Agrobacterium-Mediated Genetic Transformation Involving Hygromycin Phosphotransferase to Impart Herbicide Tolerance in Indica Rice

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Abstract: - **Hygromycin phosphotransferase (*hpt*) gene has been successfully intruded in an indica rice variety-IET 13856 through *Agrobacterium*-mediated genetic transformation to impart herbicide-tolerance. Transgenic plants were regenerated from *in vitro* developed embryogenic calli of IET 13856 through co-cultivation with *Agrobacterium tumifaciens* strain LBA 4404 (pTOK 233). The *hpt* gene was housed under the transcriptional control of CaMV 35S promoter and Nos polyadenylation signal in the super binary vector pTOK233. This protocol of *Agrobacterium* -mediated genetic transformation was found to be very efficient as evident from high (51.4% transient and 13.51% stable) transformation efficiency. Transient expression was confirmed through GUS histochemical assay, while stable transgene integration at the genomic level was confirmed by PCR, slot blot and Southern blot hybridization. Inheritance of the transgene was worked out through seed germination test of the seeds at T_1 generation on hygromycin supplemented MS medium. It showed typical Mendelian ratio for monogenic inheritance (dominant transgene) in the progenies of maximum transgenic lines except a few deviations, which might be due to epistatic interactions or small sample size (less number of seeds used in seed germination test). This study prospects amply, use of transgenic indica rice harbouring *hpt* to enhance productivity in rice by saving the crops from weed menace in Andaman and Nicobar Islands, India, endowed with prolonged high rain fall with rampant weed infestation and other rice growing areas elsewhere.**

Key words: Agrobacterium-**mediated genetic transformation, GUS, Hygromycin phosphotransferase, Glyphosate, Indica rice, Andaman and Nicobar Islands.**

I. INTRODUCTION

Rice is the most important life- line cereal crop provides staple food to about two third populations globally and 90% of this crop is grown and consumed in Asia [1, 2]. Among all rice varieties, indica rice feed more than 2 billion people predominantly in the developing countries and encompasses 80% of cultivated rice in the world [3]. Conventional breeding made significant contribution in developing a large number of highly productive varieties worldwide to ensure food and nutritional security [4] albeit the approaches are tedious, time consuming and often misfires. Weed is serious constraints in rice growing areas especially in high rainfall low lying areas, which must be managed to save heavy yield loss occurring recurrently. At the interface of discrete adverse climatic change, acute man power scarcity, bunch of anthropogenic reasons etc. diverse herbicides are being used globally to make crop husbandry more remunerative, albeit their use especially the residual effect to the environment is a serious concern.

This major concern warrants development of weedicide resistant crops and fortunately a large of such genes has been identified and herbicide resistant crops produced, which are cultivating on large-scale worldwide [5] especially in the developed countries for commercial purpose. Keeping all those in purview we launched a programme to develop transgenic lines involving *hpt* gene in well adaptive high yielding background of IET 13856 to make rice husbandry more remunerative in rice- deficit union territory of Andman & Nicobar Islands, India by combating weed menace. It is to be mentioned that in addition to conventional breeding, modern plant biotechnology and genetic engineering offers ample scope to genetically modulate crop plants especially in enhancing productivity, value addition and quality enhancement with precision, ease and confidence [6, 7, 8]. Genetic transformation is one of the most lucrative avenues for dispensing alien gene/s from heterologous sources to a recipient system by overcoming transsexual barrier for genetic improvement in rice. In India, ~ 44 million ha is cultivated with rice and provides substantial life- line support to its burgeoning population [28]. More so, population is increasing day by day; therefore, food production needs to be increased proportionately. By 2030 AD, rice production need to be

doubled for our food and nutritional security [9, 8]. Explicitly, Andaman and Nicobar Island, a union territory of Indian Republic, where rice is the principal cereal crop for livelihood albeit with exceedingly low productivity (~ 2.1 tons/ha). These islands are endowed with heavy rainfall amounting ~ 3300 mm, distributed over eight months may easily produce two rice crops consecutively. Weeds are diverse, rampant and making heavy dent in annual rice productivity [10], which is being managed to some extent by using herbicides in this labourer scarce island. This warrants cultivation of herbicide tolerant varieties well-fit to this unique agro-ecological niche. It is to be mentioned that to facilitate growth of HYVs, weed free clean husbandry is indispensable in which herbicide application is inevitable [11]. Use of herbicide has increased many times during the last 30 years [12]. Glyphosate [N-(phosphonomethyl glycine)], a non-selective broad spectrum highly efficient herbicide plays spectaculr role in weed management with minimal environmental risks [13]. In the past, rice improvement programmes for higher productivity along with herbicide tolerance were largely centred to save huge labour cost spent for weeding for remunerative crop husbandry. Cultivation of transgenic crops harbouring hygromycin phosphotransferase (*hpt*) gene in indica rice varieties has been found to be rapid and impressive worldwide and steadily continuing at commercial- scale [14, 15, 16]. This technology was found to be more environmental benign and showed more stability to receiver plant genome as compared to other weed management practices [17]. *Agrobacterium*-mediated genetic transformation has been proved to be useful for integration of the transgene to the recipient plant genome especially for developing transgenic lines with low copy numbers preferably single copy in one locus [18]. Therefore, the present experiment was designed to develop transgenic indica rice harbouring *hpt* gene to impart tolerance to antibiotic (hygromycin B) as well as herbicides (glyphosate; up to 3 mM, which is sold in trade name Round up marketed by Monsanto USA/ Canada/India) in a popular indica rice- IET 13856 from Andaman and Nicobar Island by using *Agrobacterium* strain LBA 4404 (pTOK 233).

