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Research Article

ESTABLISHMENT OF AN EFFICIENT REGENERATION SYSTEM AMENABLE TO *AGROBACTERIUM* MEDIATED TRANSFORMATION OF TWO ELITE *INDICA* RICE VARIETIES OF NORTH EAST INDIA

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Abstract

An efficient plant regeneration system from embryogenic callus of two elite *indica* rice (*Oryza sativa* spp. *indica*) varieties of Northeast India, Ketokijoha and Monoharsali is established. The effect of auxin, 2,4-dichlorophenoxy acetic acid (2,4-D) on callus induction was optimized. Friable, nodular and creamish-white embryogenic calli were induced from mature seeds on NB medium supplemented with 2.5 mg/l 2,4-D. Plants were regenerated from 40-50 days old embryogenic callus on NB medium containing 0.5 mg/l BAP (6-benzylaminopurine) and 0.25 mg/l ABA (abscisic acid). Regenerated plants with multiple tillers were rooted on half strength MS medium and rooted plants were acclimatized with 94% survival rate. Higher frequency of callus induction as well as plant regeneration was recorded in Ketokijoha as compared to Monoharsali. The calli of both the varieties were found amenable to *Agrobacterium*-mediated transformation as evident from strong GUS (β -glucuronidase) expression. The results may find wide application for genetic improvement for valuable traits these elite *indica* rice varieties of Northeast India.

Keywords: *Agrobacterium*; *Indica* rice; plant regeneration; callus

Introduction

Rice (*Oryza sativa* L.) is the main staple food for more than half of the world. In Asia, more than 90% of rice is produced and consumed (Datta 2004). Among the two major subspecies of rice, long-grained *indica* rice accounts for 80% of cultivated rice. Establishment of a highly efficient regeneration system for *indica* rice is a prerequisite for successful application of genetic engineering tools for improvement of cultivars against biotic and abiotic stress, and value added traits. Genetic transformation of rice in general and *indica* subspecies in particular is considered to be difficult due to their recalcitrance to *in vitro* manipulations. However, these constraints have been overcome in part by regenerating transgenic rice plants from transformed embryogenic calli (Hiei *et al.*, 1994) as these tissues are known for their amenability to both gene delivery and plant regeneration. Among the factors that affect frequency of callus induction and plant regeneration in *indica* rice, genotype, physiological status of the seed scutellum, type and dose of the plant growth regulators among others are very important (Ge *et al.*, 2006). Although there are several reports of successful regeneration of transgenic *indica* rice from *Agrobacterium*-mediated transformation of embryogenic calli (Ignacimuthu and Arockiasamy 2006, Mohanty *et al.*, 2002), many *indica*

varieties are still recalcitrant to cultural manipulations due to poor callus induction, proliferation and regeneration abilities (Islam *et al.*, 2004, Khaleda and Al-Forkan 2006, Zuraida *et al.*, 2011). Difficulties still exist in establishing a widely used culture system for *indica* rice applicable to wide range of cultivars. Northeast India is considered to be one of the hot spots of rice genetic resources of the world and rice is extensively cultivated in this region under extremely diverse cultivation conditions (Durai *et al.*, 2009). Among the rice varieties of north-eastern region, aromatic rice of the state Assam, Ketokijoha is most popular due to its aroma, quality and palatability (Das *et al.*, 2010) and Monoharsali is an widely adapted variety and identified as resistant to blast, bacterial leaf blight, sheath blight and brown plant hopper (Rathaiah and Das 1987). However, protocols for high frequency plant regeneration using embryogenic callus cultures have not been reported in these highly popular two local *indica* rice varieties.

Success in transformation of *indica* rice using embryogenic callus based regeneration systems depends on the factors that favor the formation of friable and high quality callus in a shorter time competent for shoot regeneration. The potential for callus induction and regeneration have been reported to be varietal dependent limiting efficient regeneration in large number of regional *indica* rice

varieties for genetic manipulation (Ali *et al.* 2007). Here, we established an efficient and reproducible protocol for plant regeneration from embryogenic callus induced from seed scutellum amenable to *Agrobacterium*-mediated transformation in two of the most popular *indica* rice varieties of Northeast India.

