2$), 200 µM of each dNTPs, 1 µl of each primer (forward and reverse) and 2 units of *Taq* DNA polymerase. The primer sequences used for amplification of *hph* gene were as follows,

Forward primer:

H1 (5' GATCTCCAATCTGCGGGATC 3')

Reverse primer:

H3 (5' ACTCACCGCGACGTCTGTCG 3')

The hygromycin sequence in total DNA was amplified in a PTC-100 minicycler (MJ research, USA) with following temperature conditions, pre-incubation at 94^{0}C for 3 min, leading to 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 55^{0}C for 1 min and synthesis at 72^{0}C for 1 min, followed by extension at 72^{0}C for 5 min. Amplified PCR product (10 µl) was subjected to electrophoresis on a 0.8% agarose gel and visualized under UV light.

gusA :

A forward primer G1 (5' GGTGGGAAAGCGC-GTTACAAG 3') and a reverse primer G2 (5' GTTTACGCGTTGCTTCCGCCA 3') were used to amplify a *gusA* gene. Temperature profile used for amplification was as follows: pre-incubation at 94^{0}C for 5 min leading to 40 cycles of melting at 94^{0}C for 45 s, annealing at 62^{0}C for 1 min and synthesis at 72^{0}C for 1 min followed by extension at 72^{0}C for 5 min. Ten microlitres of the amplified product were used for electrophoretic analysis on 0.8 % agarose gels.

cry2Ac :

A forward primer (5' ATGAACACCGT-GCTCAACAAC 3') and a reverse primer (5'TGGTACTTGAAGAGGGACCAG 3') were used to amplify a *cry2Ac* gene. Temperature profile used for amplification was as follows: pre-incubation at 94^{0}C for 2 min leading to 29 cycles of melting at 94^{0}C for 40 s, annealing at 64^{0}C for 45s and synthesis at 72^{0}C for 1 min followed by extension at 72^{0}C for 7 min. Ten microlitres of the amplified product were used for electrophoretic analysis on 0.8 % agarose gels.

Agarose gel electrophoresis :

Required amount of agarose was weighed (0.8% w/v) and melted in 1x TBE buffer (0.9 M Tris borate, 0.002M EDTA, pH 8.2). Ethidium bromide was added at a final concentration of 0.5 µg/ml of gel. After cooling to 50-55^{0}C, the mixture was poured onto a preset template with an appropriate comb. The comb was removed after solidification and the gel with template was placed in an electrophoresis chamber containing the running buffer (1x TBE). DNA to be analyzed was mixed with the gel-loading dye (6x dye contains 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) at 5:1 ratio and loaded into the well. Electrophoresis was carried out at 50V (Sambrook *et al.*, 1989).

Agrobacterium-mediated transformation :

Rice genotypes:

Two local elite rice cultivars namely IR64 and ASD16 (obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore) were used in the present investigation.

Callus induction :

Scutellum-derived embryogenic calli were used as explants (IR64 and ASD16) in the transformation experiments. Manually dehusked seeds were surface sterilized with 70% ethanol for 3 min followed by 0.1% $HgCl_2$ for 5 min and washed thrice with sterile distilled water. Sterilized seeds were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2, 4-D (2.5 mg/l), maltose (30g/l), myoinositol (100mg/l), phytagel (4 g/l) and incubated in dark at 25^0 for 16 days for callus induction (Plate 1a). After 2 wk, the proliferating calli were dissected out and subcultured on to the same medium, 4 d prior to cocultivation (Plate 1b). Fast growing nodular, healthy looking embryogenic calli were used for cocultivation.