II. MATERIALS AND METHODS

To impart herbicide resistance in indica rice, a popular high yielding and well adaptive indica rice genotype viz. IET 13856 was obtained from Directorate of Rice Research Hyderabad, India. Mature healthy seeds were dehusked manually and were immersed in 5% aqueous Teepol (Camlab, Cat No: CL640-15) solution under continuous shaking for 5 minutes at 80 rpm (Remi, Model**:** R-4C). Seeds were thoroughly washed and surface sterilized with freshly prepared 0.1% $HgCl_2$ (Qualigens, Cat No. 15564) solution under laminar airflow cabinet [LAF (Klenzaids, Model: 1590-R-48-24-30)] for 10 minutes. Those seeds were washed thrice with sterile distilled water and blot dried with autoclaved tissue paper. Surface sterilized 20 seeds were cultured on callus induction medium (CIM) constituted of MS [19] containing 2 mg/l 2, 4-D. The pH of the medium was adjusted to 5.8 before autoclaving at 121^0C under 15 lbs/inch pressure for 15 min. 20 ml of molten medium was dispensed into rimless culture tubes (25x150mm Borosil Cat. No: 9820U08) and plugged with non-absorbent cotton caps wrapped in cheese cloth. Single seeds were cultured in each culture tube, closed with cotton plugs under LAF and were incubated in dark at 25 ± 2^0C. After 10 days, loose calli started appearing at the swollen junction of radical and mesocotyl. 10 days old calli were excised out from the germinating seedlings and placed on callus maintenance medium consisting of MS with half dose of 2, 4-D of CIM (1mg/l 2, 4-D). After 1 month, all calli were shifted to embryogenic induction medium (EIM) to develop highly embryogenic calli with large number of somatic embryos. EIM was made up of MS fortified with 0.1% casein hydrolysate (Sigma-Aldrich, Cat No: 91079-40-2), 1 mg/l kinetin (Sigma-Aldrich, Cat No: K-0753), 1 mg/l abscisic acid (Sigma-Aldrich, Cat No: 14375-45-2) following previous authors [20]. After 7 day of culture, somatic embryo masses were shifted onto regeneration medium (RM), made up of MS containing 1 mg/l kinetin (Sigma-Aldrich, Cat No: K-0753), 0.5 mg/l NAA (Sigma-Aldrich, Cat No: N-0640), 1 mg/l BAP (Sigma-Aldrich, Cat No: B-9395). Cultures were kept on regeneration medium under 16h light (130 Einstein/m^2/s) / 8h dark cycle.

Effect of callus age:

Since age of calli plays key role in governing transformation efficiency [21] so it's effect in influencing genetic transformation of IET 13856 was assessed by undertaking this experiment. Generally within 10 days, small calli were developed at the junction of mesocotyl and radical and subsequently used for subculture for 15 days were used for genetic transformation. Remaining calli were maintained for 30, 60 and 90 days with subcultures at 30 days interval and subsequently transformed with LBA 4404 (pTOK233) following the standard transformation protocol outlined by previous authors [22] and GUS expression was determined following previous authors [23] was used to determine transformation efficiency at transient level..

Organ specificity in governing Agrobacterium-mediated genetic transformation:

Mature healthy seeds were dehusked manually and immersed in 5% aqueous Teepol solution for 5 minutes at 80 rpm on a shaker (Remi, Model**:**R-4C). Seeds were thoroughly washed and surface sterilized under LAF with freshly prepared 0.1% $HgCl_2$ for 10 minutes and washed thrice with sterile distilled water. Seeds were used for development of calli from coleoptiles and root. Calli from anthers were also induced from glass house grown plants of the same variety following the standard protocol outlined by previous authors [24]. Fully developed calli grown in callus induction medium (CIM) were exercised from the surface of the coleoptiles and roots; placed on the callus maintenance medium whereas anther derived calli of same age were also used in similar way. All calli regardless sources were transformed with LBA 4404 (pTOK 233) following previous authors [22]. Root and coleoptile

derived calli were compared with anther and mature seed derived calli for comparative assessment of genetic transformation efficiency at transient level based on GUS expression [23].

Determination of generation time in Agrobacterium strain LBA 4404 (pTOK 233):

To facilitate efficient *Agrobacterium*-mediated genetic transformation, bacterial cells are generally used in active "log" growth phase. 20 ml of Agrobacterial suspension [strain LBA 4404 (pTOK 233)] was inoculated in AB medium [25] containing 50 mg/l kanamycin and 50 mg/l hygromycin (Sigma-Aldrich, Cat No: H0654) aseptically in 150 ml capacity Erlenmeyer Conical Flasks (Borosil, Cat No 4980). Flasks were shaked on a shaker (Remi, Model**:** R-4C) at 250 rpm at 28^0C. UV-vis spectrophotometer (Chemito 1200) was calibrated for 100% transmittance (0-absorbance) using un-inoculated control. Absorbance of sample bacterial suspension was measured spectrophotometrically at 600 nm. At the mid log phase, 1 ml of the inoculum at two definite timings were withdrawn. Following serial dilution procedure, spread plate were made by taking 50 µl of bacterial suspension in each dilution for plating out at 10^{-5} and 10^{-6} dilutions in a radio sterile petri plate (Tarsons , 30 mm dia) containing semi- solid agar medium. Cultures were incubated at 28^0C by keeping in inverted position. After 48 h colony forming units (CFU) were counted and the data was used for calculation of generation time for the strain LBA 4404.

Determination of minimum inhibitory concentration:

To remove *Agrobacterium* cells after co-cultivation, use of appropriate dose of antibiotics is essential in transgenic development process. Minimum concentration of the antibiotic at the edge of zone of inhibition halting bacterial growth denotes minimum inhibitory concentration (MIC) of an antibiotic. LBA4404 (pTOK233) was inoculated on AB agar plates, followed by preparing four circular wells of 5 mm diameter following standard bacteriological procedure. Subsequently those wells were filled with 10-50 µg/ml concentrations of cefotaxime (Sigma-Aldrich, Cat No: C7912) and after two days of incubation, the zone of inhibition on the plate was measured in metric units.

Optimization of hygromycin level for selection of transformants:

To detect the appropriate dose of hygromycin (Sigma-Aldrich, Cat No: H0654), for selection of transformed rice calli, an experiment involving MS medium supplemented with 0, 25, 50, 75 and 100 mg/l of hygromycin was undertaken. Twenty five pieces of embryogenic cell clumps (calli) after co-cultivation with *Agrobacterium* were transferred to those plates containing hygromycin supplemented medium along with untransformed control callus and grown for 10 days. Thereafter, number of calli survived and plantlet regeneration was recorded to calculate the appropriate dose of hygromycin in which transgenic plantlet regeneration was not affected and simultaneously non-transformed calli were also not allowed to escape for regeneration of false transgenic plantlets.

Optimization of glyphosate level for herbicide tolerance:

Level of glyphosate (Sigma-Aldrich, Cat no: 45521) tolerance was done through regeneration of plantlets from hygromycin resistant calli. 20 pieces of hygromycin resistant calli in each treatment were transferred on to MS medium containing 50 mg/l hygromycin (obtained from the previous experiment) and different levels of glyphosate (0, 1, 2, 3, 4 and 5 mM). Concurrently non-transformed (control) calli were also tested on similar MS medium containing different levels of glyphosate (0, 1, 2, 3, 4 and 5 mM) without hygromycin. After one month of culture at 28 ± 1^0C and photoperiod of 16/8 h light/dark, the growth was evaluated [26, 13] to determine the optimum level of glyphosate tolerance.

Rice Variety, Bacterial Strains and Plasmids:

In the present experiment, transformation experiments were performed involving an indica rice var. IET 13856 using A *tumefaciens* strain LBA4404 harbouring the super binary vector pTOK233 [27], which contained *npt, hpt* and *intron-gus* gene [28] (kind gift from Prof.T. Komari, Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., 700 Higashibara, Toyoda, Iwata, Shizuoka 438-0802, Japan).