Materials and Methods

Plant Material and Callus Induction

Mature seeds of rice (*Oryza sativa* L) varieties, Ketokijoha and Monoharsali were obtained from Krishi Vigyan Kendra Regional Agricultural Research Station Akbarpur, Karimganj, India. Mature seeds were dehusked, surface sterilized with 70% ethanol for 30 s, rinsed with 1% bavistin and 0.2% HgCl₂ (w/v) for 5 min each. The seeds were subsequently rinsed five times with sterile double distilled water and cultured on NB medium (Chu *et al.*, 1975) containing proline (500 mg/l), glutamine (500 mg/l), casein hydrolysate (300 mg/l) with varying concentrations of 2,4-D (0, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/l) for callus induction. The proliferating calli were subcultured twice onto fresh media at an interval of 30 and 15 days respectively. The frequency of callus induction was determined as the number of seeds producing calli per number of seeds cultured on callus induction media. Each treatment consisted of 30 seeds, and each experiment was repeated thrice. Visual observations of the cultures were made periodically in every week, and the frequency of callus induction was recorded after 30 days. The relative frequency of callus induction was compared between the two rice varieties.

Multiple Shoot Induction and Plant Regeneration

Embryogenic calli, after two subcultures, were transferred to regeneration medium NB medium containing different concentration of BAP (0.5, 1.0 and 1.5 mg/l) in combination with 0.25 mg/l of ABA. The efficiency of multiple shoot induction was evaluated by scoring the mean number of shoots induced from responding embryogenic callus, and measuring the mean shoot length. The regeneration frequency was calculated based on the number of callus responding to regeneration by total number of callus cultured. The regeneration frequency and efficiency of multiple shoot induction were compared among the two cultivars.

Culture Conditions

In all the case, the pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl and 0.8% agar (Hi-Media, Mumbai) was added prior to autoclaving at 121°C at 15 psi for 15 min.

All the cultures were maintained at 25±2⁰ C under 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 50 μmol m⁻² s⁻¹ provided by 40 W cool white fluorescent lamps (Philips, India). Seeds cultured in hormone free media were considered as control.

Rooting and Transplantation

The shoots (5-8 cm) were separated from multiple shoot clumps and transferred individually to MS medium (Murashige and Skoog 1962) devoid of growth regulator for two weeks for root formation. Subsequently plantlets with well-developed root system were washed in tap water and acclimatized in polybags containing soil and vermicompost (1:1), covered with transparent polybags at 27°C and 16 h photoperiod for 14 days. Finally, the acclimatized plantlets were transferred to pots containing soil and established in a green house.

Transformation Procedure and GUS Assay

Agrobacterium tumefaciens strain EHA105 harboring a binary vector pCAMBIA1301 with β-glucuronidase (*gus*) interrupted with an intron in the coding region and neomycin phosphotransferase (*nptII*) genes, and plant selection marker hygromycin phosphotransferase (*hptII*) driven by CaMV35S promoter (Fig. 1) was used for transformation studies. The bacteria was grown on YEP (10 g/l yeast extract, 10 g/l peptone, 50 g/l NaCl, 15 g/l agar and pH 7.0-7.2) solid medium containing 50 mg/l kanamycin and 10 mg/l rifampicin at 28° C. A single bacterial colony was inoculated into 2 ml of liquid AB medium containing 5 mg/l rifampicin and 25 mg/l kanamycin and grown overnight on a rotary shaker at 200 rpm at 28° C. Bacteria were pelleted at 5000 rpm for 5 min and resuspended in liquid NB medium supplemented with 2.5 mg/l 2,4-D, containing 100 μM acetosyringone at a density of OD₆₀₀=1. Embryogenic callus from 45-d-old calli were used in transformation being immersed in bacterial suspension for 30 min with shaking at 80 rpm at 25° C. Inoculated explants were blotted on sterile filter paper and co-cultivated on solid NB medium supplemented with 2.5 mg/l 2,4-D containing and 100 μM acetosyringone for 3 days at 25° C under dark condition. After co-cultivation, the embryogenic calli were washed three to four times with sterile double distilled water by constant stirring and blotted dry on sterile filter paper. Approximately 20 explants were tested for transient *gus* expression by histochemical assay using 5-Bromo-4-Chloro-3-Indolyl Glucuronide (X-Gluc) as a substrate (Jefferson 1987). The explants were then visually scored for transient *gus* activity.