Cocultivation :

Agrobacterium strain LBA4404 (pS2AcP2) was streaked onto AB (Chilton *et al*., 1974) agar plate supplemented with rifampicin (10 mg/l), kanamycin (100mg/l) and tetracycline (5mg/l) and grown at 28^{0}C in the dark for 2-3 d. Three millilitre culture of bacteria were grown overnight at 28^{0}C in a rotary shaker set at 200 rpm. From overnight culture, 0.5 ml was transferred to 30 ml of AB broth containing rifampicin (10 mg/l) and kanamycin (100 mg/l) and tetracycline (5mg/l). The culture was grown overnight and bacteria were collected by centrifugation at 4000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 30 ml of AAM medium (Hiei *et al*., 1994) containing 100 µM acetosyringone. Embryogenic calli were immersed in the bacterial suspension for 5-10 min. After immersion, excess bacterial suspension was removed by placing calli on sterile tissue paper and the calli were transferred to an MS cocultivation medium (MS medium supplemented with 30 g/l sucrose, 10 g/l glucose, 700 mg/l casein hydrolysate, 100 µM acetosyringone, 3.5 g/l phytagel, pH 5.6) (Plate 1c) overlaid with Whatman No.1 filter paper (wetted with 1.0 ml of AAM medium containing 100 µM acetosyringone) and incubated at 25^{0}C in dark for 3 d (Rashid *et al*., 1996).

Selection and regeneration :

After cocultivation, calli were washed thoroughly in sterile distilled water followed by two washes in sterile water containing 500 mg/l cefotaxime (Nicolas-Piramal, India) and placed on a selection medium (MS callus induction medium supplemented with 500 mg/l cefotaxime and 50 mg/l hygromycin B) (Plate 1d). After 3 weeks of culture, proliferating calli were subcultured onto a fresh selection medium. Proliferating calli, that survived 3 rounds of selection each at 2 week's interval (Plate 1e) were transferred to regeneration medium (Plate 1f) (MS basal medium with 3 mg/l BAP and 1.0 mg/l NAA) and incubated at 25^0 C under 16 h photoperiod cycle (Datta *et al*., 1992). The emerging shoot buds were transferred

to half MS medium containing hygromycin B (30 mg/l) for rooting (Plate 1g). The rooted plants were transferred to soil and maintained in transgenic greenhouse (Plate 1h).

Particle bombardment mediated transformation :

All bombardment experiments were conducted using Biolistic gun, Model PDS-1000/He system (BioRad Laboratories, Hercules, USA) (Plate 2c). Vector pS_2AcP_2 harbouring *cry2Ac* gene driven by *CaMV35S* promoter, hygromycin resistant gene (*hph*) as plant selectable marker and *gusA* gene as reporter gene was used for coating gold particles.