Agrobacterium-mediated genetic transformation methodology:

Agrobacterium tumefaciens strain LBA 4404 (pTOK233) were grown for 2 days in LB medium with 50mg/l hygromycin (Sigma-Aldrich, Cat No: H0654) at 28^oC in a BOD shaker incubator (Lab-Line, Model: 3527) at 250 rpm. OD of the bacterial suspension was periodically checked using a UV-Vis spectrophotometer (Chemito, 1200). Then, the bacterial cells were resuspended in the induction medium when OD reached to 1 at 600 nm [29]. Resuspended medium was prepared with MS salts, 12.5 mM phosphate buffer, (Sigma-Aldrich, Cat No: P381), 3% sucrose (Sigma-Aldrich, Cat No: S038) 4µM BAP (Sigma-Aldrich, Cat No: B-9395), 0.5 µM NAA (Sigma-Aldrich, Cat No: N-0640), 0.1 mg/l nicotinic acid (Sigma-Aldrich, Cat No:59-67-6), 1 mg/l thiamine HCl (Sigma-Aldrich, Cat No: T1270), 10 mg/l myoinositol (Sigma-Aldrich, Cat No: I5125) and acetosyringone 100 µM (Sigma-Aldrich, Cat. No: D134406), pH of the medium was adjusted to 5.5. Embryogenic calli of IET 13856 (freshly subcultured; 7d - old) were taken out from the culture tubes and immersed in *Agrobacterium* suspension for 10 min under LAF. Those were blot dried with sterile tissue papers to remove excess bacterial cells and were transferred onto semi-solid MS medium containing 50 mg/l hygromycin. Those calli were incubated at 25 ± 2^0C for 3 days for co- cultivation with *Agrobacterium* cells. After 3 days, the calli were rinsed thoroughly in filter sterile 200 mg/l cefotaxime (Sigma-Aldrich, Cat No: C7912) followed by 5-6 washes in sterile distilled water. Calli were further blot dried on sterile tissue paper and transferred onto MS medium

containing 250 mg/l cefotaxime and 50 mg/l hygromycin. Survivor calli were selected and transferred onto regeneration medium containing ½ MS basal with 3% sucrose, 0.5 mg/l BAP, 1 mg/l kinetin, 0.5 mg/l NAA, 50 mg/l hygromycin and 250 mg /l cefotaxime at 25±2^{0}C under 16 h light provided by 4′ long tube lights (Philips make) (intensity 130 μ Einstein /m^2/s) /8 h dark cycle. Regenerated plantlets were transferred to culture tubes containing Hoagland solution (pH 5.5) [30] for 5 days to acclimatize the plants and finally shifted to small plastic pots containing autoclaved soil covered with polyethylene bags to maintain desirable relative humidity during hardening in the glass house. When the survivor plants attain a height of 12-15 cm, those were shifted to big cement pots (1′ X 1′ X 1.5′) filled with soil and kept in the Transgenic Rearing Facility (Category II, DBT approved) and were allowed to grow under standard agronomic practices till maturity. During anthesis, the panicles were covered with butter paper bags to prevent pollen escape and cross pollination. Seeds were collected and kept separately plant wise and T_1 generation was raised in the succeeding season to monitor transgene segregation and to assess transgenic status through different physiological and biochemical assays

GUS Histochemical Assay:

Expression of the transgene in putative transformed calli and different plant parts of the primary regenerants was assayed following previous authors [23]. Transformed calli were incubated for 1 h at 37^{0}C in 50mM phosphate buffer (pH 7.2) containing 1% Triton-X (Sigma-Aldrich, Cat No: MFCD00128254). Buffer was removed and fresh buffer containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronidase) [Sigma-Aldrich, Cat No: B6650] and 20% methanol (Bengal Chemical) was added and incubated overnight at 37^{0}C. Following day, the experimental tissues were examined and blue foci/stained area were counted / observed under stereomicroscope (Nikon make, Model MSZ-10 DIA STAND). Expression of GUS stained calli were photographed as evidences of transient expression of *gus* gene.

Extraction of Genomic DNA:

Extraction of genomic DNA from fresh leaf tissues of putative transgenic rice plants var. IET 13856 was made following CTAB method [31]. Extracted DNA was purified using RNase (Amersham Pharmacia Biotech Inc., USA, Cat. No: 27-0994-01) and quantified both through spectrophometrically (UV-Vis spectrophotometer, Chemito 1200) as well as through co-electrophoresis in a horizontal electrophoresis apparatus (Bio-Rad) using *Bam*HI- *Hind*III (Sigma-Aldrich, Cat No: D9780) cut λ DNA as molecular ruler. Gel was made by mixing ethidium bromide (EtBr) (BDH, Cat No 214-984-6) with 0.8% agarose. Lanes were loaded with genomic DNA along with the aforesaid λ marker (Sigma-Aldrich, Cat No: D9780) and electrophoresed at 100V in TAE buffer, pH 8.0 till the tracking dye reach the opposite end of the gel. Purified genomic DNA was used for PCR analysis slot blotting and Southern hybridization.

PCR analysis:

Transgene integration at DNA level was also detected by PCR analysis in which genomic DNA isolated from putative transformed calli and regenerated transgenic plantlets were used [32, 10]. Reaction mixture was containing 1 μg / 25 ng template DNA, 1mM dNTPs (Pharmacia Biotech, Cat No: 27-2035-01), 1 unit of Taq DNA polymerase (Amersham Cat No: 27-0799), 2mM $MgCl_2$, PCR buffer and 10 pmol of each specific primers (forward:5'-GCTGGGGCGTCGGTTTCCACTATCCG-3'and reverse: 5'-CGCATAACAGCGCTCATTGACTGGAGC-3') for amplification of the *hpt* gene. Thermal cycling was done for 1 min at 94^{0}C for denaturation, 1 min at 55^{0}C for annealing and chain extension step was performed for 2 min at 72 °C for 30 cycles (Bio-Rad, C1000 Touch™ Thermal Cycler). PCR products were electrophoresed in 0.8% agarose gel containing EtBr and obsereved under UV-vis transilluminator (Bio-Rad, SI No. 465 BR1056) and subsequently photographed using a gel documentation system (Bio-Rad, Gel Doc™ EZ System 1708270).