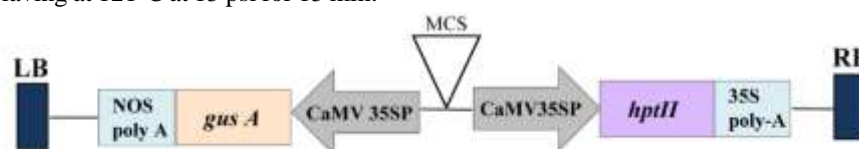


Fig. 1: Schematic representation of the T-DNA region of plasmid pCAMBIA1301 showing the position of the *gusA* and hygromycin phosphotransferase (*hptII*) genes. CaMV35S - Cauliflower Mosaic Virus 35S promoter, NOS PolyA - Nopaline Synthase (nos) gene terminator, GUS - *E.coli* *gus* reporter gene, LB – Left border, RB – Right border

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean separation by Duncan's multiple-range test (DMRT) using single-factor completely randomized block design in order to study the effect of different treatments on shoot proliferation and frequencies of transient expression. All experiments were performed at least three times with a minimum of 30-40 calli per treatment.

Results and Discussion

Efficient transformation of rice using *Agrobacterium* mediated approach and subsequent plant regeneration are dependent on crucial factors such as the choice of explant, hormonal composition of the medium used and nutritional supplements for effective induction of callus and regeneration (Ratnayake and Hettiarachchi 2010). Among the different explants, scutellum derived callus has been reported to be the most amenable explant for *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). However, success in transformation of *indica* rice using scutellum derived callus depends on the factors that favor the formation of friable and high quality callus in a short time competent for shoot regeneration. Moreover, the potential for callus induction and regeneration are often variety-dependent and therefore need establishment of efficient regeneration for specific varieties for genetic manipulation (Ali *et al.*, 2007). Moreover, poor regenerative ability of a cultivar can be a bottleneck in the establishment of an efficient system for genetic transformation (Carsono and Yoshida 2006, Nishimura *et al.*, 2005).

In the present study, factors affecting efficient embryogenic callus induction and plant regeneration were presented prior to the investigation of the amenability of explant to *Agrobacterium*-mediated transformation in two of the most popular *indica* rice varieties of rice diversity-rich Northeast India.

Effect of 2,4-D on Callus Induction

Most of the seed explants responded to callus induction on medium containing 2,4-D. The callus induction appeared from the scutellar region of the seeds within 7-10 days of culture in 2,4-D containing media in both Ketokijoha and Monoharsali varieties (Fig. 2). However, significant differences were recorded among the 2,4-D concentrations tested with respect to percentage of callus induction (Fig. 2). The highest percentage of callus induction was achieved in medium containing 2.5 mg/l 2,4-D in both Ketokijoha (84%) and Monoharsali (82.87%). Apparently, no significant difference in percentage of callus induction was observed in the two varieties tested (Fig. 2). It was also observed that 2.5 mg/l 2,4-D was ideal concentration for further proliferation of embryogenic callus in the sub cultured medium. Callus induction was poor on medium with lower (1.0, 1.5 and 2.0 mg/l) concentrations of 2,4-D while necrosis of callus was visible with increasing concentration of 2,4-D (3.0 and 3.5 mg/l) (Fig. 2). The browning in most of the initiated callus at higher 2,4-D concentrations affected further proliferation in the sub-cultured medium. No callus formation was observed in control NB medium devoid of 2,4-D (Fig. 2).

The explants were cultured on callus induction medium for 4 weeks and the calli were sub-cultured onto the same fresh medium thrice. Compact, nodular creamish-white embryogenic calli were formed within 24 days of culture initiation. The high-quality calli was yellow or light yellow in color and was dry, compact, and globular in appearance (Fig. 3a). The stimulatory effect of 2,4-D at 2.5 mg/l on induction of embryogenic callus from seed scutellum has been reported in a number of *indica* rice genotypes (Karthikeyan *et al.*, 2009, Sabir *et al.*, 2010, Saharan *et al.*, 2004). The 2,4-D at concentrations between 2.0-2.5 mg/l facilitated embryogenic callus induction in somatic tissues of *indica* and *japonica* rice varieties (Tyagi *et al.*, 2007).