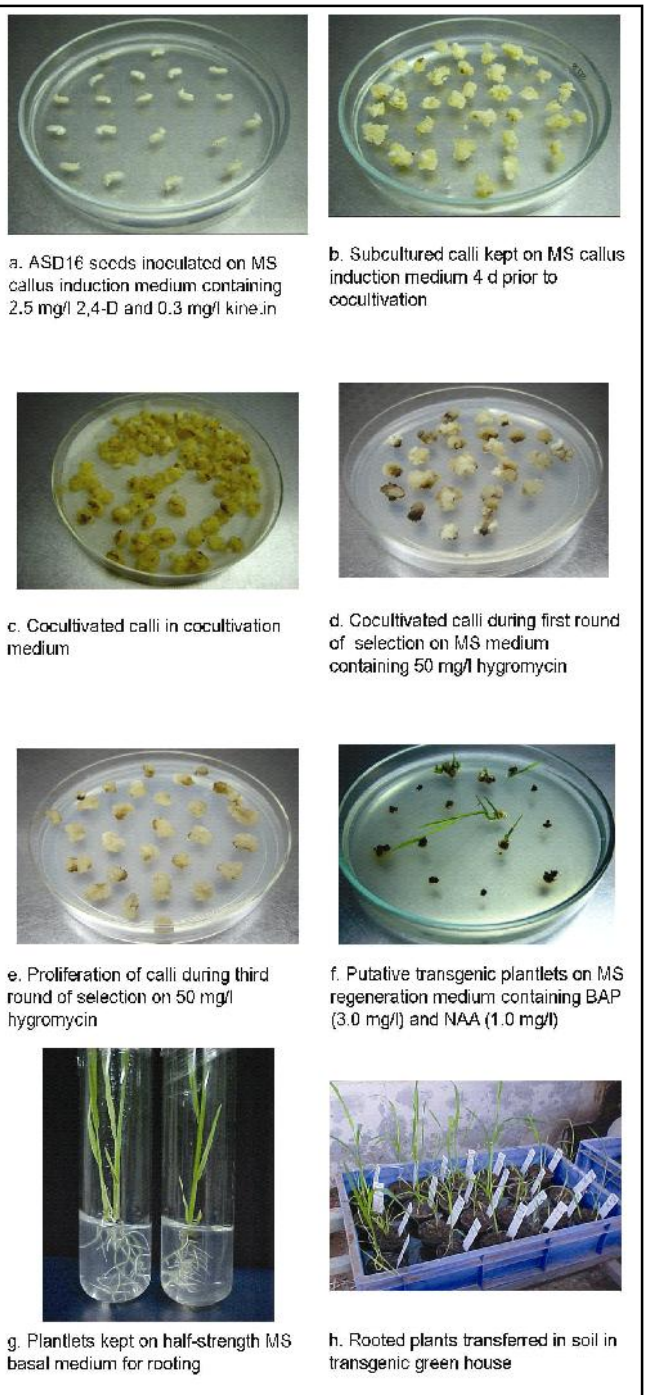

Plate 1:Agrobacterium-mediated transformation using mature seed derived calli of ASD16 with cry2AC gene

Coating gold particles with plasmid DNA :

Gold particles of size 0.9 µm dia (BioRad, USA) were used as microcarriers to deliver the genes harboured on the plasmids into target tissues. Ten micrograms of pS2AcP2 was used for coating 5 mg of gold particles as per the manufacturer's instructions.

Preparation of explants :

Immature seeds of IR64 and ASD16 were collected 12-14 d after pollination. After the removal of glumes, immature seeds were surface-sterilized with 70% ethanol for one minute and 0.1% $HgCl_2$ for 5 min followed by three washes with sterile distilled water. Embryos were excised aseptically under a microscope (Leica, Switzerland) and incubated with their scutellar region facing up for 2 d in dark at 25±2^{0}C on CC callus proliferation (CC medium containing 2 mg/l 2,4-D) medium (Plate 2a) (Potrykus *et al*., 1979)

Bombardment :

Four hours prior to bombardment, precultured immature embryos were transferred to CC osmoticum medium supplemented with mannitol and sorbitol, each at 36.4 g/l. The embryos were arranged in a 10 cm dia circle at the centre of the Petriplate in such a way that their scutellar region was facing up (Plate 2b). About 16 µl of gold suspension (suspended in 99.9% v/v ethyl alcohol) was placed at the centre of the macrocarrier and allowed to dry for 2-3 min. Then, the explants were bombarded twice using rupture discs with 1100 pounds per square inch (psi) specification at 25 inch of Hg vaccum at an interval of 4 h. Four hours after the second bombardment, immature embryos were transferred to CC proliferation medium and incubated in dark at 25±2^{0}C for 2 d (Plate 2d).

Transient GUS expression :

Transient GUS assay was carried with a few randomly selected embryos 48 h after second bombardment. The embryos were incubated overnight in X-Gluc staining solution at 37^{0}C.

Selection on hygromycin B :

Two days after bombardment, immature embryos were transferred to selection medium (CC proliferation

medium containing 50 mg/l hygromycin B) and incubated in dark at 25± 2^{0}C. The germinating shoots of bombarded embryos were removed after 2-3 d of culturing in the selection plate (Plate 2e). After 2 wk, the calli on selection medium were subcultured onto a fresh selection medium containing 30 mg/l hygromycin B. Surviving embryogenic calli were subcultured thrice on 30 mg/l hygromycin B at 2 wk intervals (Plate 2f).