Slot blotting:

DNA from putative transformed embryogenic calli and primary regenerants as extracted above was also used for slot and Southern hybridization. Charged NYLON membrane (Pharmacia-Biotech, Cat No: 80-6221-93) was used as the principal matrix in this process. Membrane was cut and soaked in water and pre-treated in 10XSSC for 10 min, then placed in slot blot instrument (Hoefer make, Cat No: PR 648), which was connected with a vacuum pump. Before spotting, the DNA was denatured at 90^{0}C in precision water bath (Thermo Electron Corporation, Model No: 2854) for 10 min and snap quenched on ice for 10 min. After a brief centrifugation, 10μg of DNA from individual entries was spot on the slot blot instrument and the pump was adjusted to 25-50 mbar pressure till DNA was completely absorbed. Blot was removed from the instrument and treated with depurination solution [33] for 10 min. Membrane was air dried and cross linked under UV light facing the DNA side towards the UV light (Bio- Rad, GS Gene Linker UV Chamber, Cat No: 165-5031).

Southern Blotting and Hybridization:

Localization of specific transgene sequences in the recipient genomic DNA was done according to previous author [34]. Isolated genomic DNA from putative transgenic plants was digested with *SalI* (Sigma-Aldrich, Cat No, 10348783001) and the resulting fragments were fractionated on 1% agarose gel (Merck, Cat No. 1012360100) with EtBr in a horizontal electrophoresis set (Bio-Rad, Sub-Cell® Model 192 Cell) in TAE buffer (pH 7.8). The *hpt* gene measuring 778 bp was extracted from the gene construct pTOK 233 by using *Bam*HI (Sigma-Aldrich, Cat No, 10220612001) restriction enzyme.

25 ng *hpt* DNA was denatured *in situ* at 95-100^{0}C for 5 min in a boiling water bath, then it was added to the labelling mixture by using Rediprime DNA labelling system (Amersham Life Science, Cat No: 16331). Probe was labelled and mixed well by pipetting up and down for 5-6 times, subsequently spun and incubated at 37^{0}C for 10 min. Reaction was terminated through addition of 5µl of Na-EDTA. Labelled DNA was denatured by heating at 95-100^{0}C in water bath for 5 min followed by snap chilling on ice. In a hybridization bag (made up of thick polyethylene sheets) radio-labelled probe was added to the hybridization buffer and sealed properly to avoid leakage using a heat sealer. Utmost care was taken to add the probe solely to the hybridization solution (30 ml buffer) and not directly on to the NYLON membrane. Blot was left with gentle shaking in a water bath for 65^{0}C overnight. Subsequently the DNA attached to the membrane (Hybond H+, Pharmacia-Biotech, Cat No: 80-6221-93) was hybridized to radio labelled dCTP *hpt* probe (α^{32}P was obtained from Jonaki, BRIT, BARC, Hyderabad). Next day the membrane was washed with washing solution, covered in Saran wrap and exposed to X-ray film [KODAK, Cat No: 200X) at -86^{0}C facility in 6″ X 6″ cassette with intensifying screen and kept in deep freeze at – 20° C till the X- ray film displayed distinct bands.

Inheritance of transgene based on seed germination test:

Transgenic status was confirmed through GUS histochemical assay, PCR, slot and Southern analyses at T_0 generation involving putative primary transgenic plants. Seeds from T_0 generation plants were harvested after maturity plant wise, cleaned and kept individually. Those seeds were used for seed germination test to assess the incorporation, expression and transmission of transgene(s) over generation more stringently. Twenty five seeds were dehusked manually, cleaned and surface sterilized with aqueous Teepol solution (5%) and washed thrice. Those were further immersed in freshly prepared aqueous 0.1% $HgCl_2$ (Merck, Cat No: 104419) solution for 10 minutes and then thoroughly washed with autoclaved dH_2O four times under LAF. Twenty five surface sterilized seeds were cultured on seed germination medium containing ½ MS supplemented with 50 mg/l hygromycin for two weeks along with seeds obtained from untransformed (control) plants and kept in dark at 25±2ºC in the culture room. After 5 days, number of germinated seeds was recorded. Data was analysed through χ^2 test to unzip the mode of inheritance. After 15 days survivor seedlings were grown in soil in the Transgenic Rearing Facility till maturity for study of transgene behaviour in the succeeding generations.

III. RESULTS AND DISCUSSIONS

Weeds are big menace in any crop cultivation; incur huge yield loss annually worldwide. Those are conventionally controlled by various ways like mechanical tools viz. tillage, mowing, hand-weeding by applying chemicals like herbicides and to some extent by grazing animals *in natura* [35, 36, 37]. However, at the interface of acute manpower scarcity now-a-days in the agricultural sector, herbicides are widely used to avert weed problem although residual effect of herbicides is eco-offensive. Transgenic development and stitching of alien gene/s in a recipient system has become promising, therefore we have undertaken this piece of research work by dove-tailing *hpt* gene in development of herbicide tolerant transgenic indica rice. It is to be mentioned that transgenic lines harbouring *hpt* gene found to confer resistance to hygromycin, an aminoglycosidic antibiotic as well as to glyphosate, an extensively used herbicide [13]. In India Round up is one of the most popular glyphosate containing herbicide and is marketed by Monsanto India. Results of the present experiments discussed in detail, below.

Determination of generation time:

Culture plates containing semi-solid AB medium were observed after 36 h of incubation at 28^{0}C and found 224x10^6 colonies incubated at t_1 and 248 x 10^6 colonies at t_2. Difference of time interval was 30 min between t_2 and t_1. Generation time calculated was found to be 125 min at 28^{0}C for the *Agrobacterium* strain LBA 4404 containing *gus* and *hpt* genes.

Determination of minimum inhibitory concentration (MIC):

MIC for cefotaxime was found to be 50 mg /l in the present experiment. Previous studies [38] indicated that cefotaxime was not much sensitive and was fairly stable under normal *in vitro* tissue culture growth room conditions up to 22 days. So, transformed materials were placed on to fresh medium at every 20 days. We have conducted the present experiment with four replications in determining MIC of this antibiotic for extent possible accuracy, so that the correct dose can be used in the culture medium for successful elimination of Agrobacterial cells after co-cultivation efficiently.

Optimization of hygromycin level for selection of transformed callus:

To detect the appropriate level of the antibiotic-hygromycin B (Sigma-Aldrich, Cat No:H0654) for efficient selection of true transformants from the calli co-cultivated with *Agrobacterium*, an experiment involving MS medium supplemented with 0, 25, 50, 75 and 100 mg/l of hygromycin B was undertaken as performed by previous authors [32]. Twenty five pieces of embryogenic cell clumps (calli) from the variety IET 13856 was used for *Agrobacterium*-mediated genetic transformation. Putative transformed calli were transferred onto MS medium along with an untransformed control set. Untransformed calli could not survive on any treatments containing hygromycin. However, the calli regenerated into healthy plantlets at 25 and 50 mg/l of hygromycin is indicative of their suitability in facilitating plantlet regeneration (Table 1).Interestingly, in 75 mg/l hygromycin supplemented medium, only a very few calli survived, grew marginally but failed to regenerate in to complete plantlets. Selective medium supplemented with 100 mg/l showed no survival of any transformed calli and thus

scope of plantlet regeneration did not arise. At the concentrations of 25 and 50 mg/l hygromycin, calli survived well. So, 50 mg/l of hygromycin level was finally selected for safer use in selection of cells transformed with *hpt* in the subsequent experiments.