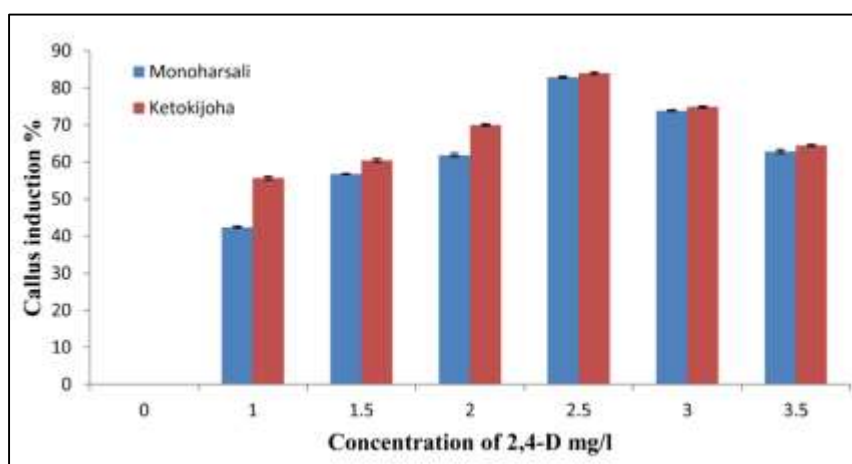


Fig. 2: Effect of various concentration of 2,4-D on percentage of callus induction of *indica* rice varieties, Monoharsali and Ketokijoha, where X-axis showing percentage of callus induction and Y-axis showing different concentration of 2,4-D in NB medium.

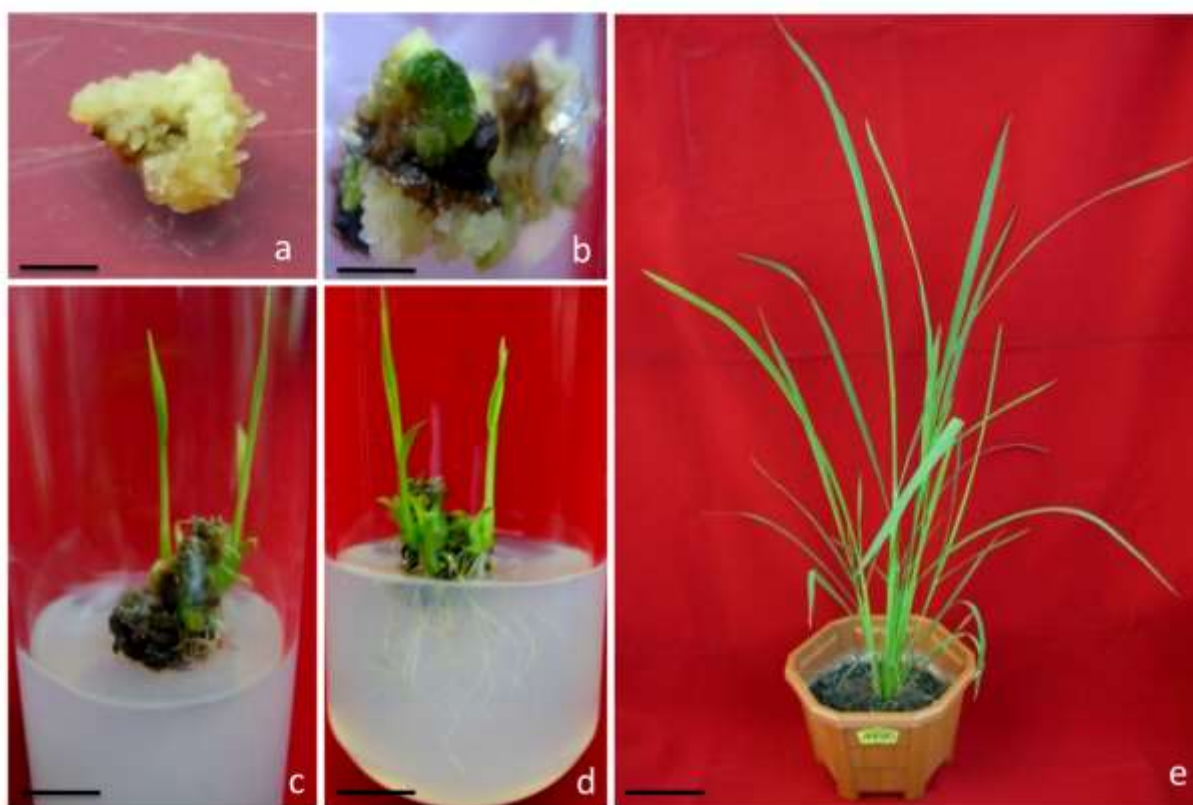


Fig. 3a-e *In vitro* plant regeneration from embryogenic callus of *Oryza sativa* spp. *indica* cv. Monoharsali. (a) Embryogenic callus, (b) Induction of shoots from callus in NB medium supplemented with 0.5 mg/l BAP and 0.25 mg/l ABA after 4 weeks in culture, (c) Callus showing multiple shoot formation after 4 weeks in culture, (d) Rooting in MS medium, (e) Regenerated plants growing in greenhouse. Bar represents 2 mm (a); 4 mm (b); 1 cm (c); 2 cm (d).