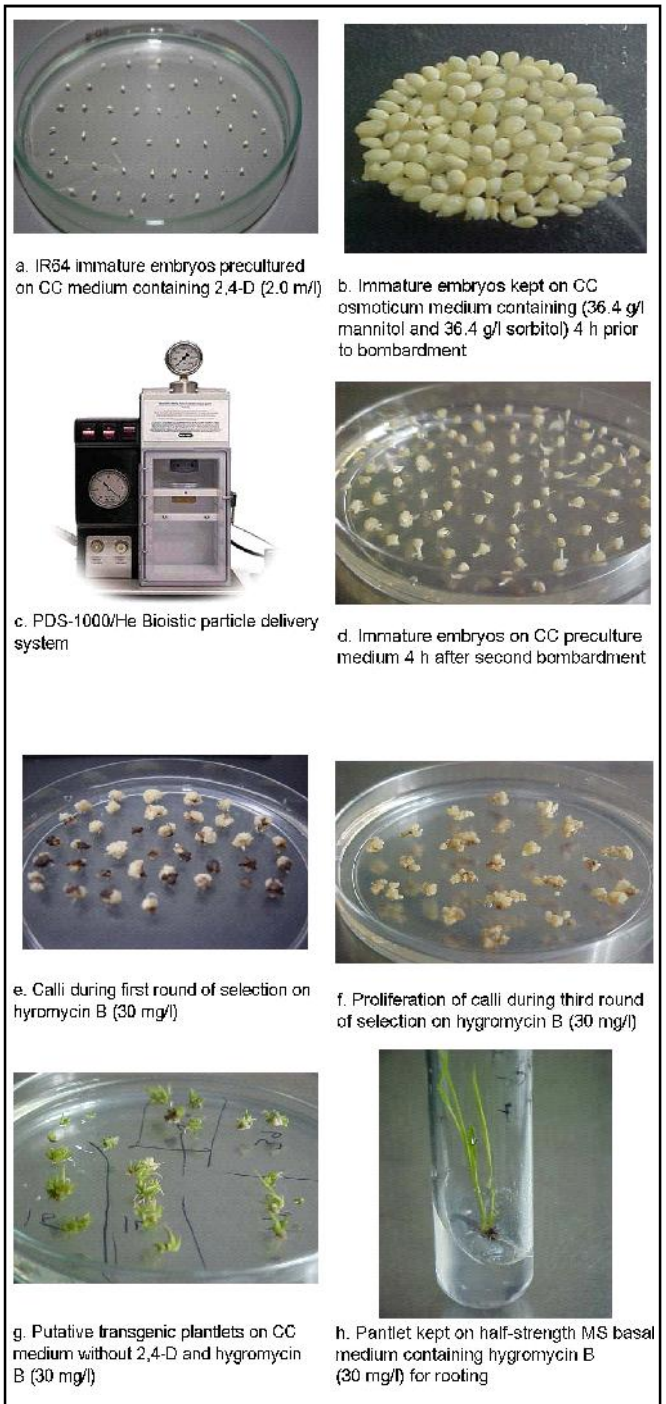

Plate 2 : Biolistic mediated transformation using immature zygotic embryos of IR64 with cry2AC gene

Regeneration of putative transgenic plants :

Established embryogenic calli derived from immature embryos after 3 rounds of selection were transferred to CC basal medium with 30 mg/l hygromycin B and incubated at 25±2^{0}C with a photoperiod of 16 h for the regeneration of shoots (3-4 wk) (Plate 2g). The emerging shoot buds were transferred to half strength MS medium containing hygromycin B (30 mg/l) for rooting (Plate 2h). The rooted plants were transferred to soil and maintained in transgenic greenhouse.