Optimization of glyphosate level for herbicide tolerance:

Tolerance to hygromycin level is the most important part for determination of transformation efficiency, concomitantly; measurement of glyphosate tolerance level was equally given emphasis in the present experiment too since the ultimate target is to engineer herbicide toterance. It was assessed under *in-vitro* condition involving 25 putatively transformed calli of indica rice var. IET 13856 harbouring *hpt* gene by growing them on MS mediun containing 0, 1, 2, 3, 4 and 5 mM of glyphosate along with 50 mg/l hygromycin [13]. Putatively transformed calli were transferred onto MS medium with an untransformed control set. Untransformed calli did not survive in any of the treatments containing hygromycin and glyphosate. However, many putatively transformed calli regenerated into healthy plantlets even at 3 mM glyphosate level, indicates suitability of this dose to obtain plantlet regeneration (Table 2). However, on medium supplemented with 4 mM glyphosate, a very few calli survived, grew marginally but could not regenerate into plantlets. Medium fortified with 5 mM glyphosate did not show survival of any transformed calli and thus scope of plantlet regeneration does not arise. At 2 and 3 mM glyphosate concentrations, calli survived well. So, 3 mM of glyphosate level was chosen as appropriate dose for selection of cells transformed with *hpt* in the succeeding experiments. Moreover, callus from control set did not grow at all on medium containing 3 mM glyphosate. In essence this experiment confirmed that the *hpt* gene in transformed plant cells/calli confers tolerance upto 3 mM glyphosate *in vitro*, and under containment facility moderate tolerance indicates suitability of its use in field trial to assess its potential to weedicides

Effect of calli of different ages in governing transformation efficiency:

Calli induced from mature seeds of IET 13856 of different ages - 10, 30, 60 and 90 days (Table 3) were used in genetic transformation with LBA4404 (pTOK233) (Fig. 1). After 72h of transformation, transient expression of GUS was confirmed through histochemical assay of β-glucoronidase. It was observed that 10 days old calli were not suitable explants for *Agrobacterium*-mediated genetic transformation and 15% calli showed GUS stained spots/foci in IET 134856. Maximum transformation was obtained from 60 and 90 days old embryogenic calli. IET 13856 showed 96% full calli transformation in 90 days old calli as evident from complete blue stainingof whole calli. Difference in transformation efficiency was also evident through differential blue foci intensity as observed in this investigation. 10 days old calli during subsequent subcultures developed fairly well developed somatic embryos and pro-embryoids whereas 60 and 90 days old calli found to be highly suitable for *Agrobacterium*-mediated genetic transformation (Fig. 2). Among several factors governing the transfer of T-DNA, mostly age and physiological state of plant tissues play major role [39]. 2-4 days old rice embryos were found to be the best material for genetic transformation [40]. To the contrary present findings firmly advocate suitability of 60 and 90 days old calli and thus may be used in undertaking future transformation works confidently.

Organ specificity in facilitating genetic transformation:

Calli developed from root, coleoptiles and anthers were compared in respect of genetic transformation efficiency along with mature seed derived calli. Maximum transformation was observed in anther-derived calli (100%) followed by coleoptile- derived calli (96.36%) and mature seed-derived calli, respectively (95.48%) (Table 4). Root-derived calli did not respond well towards *Agrobacterium*-mediated genetic transformation (5.38%). Anther calli showed full transformation with deep blue GUS stain. About 80.9, 10.9 and 4.54% coleoptile-derived calli showed full, ½ and ¼ GUS expression (on the basis of GUS stained area of calli), respectively (Fig. 3). Similarly, 69.17, 14.28 and 12.03% of mature seed- derived calli showed full, ½, ¼, GUS expression. On root explants, directly globular somatic embryos were found to develop within a short time; calli like masses with wet surface and considerable number of long tubular non-embryogenic cells developed subsequently. This perhaps attributes towards low genetic transformation efficiency in case of root calli. It can be concluded that mature seed- derived calli or coleoptile-derived calli are far superior and offer greater scope for use in *Agrobacterium*-mediated genetic transformation.

Transgenic development and molecular analysis:

Massive efforts were mounted earlier by many investigators worldwide and a large number of transgenic lines governing diverse traits in rice were successfully achieved [41, 42], however, the transformation efficiency was found to be very less especially in indica rice. A paradigm shift in augmenting transformation efficiency and development of large number of transgenic lines become possible after the landmark experimentation through *Agrobacterium*-mediated genetic transformation involving super binary vector pTOK 233 [22, 43]. The same protocol was adopted in this study with minor modification where calli were induced with an optimised medium and supplements (Fig. 2). The experiments were conducted with two replications and the results are highlighted in Table 5.

Maximum transformed calli died during the course of selection on hygromycin supplemented medium. However, many survivor calli were regenerated and green plantlets (putative transgenic lines) were recovered in IET 13856 transformed with LBA 4404 (pTOK233) containing *npt, hpt* and intron–gus in the T-DNA region (Fig. 2h). The hpt primers amplified 350 bp internal *hpt* sequences in majority of the plants. Mode of plantlet regeneration was found to be both

somatic embryogenesis as well as organogenesis. Interestingly, percent plantlet regeneration was found to be drastically reduced in comparison to control (data not presented). Transgenic gus expression in co-cultivated embryogenic calli was found to vary between the two experiments conducted. Experiment II was found to be superior (similar observation was made by previous authors [22] as average of two replications amounting to 61.03% in comparison to average 41.78% as observed in Experiment 1 (Table 5). Plantlets at the height of 8-10 cm were transferred to Hoagland solution (pH 5.5) on filter paper bridges for 7 days. Subsequently those were shifted to experimental glass house and planted on soil in plastic pots covered with polyethylene bags for hardening. Hardened plantlets were further shifted to big cement pots as mentioned in Materials and method and kept in Transgenic Rearing Facility (Category II, DBT approved) under appropriate agronomic practices till maturity. Out of 84 regenerated plantlets, only 74 plants survived and were grown to maturity (Table 6). By using *hpt* specific primers, presence of transgene was evaluated (Fig. 3). Different plant parts viz. roots, leaves and spikelets (Fig. 3) were used for GUS histochemical assay following previous authors [23] by taking samples from the primary regenerates at actively vegetative and mature stages. GUS stain was observed within 2 h of staining.