Plant Regeneration

In order to evaluate the efficacy of regeneration medium differing in concentrations of BAP on differentiation of shoots, the embryogenic calli were shifted to regeneration medium after 40-50 days of callus initiation. The preembryogenic clumps on calli turned green in colour within 3 weeks of culture on regeneration medium. The emergence of shoot buds appeared from the green clumps within 3-4 weeks of subculture on fresh medium (Fig. 3b). We found that the concentration of BAP in regeneration medium containing 0.25 mg/l of ABA significantly influenced the regeneration frequency. Among the different concentrations of BAP tested, medium fortified with 0.5 mg/l BAP showed highest regeneration frequency in both Ketokijoha (84%) and Monoharsali (71%). The results revealed significant difference in regeneration frequency between the two *indica* varieties, Ketokijoha and Monoharsali tested (Fig. 3 and 4) indicating the effect of interaction of genotype and regeneration medium on regeneration frequency. Within *indica* subspecies, significant variations of *in vitro* culture response have been reported in different genotypes (Seraj *et al.*, 1997). Regeneration medium containing 2.5 mg/l BAP resulted in an average of 4.33 and 4.21 shoots from embryogenic callus cultures of Ketokijoha and Monoharsali respectively (Table 1; Fig. 3c and 4c).

Table 1. Effect of BAP in combination with 0.25 mg/l ABA on *in vitro* plant regeneration from matured seed derived embryogenic callus of *indica* cv. Monoharsali and Ketokijoha on NB medium

BAP (mg/l)	Regeneration frequency ^a (%)	Average no of shoot	Average shoot length (cm)
	<i>Monoharsali</i>		
0	0	0	0
0.25	40	2.35±0.45	5.7±0.24
0.5	71	4.21±0.37	8.5±0.28
1.0	60	3.17±0.29	7.0±0.43
1.5	50	4.34±0.57	5.5±0.34
	<i>Ketokijoha</i>		
0	0	0	0
0.25	50	3.57±0.73	6.5±0.29
0.5	84	4.33±0.25	9.5±0.45
1.0	55	3.22±0.41	7.0±0.43
1.5	40	2.47±0.36	5.5±0.64

^a Regeneration frequency (%) = (Number of embryogenic callus shoots / Total number of callus cultured) X 100
Values represent means ± SE.