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been presented under following heads:

Back-transformation of E.coli DH5~ :

The plasmid isolated from the back transformed DH5á, on digestion with the enzyme *Hin*dIII, produced a single band (linearised plasmid). Digestion with *Sac*I resulted in the release of a band (2.5 kbp) corresponding to the *CaMV35S* promoter and *cry2Ac* coding sequence. Digestion with *Pst*I resulted in a 2.6 kbp fragment corresponding to the gene expression cassette of *cry2Ac*. The digestion with *Xho*I resulted in release of three bands (1.5, 1.1 and 0.7 kbp) as expected (Plate 3).

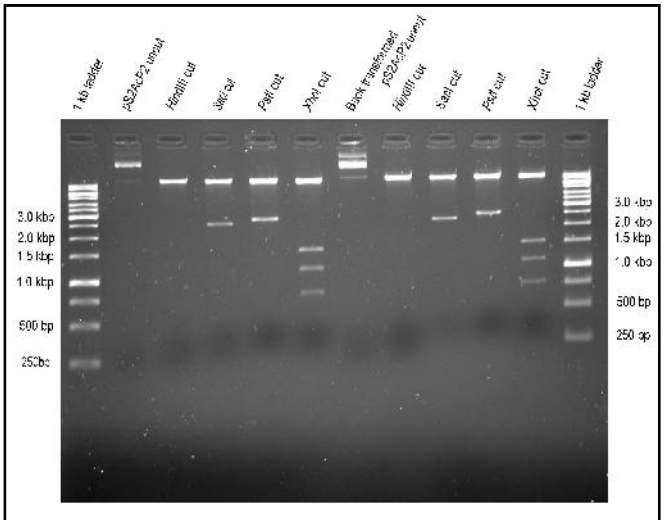

Plate 3 : Restriction digestion analysis of plasmid DNA (pS2AcP2) isolated from back transformed *E. coli*

PCR analysis

hph gene

The analysis showed an amplification of a 955 bp long internal sequence of *hph* gene in the plasmid isolated from back-transformed DH5α cells and pS_2AcP_2 (Plate 4).

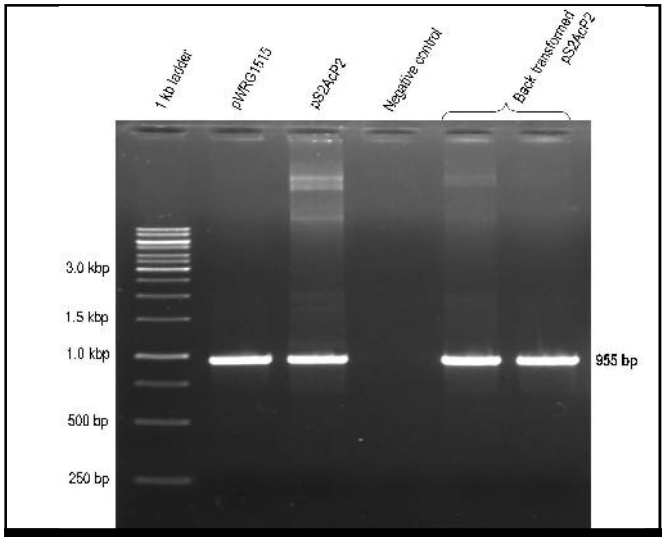

Plate 4 : PCR analysis of hph gene sequence in plasmid DNA (pS2AcP2) isolated from back transformed E. coli

gusA gene :

The analysis showed an amplification of a 639 bp-long internal sequence of *gusA* gene in the plasmid isolated from back-transformed DH5α cells and pS2AcP2 (Plate 5).