Hybridization experiments were carried out to check the presence of introduced *hpt* gene in the putative transgenic plantlets and its stable integration in the recipient's genome [44]. DNA was isolated, purified and spotted on charged NYLON membrane and hybridized as described in Materials and method. Fig. 4 showed spotted DNA were able to hybridize with the radio labelled *hpt* probes, while no hybridization took place with DNA from negative control (untransformed plant). PCR amplification confirmed integration of T-DNA harbouring *hpt* gene to the putative transgenic plantlets. Amplification of DNA from control plant (normal IET 13856) produced no amlicon, whereas 14 plantlets (Table 6) were found to be GUS positive (histochemical assay) by and 16 plantlets were found to be *hpt* positive. Those were further analysed through slot blot and Southern blot hybridization. By using *gus* histochemical assay, 14 plants were found to be GUS positive, where as only 10 plants were found to be *hpt* positive (Table 6). While analyzing the band size, among the *hpt* positive plants, 9 were found to be carrying expected 0.77 kb size bands, which indicate no alteration, occurred in the transgene size, before integration into the rice genome (Table 7). In *Agrobacterium*-mediated genetic transformation this advantage is well evident in many cases [45].

Southern hybridization confirmed stable integration of the transgene into rice plantlets var IET 13856. GUS $^{+}$ and *hpt* resistant nature of the transformed tissues confirmed expression of the transgene in different plant parts [44]. Southern blot further displayed occurrence of three independent transformation events in six plants, which were the sister clones developed from one embryogenic cell group co-cultivated in Experiment I following Protocol II [22] the 4 loci showed identical hybridization pattern without any change in molecular weight of the intruded transgene as evident from similar banding pattern. This observation was found be in contradiction with earlier results of Li et al., 1990. Transgene integration in more than one site of the genome was earlier reported by many investigators including [43]. However, as previous authors [46] reported insertion of transgene at one and two loci using LBA 4404 (pTOK 233) housing *hpt* gene in scented indica cv Karnal local and in one very high yielding modern var. IR 64. It is to be mentioned that no pre-insertional change in transgene seems to be advantageous in its expression at transcriptional and translocational levels.

It is noteworthy that the efficiency of *Agrobacterium*-mediated genetic transformation was reported and transgenic lines were also developed by previous authors [47, 32] in many rice cultivars/varieties. However, conventionally, indica rice was found to be refractory to *Agrobacterium*-mediated genetic transformation. In this case, difficulties in regenerating plantlets from transformed calli after selection/screening on hygromycin (mg/l) supplemented medium were experienced. Among 1794 calli used, only 84 survived from which ultimately 74 plantlets were produced. Three plantlets showed GUS expression at the whole plant level. This demands fine tuning of optimization of the physico-chemical environment to regenerate more plantlets from survivor calli after *Agrobacterium*-mediated genetic transformation and selection on hygromycin supplemented medium [43]. Synoptically the above finding indicates that *Agrobacterium*-mediated genetic transformation holds enormous promise in indica rice, if an efficient plantlet regeneration system of post- transformed calli is developed. An effective decontamination procedure to halt over growth of *Agrobacterium* after co-cultivation without affecting the regeneration potential based on the minimum bacterial count (MBC) is indispensible. The transgenic plants of IET 13856 were apparently looking normal without expression of any ectopic characters or abnormalities and they were found to produce fully set fertile seeds in the Transgenic Rearing Facility.

Inheritance of the inserted transgene:

Once the transgene is integrated it is essential to study the inheritance pattern of the inserted transgene. Seed germination test for the presence of *hpt* gene was done involving seeds from the transgenic plants at T_1 generation (Fig. 2). Results (Table 8) showed in majority of the cases 3:1 ratio was discernible following Mendelian ratio of monogenic inheritance of dominant transgene (Table 8). Southern hybridization for *hpt* gene showed that this gene was transmitted to the next generation in 3:1 ratio. The *hpt* gene was inherited in the mode of 3:1 (p=0.10-0.90) based on germination test and 3:1 ratio (p=0.01-0.50) based on Southern blot analysis / seed germination (Table 9).

IV. CONCLUSION

In essence, development of transgenic indica rice, which is considered to be being relatively less responsive to *in vitro* culture system in comparisons to Japonica and tropical Japonica (erstwhile Javanica) is possible by dispensing appropriate genes into their embryogenic calli and their subsequent dedifferentiation into plantlets. Molecular analysis of the putative transgenic plants is essential to confirm their transgenic status and inheritance study of the transgene is indispensible to understand the mode of inheritance of transgene. However, from the present experiment we firmly believe that *hpt* gene can significantly increase the glyphosate tolerance of IET 13856 var. through *Agrobacterium*-mediated genetic transformation. Regenerating calli with transgene followed by selection on hygromycin B exhibited successful growth and significant tolerance level (up to 3 mM) in glyphosate containing medium. These approaches are very much feasible, effective, simple and moreover less expensive to develop herbicide tolerant rice varieties, especially for niche specific popular varities like IET 13856 which grows well in Andaman and Nicobar Islands in Indian Republic. In future elaborate experimentation will be undertaken with different crops so that the food and nutritional security may be restored in demandable area with less investment and simpler way. It is to be mentioned that the herbicide Roundup (w/w 41 % glyphosate) marketed by Monsanto India is a very popular herbicide used in rice husbandry in high rainfall areas like Andamans where weed is a big menace and labour scarcity is making the rice cultivation non-remunerative such transgenic line harbouring *hpt* may paves an way out to augment rice productivity and also in rice growing low lying areas with similar agro climates elsewhere.

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Fig 1 Gene construct. Legends: Physical map (partial) with restriction enzyme sites and transgene/reporter gene locations under up and down stream regulatory elements.

Fig 2 Different steps of *Agrobacterium*-mediated genetic transformation through LBA4404 (pTOK233) in an indica rice var. IET13856. Legends: a. Germinating seeds on callus induction medium (MS+ 2mg/l 2-4-D) showing successful callus induction; b. Stereomicroscopic view of the calli initiation from mature seed; c. Sub-cultured calli proliferating on callus maintenance medium containing MS+1mg/l 2-4-D; d. Close view of an embryogenic calli harbouring plenty of embryo-like structures; e. Calli co-cultivated with LBA4404 (pTOK233) for 72h; f. Putatively transformed calli multiplying on selection medium containing MS+50mg/l hygromycin; g. Emerging plantlets from survived calli after co-cultivation with *Agrobacterium*; h. Regenerating plantlets from putatively transformed calli; i. Putative transgenic plants in soil-filled cement pots at T_0; j. Many putative transgenic lines generated from different experiments; k. Transgenic plants in T1 generation after confirmation through molecular analysis and seed germination test.

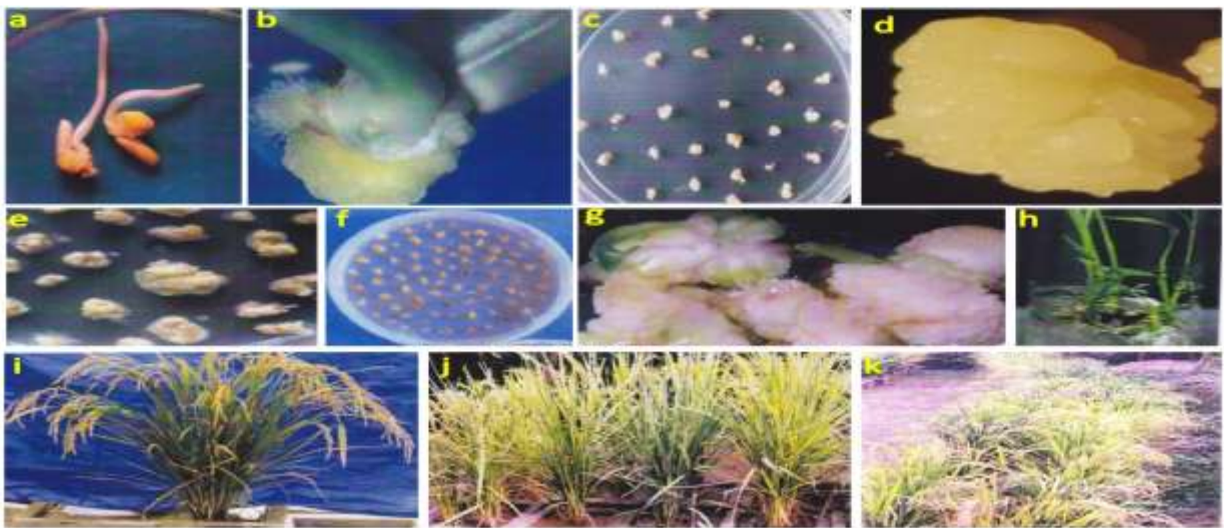

Fig 3 Complete and chimeric GUS expression in spikelets of a putative transgenic line R 20-5 Legends: a. Transformed embryogenic calli; b. Transformed and non-transformed leaf; c. Leaf-sheath; d. close view of primary root; e. Peduncle; f. Lemma; g. Palea; h. Germinating seeds at T_1 generation; i. Close-view of h; j. Germinating seeds at T_1 generation on MS+50mg/l hygromycin I. Seeds from non-transformed plant II. Seeds from a transgenic line (R 5-32).

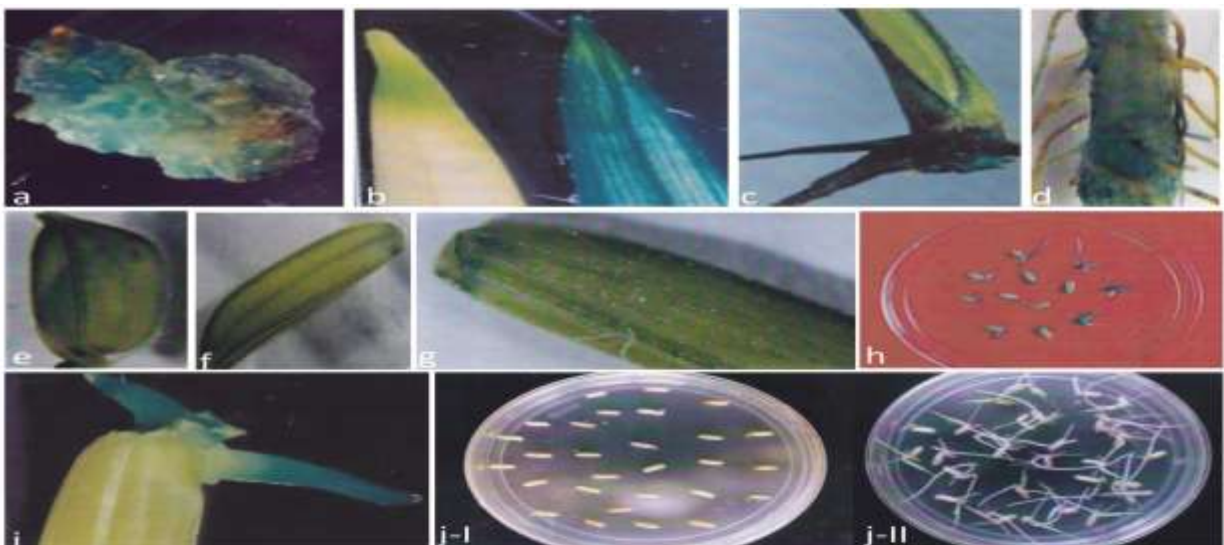

Fig 4 Molecular evidences confirming transgenic status of a few lines carrying *hpt* gene Legends: N and P denotes Negative and Positive control a. PCR amplicon profile involving reverse and forward primers of *hpt.* b. A representative slot blot involving genomic DNA isolated from putative transgenic plants hybridyzed with radio labeled (p^{32}) *hpt* probe. *(after first round of slot blot, DNA) from promising lines [positive slots] were subjected to re-slot blot hybridization c. Southern hybridization pattern involving genomic DNA restricted with *Sal*I from six tillers derived from R20-5 indicating insertaion of *hpt* gene at three major locus d. Southern profile of genomic DNA restricted with *Bam*HI and hybridized with *hpt* probe.