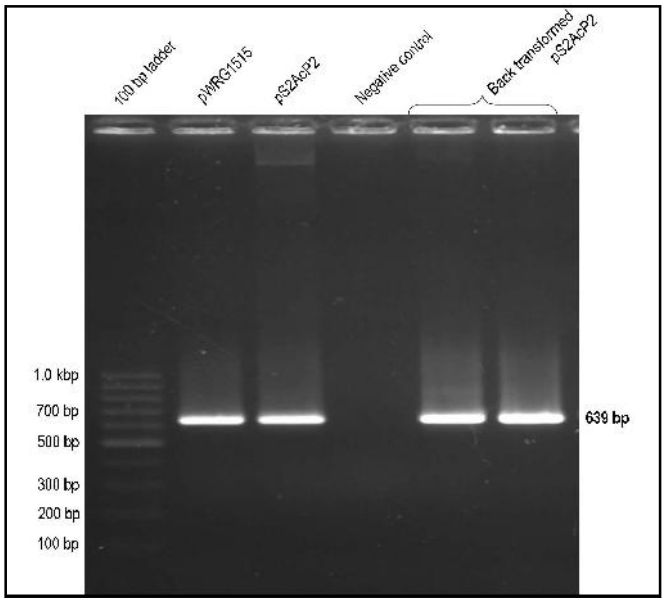

Plate 5 : PCR analysis of gusA gene sequence in plasmid DNA (pS2AcP2) isolated from back transformed *E. coli*

cry2Ac gene :

PCR analysis showed an amplification of 800 bp-long internal sequence of *cry2Ac* gene in the plasmid DNA isolated from back-transformed DH5α cells and pS2AcP2 (Plate 6).The results of the PCR analysis with plasmid DNA isolated from back-transformed DH5α using *hph*, *gusA* and *cry2Ac* gene-specific primers showed amplification of expected gene sequences. However, no amplification was observed in control plasmid used as negative control.

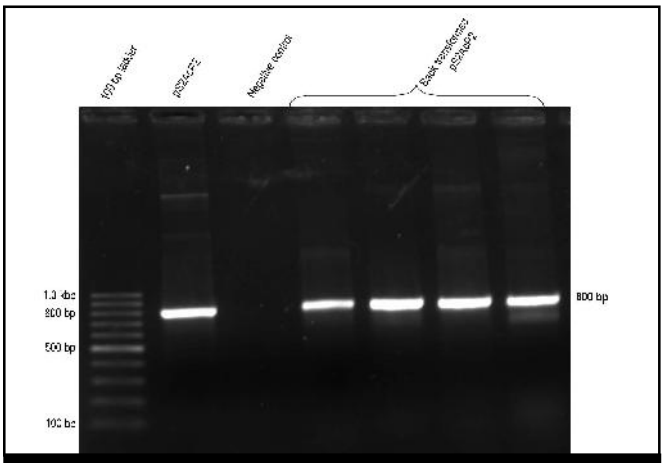

Plate 6 : PCR analysis of cry2Ac gene sequence in plasmid DNA (pS2AcP2) isolated from back transformed *E. coli*

Agrobacterium-mediated transformation :

Transformed embryogenic calli grew well on selection medium containing 50 mg/l hygromycin while, untransformed calli turned black and eventually dried. After three rounds of selection, each at 2 wk intervals, proliferating and healthy embryogenic calli were transferred to regeneration medium for shoot induction. At the end of three rounds of hygromycin selection, 14 calli lines in ASD16 and 22 in IR64 were obtained. Two of the ASD16 calli lines regenerated on regeneration medium with a regeneration efficiency of 1.1% (Table 1). None of the IR64 calli regenerated into plants.

Particle bombardment mediated transformation :

Particle bombardment mediated transformation using immature embryos :

After 48 h of bombardment, a few embryos were incubated in X-Gluc solution for transient GUS expression (Plate 7). The embryos exhibited transient GUS

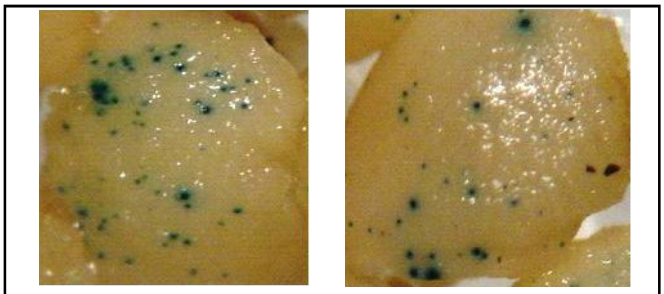

Plate 7 : Transient GUS expression in immature zygotic embryos 2 days after second bombardment