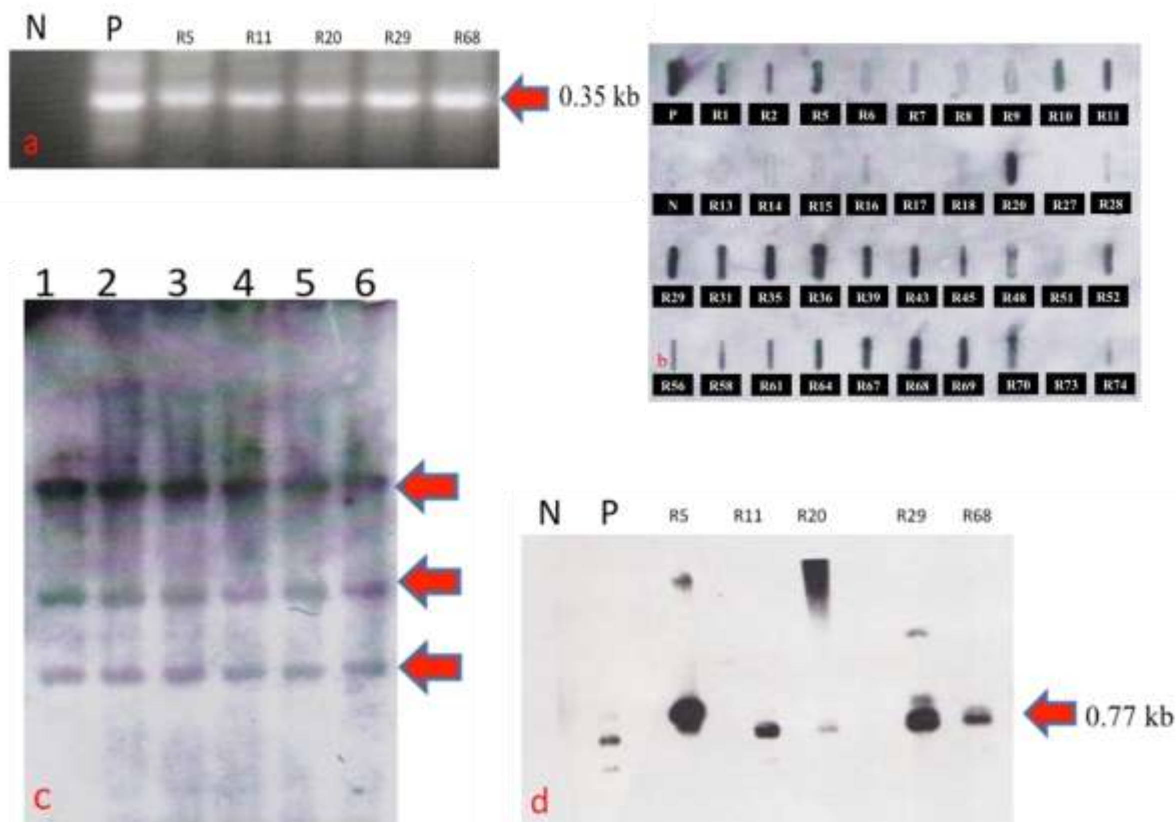

Table 1: Optimization of hygromycin dose for use in the selection medium

Hygromycin (mg/l)	Cell clumps (calli) implanted	Survival (%)	Plantlet regeneration (%)
0	30	100	71
25	30	94	63
50	30	89	58
75	30	3	0
100	30	0	0

Table 2: Optimization of glyphosate dose for use in selection medium

Glyphosate (mM)	Cell clumps(calli) implanted	Survival (%)	Plantlet regeneration (%)
0	25	100	88
1	25	90	79
2	25	86	74
3	25	81	65
4	25	4	0
5	25	0	0

Table 3: Effect of callus age in influencing *Agrobacterium*-mediated genetic transformation in rice.

Designation	Age of calli (days)	No. of calli used	No. of calli showing GUS expression	% transformation
IET 13856	10	50	5	15(a few spot)
	30	50	31	63(half)
	60	50	47	95(half)
	90	50	48	96(half)

Table 4: *Agrobacterium*-mediated genetic transformation of calli induced from different plant parts in indica rice.

Organ used for callus induction	% transformation		GUS expression*			
	Calli used	GUS +ve	Full	½	¼	Nil
Root	130	7(5.38)	-	-	7(5.38)	123(94.61)
Coleoptile	110	106(96.36)	89(80.9)	12(10.9)	5(4.54)	4(3.63)
Anther	80	80(100)	70(87.5)	10(12.5)	-	-
Mature seed	133	127(95.48)	92(69.17)	19(14.28)	16(12.03)	6(4.51)

* part of calli showing GUS expression

Table 5: Transient and stable GUS expression and plantlet regeneration in an *indica* rice var. IET 13856 with LBA 4404 (pTOK 233)

Experiment *	Replication	No. of calli co-cultivated	Transient GUS expression (%GUS positive calli)	No. of calli placed on selective medium (with 50mg/l	No. of *hpt* resistant calli (%)	No. of *gus* positive calli	No of calli showing plantlet regeneration	No. of plantlets regenerated	gus+ plantlets
I	1	494	224 (45.34)**	185	290 (80.0)**	196 (67.50)	22	24	18
	2	450	172 (38.22)	402	260(64.67)	152 (58.46)			
	Average		41.78		72.33	62.98			
II	1	378	213 (56.61)	270	214(72.25)	152 (71.02)	16	16	12
	2	472	3.9(65.46)	338	230(68.04)	63(60.50)	15	13	14
	Average		61.03		70.14	65.76			

*Exp.I Callus induced on MS with 2 mg/l 2,4-D and 10% coconut water; however, transformation protocol and culture media used after co-cultivation were same as prescribed by Hiei et al, 1994.

Exp.II Media and protocol were same as outlined by Hiei et al, 1994.

**Figures indicate percent values

$$\textit{hpt}\text{ resistant calli (\%)} = \frac{\text{No. of hygromycin resistant calli}}{\text{No. of calli plated on selection medium with 50 mg/l hygromycin}} \times 100$$

$$\text{GUS positive calli (\%)} = \frac{\text{No. of selected calli showing GUS expression}}{\text{No. of calli showing resistance to hygromycin}} \times 100$$

Table 6: Synopsis of *Agrobacterium*-mediated genetic transformation with LBA 4404 harbouring pTOK 233 in indica rice var. IET 13856

Number of regenerated plantlets	84
Number of survivors and analysed plantlets	74
Number of PCR positive plantlets *hpt*	 16 (21.62%)
Number of GUS** plantlets	14 (18.91%)
Slot / Southern positive *hpt*	 10 (13.51%)

*values in parentheses indicate percent values.

**based on histochemical staining

Table 7: Frequency and grouping of different band patterns of *hpt* gene in transgenic plants at T_0 from *Agrobacterium* - mediated genetic transformation

Group	Total	Number of bands			Number of plants
		Expected size 0.77kb	Smaller size <0.77kb	Large size >0.77kb	
I	1	9	-	-	9
II	2	1	-	1	2
III	3	1	2	-	3

Table 8: Germination test of seeds at 50mg/l hygromycin B concentration at T_1 generation

Transformant	Resistant	Sensitive	Expected ratio	χ^2	P
R5-17	18	18	3:1	0.889	0.50-0.30
R5-32	42	27	3:1	1.753	0.20-0.10
R11-7	75	45	3:1	2.133	0.20-0.10
R20-4	81	27	3:1	0.037	0.90-0.70
R20-5	39	15	3:1	0.017	0.90-0.70
R20-18	69	30	3:1	0.600	0.50-0.30
R29-21	68	48	3:1	1.846	0.20-0.10
IET 13856 (control)	0	30	-	-	-

Table 9: Inheritance of *hpt* gene at T_1 generation based on Southern analysis

Transformant	Southern blot		Expected ratio	χ^2	P
	+ve	-ve			
hpt gene					
R 5-32	6	10	3:1	0.259	0.05-0.01
R 11-7	24	8	3:1	0.167	0.50-0.70
R 31-16	18	2	3:1	0.533	0.30-0.50
R 20-4	15	5	3:1	0.167	0.50-0.70