Table 1 : *Agrobacterium*-mediated transformation using mature seed derived calli of ASD16 and IR64 with *cry2Ac* gene

Variety	Experiment no	No. of explants cocultivated	No. of lines survived after third selection	No. of lines regenerated	Regeneration efficiency (%)	No. of positive lines		Transformation efficiency (%)
						hph	GOI	
ASD16	1.	120	-	-	-	-	-	-
	2.	57	-	-	-	-	-	-
	3.	57	-	-	-	-	-	-
	4.	150	6	1	0.6	-	-	-
	5.	65	8	1	1.6	-	-	-
	6.	44	-	-	-	-	-	-
	7.	65	-	-	-	-	-	-
	8.	41	-	-	-	-	-	-
IR64	1.	150	-	-	-	-	-	-
	2.	64	-	-	-	-	-	-
	3.	53	-	-	-	-	-	-
	4.	68	-	-	-	-	-	-
	5.	154	22	-	-	-	-	-

Table 2 : Particle bombardment mediated transformation using immature embryos of ASD16 and IR64 with *cry2Ac* gene

Variety	Experiment no.	No. of explants bombarded	No. of lines survived after third selection	No. of lines regenerated	Regeneration efficiency (%)	No. of positive lines		Transformation efficiency (%)
						hph	GOI	
IR64	1.	84	11	6	7.14	-	-	-
	2.	82	31	4	4.87	-	-	-
	3.	80	38	5	6.25	-	-	-
	4.	67	16	-	-	-	-	-
	5.	70	12	-	-	-	-	-
ASD16	1.	78	-	-	-	-	-	-
	2.	104	-	-	-	-	-	-
	3.	107	-	-	-	-	-	-
	4.	110	-	-	-	-	-	-
	5.	85	-	-	-	-	-	-
	6.	180	35	-	-	-	-	-
	7.	60	16	-	-	-	-	-

expression on an overnight incubation at 37^{0}C. Hygromycin resistant embryogenic calli on selection media grew well, whereas untransformed calli turned necrotic and later dried. At the end of three rounds of hygromycin selection, 108 calli lines in IR64 and 51 in

ASD16 were obtained (Table 2). Some of these calli showed greening on regeneration medium. A total of fifteen putative transgenic lines were recovered in IR64 with a regeneration frequency of 6%. A few batches of bombarded calli are in regeneration stage at the time writing this report.

This crop is a major target in crop improvement programmes. Biotic and abiotic stresses are the major constraints for rice production. Among insect pests, lepidopterons are the major ones in rice producing regions, for which about 50% of insecticidal sprays are targeted in Asian countries (Heong *et al.*, 1994).

As the cryptic habits of stemborers protect them from insecticidal sprays, making careful timing of systemic insecticide applications at high rates necessary for effective control. Resistance development to the major classes of insecticides in rice stemborers further decreased their efficacy in rice fields (Jiang *et al.*, 2005; Li *et al.*, 2001; Chi *et al.*, 2005; Peng *et al.*, 2001). Use of toxic chemicals not only increases the rice production cost but also causes health hazards to rice farmers as well as deteriorates the rice field environment (Pingali and Roger, 1995). Biological control of insects is more popular as it has several advantages over the chemical pesticides. Among various biocontrol agents, *B. thuringiensis* (Bt) offers greater scope for controlling insect pests. The development of transgenic crops that

produce Bt cry proteins has been a major break through in the substitution of chemical insecticides by environmental friendly alternatives. In transgenic plants the cry toxin is produced continuously, protecting the toxin from degradation and making it reachable to chewing and boring insects.

Application of genetic engineering to transform rice plants with agronomically useful genes has become an important component of rice improvement programmes. Genetic manipulation has several advantages over conventional plant breeding methods. It overcomes the incompatibility barriers among different crop species, a major limiting factor in any breeding programme. Considerable studies and field trails of Bt rice have been reported since 1993 (Fujimoto *et al.*, 1993; Wunn *et al.*, 1996; Ghareyazie *et al.*, 1997; Nayak *et al.*, 1997; Wu *et al.*, 1997; Cheng *et al.*, 1998; Datta *et al.*, 1998; Maqbool *et al.*, 1998; Alam *et al.*, 1999, Tu *et al.*, 2000, Maqbool *et al.*, 2001, Ye *et al.*, 2001, Khanna and Raina 2002; Bashir *et al.*, 2004; Ramesh *et al.*, 2004; Chen *et al.*, 2005; Tang *et al.*, 2006). Overall, *cry1Ab, cry1Ac, cry1Ab/c* (fusion gene), *cry1C* and *cry2A* have been transformed and evaluated in transgenic rice. Bt rice was released in Iran in 2005 for commercial cultivation (James, 2005).

Extensive greenhouse and field studies documented that most of the Bt rice lines could effectively control the target lepidopteran pests and reduce chemical insecticide application both in China and some other countries, with important benefits to environmental quality and the health of rice growers and consumers. Ecological risk assessments of insect-resistant transgenic rice confirmed that transgenic Bt rice has no markedly negative or unintended impact on non-target organisms, compared with non-transgenic rice, and is safer than chemical insecticides (Chen *et al.*, 2006). Since 1996 when the first insect resistant crop was released, the farmers planting GM crops have reduced pesticide inputs in their fields by 6.3 per cent or over 172.5 million kg which led to an overall reduction in the environmental effects of pesticides by 14% (Brookes and Barfoot, 2005).

The introduction of cloned genes governing traits of interest into plant cells could be achieved either by direct DNA transfer (particle bombardment) or *Agrobacterium*-mediated gene transfer methods. In the present study, *cry2Ac* gene was used to transform two local elite cultivars, IR64 and ASD16 by both *Agrobacterium* and particle bombardment mediated methods with a view to imparting resistance against major lepidopteron pests such as yellow stemborer and leaffolder.

Agrobacterium-mediated transformation of target genotypes IR64 and ASD16 mature seed derived calli with the construct harbouring *cry2Ac* under the control of *CaMV35S* promoter resulted in the generation of 14 callus lines in ASD16 and 22 in IR64 were obtained after three round selections. Two putative transgenic ASD16 lines were recovered.

In yet another study, immature embryos of ASD16 and IR64 were transformed with pS2AcP2 construct using particle bombardment. The transient GUS expression in the calli 48 h after bombardment has demonstrated the efficiency of the system in delivering gene constructs into cells. In this experiment, 108 independent callus lines in IR64, 51 callus lines of ASD16 were obtained after three rounds of selection on hygromycin. These callus lines when transferred to regeneration medium, 15 lines were recovered in IR64 and none in ASD16.

Though several methods of genetic transformation of rice have been developed, particle bombardment and *Agrobacterium* mediated methods are widely used methods for transformation of rice. There is, however, an undercurrent of feeling that *indica* rice is recalcitrant to high efficiency transformation and some genotypes could not be tamed by employing common protocols. This entails search for better transformation protocols particularly for *indica* rice (Bajaj and Mohanty, 2005). There are several reports on transformation of recalcitrant *indica* rice cultivars, for example. 1R64 and IR72 through *Agrobacterium* (Datta *et al*., 2001; Khanna and Raina, 2002; Tu *et al*., 1998; Aldemita and Hodges, 1996) with a relatively low frequency of transformation. This problem could be overcome by developing genotype specific protocols to obtain high transformation frequency. Alternatively, by increasing the number of target tissues, more number of transgenic plants could be generated.

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