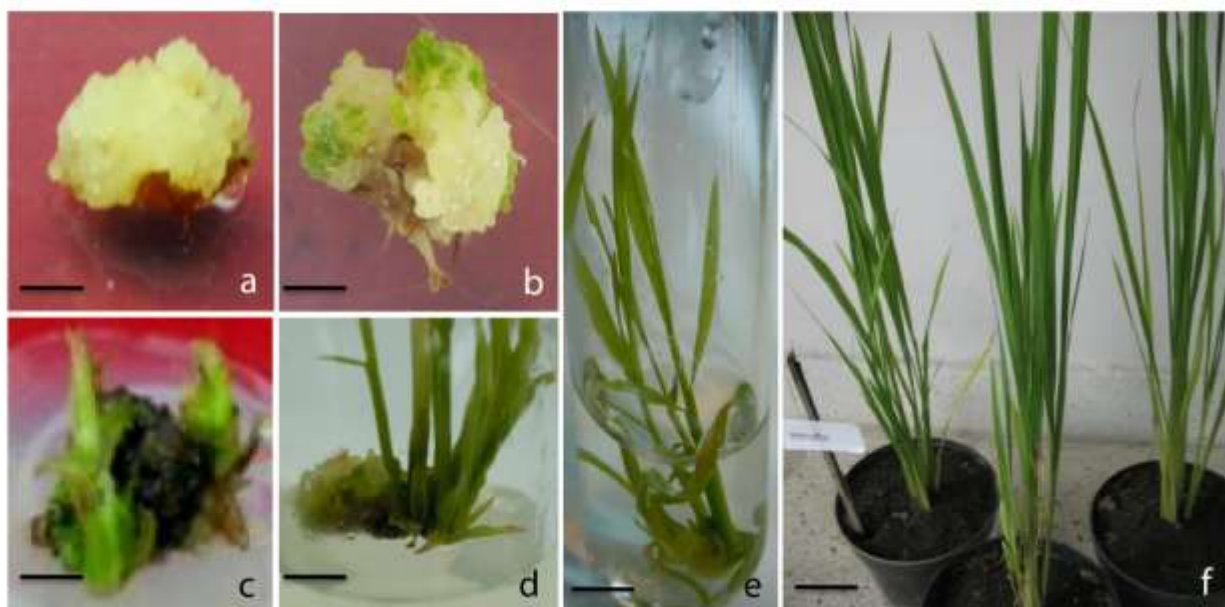


Fig. 4a-f Multiple shoot induction and plant regeneration from embryogenic callus of *Oryza sativa* spp. *indica* cv. Ketokijoha. (a) Embryogenic callus, (b) Induction of shoots from callus in NB medium supplemented with 0.5 mg/l BAP and 0.25 mg/l ABA after 4 weeks in culture, (c) Callus showing multiple shoot formation after 4 weeks in culture, (d) Callus showing multiple shoot in NB medium, (e) Rooting in MS medium, (f) Regenerated plants growing in greenhouse. Bar represents 3 mm (a); 5 mm (b); 1 cm (c); 3 cm (d).

The stimulatory effect of BAP has been reported to facilitate regeneration from embryogenic callus cultures in rice (Hoque and Mansfield 2004, Rachmawati and Anzai 2006). On the contrary, BAP in combination with NAA have been reported to induce differentiation of shoots from embryogenic callus cultures (Karthikeyan *et al.*, 2009). Most of the shoot buds developed to shoots within 15-18 days and complete plants with multiple tillers (Fig. 3d and 4d).

Upon separation and transfer of individual shoots to half strength MS medium, numerous roots were developed within 2 weeks of culture (Fig. 3d and 4e). Over 94% of rooted plantlets survived when established in soil (Fig. 3e and 4f). The plants resumed growth in greenhouse reaching maturity and represented no phenotypic variation or sterility, irrespective of two cultivars tested.

Transformation and GUS Expression

It is generally known that genotype remains the major limiting factor restricting successful transformation in *indica* rice using seed scutellum derived callus (Ge *et al.*, 2006). In order to determine the competency of the regeneration competent scutellum derived callus of two varieties, Ketokijoha and Monoharsali to *Agrobacterium*-mediated genetic transformation, experiments were carried out to inoculate explants with *A. tumefaciens*. Strong transient *gus* expression was detected in the scutellum derived callus in both the varieties, Ketokijoha and Monoharsali (Fig. 5). The endogenous GUS activity (color) was not detected in non-transformed (control) explants.

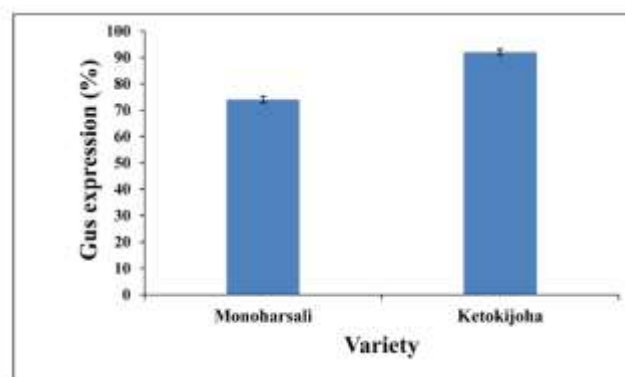


Fig. 5: Transient GUS expression in embryogenic callus of *Oryza sativa* spp. *indica* cv. Ketokijoha and Monoharsali after 3-days of co-cultivation. (a) Control (untransformed). Bar represents 1 mm, (b) *Agrobacterium*-cocultivated callus of Monoharsali (transformed) after 3 days of co-cultivation showing low GUS expression, (c) callus of Ketokijoha after 3days of co-cultivation showing intense GUS expression.

GUS activity at the regenerating sites indicated the amenability of explants to *Agrobacterium* mediated transformation. Transformation efficiency was determined by measuring the number of GUS positive calli per total calli inoculated. Highest transient *gus* expression efficiency was recorded in variety Ketokijoha (92%) whereas 74% transient GUS expression efficiency was recorded in Monoharsali (Fig. 6).

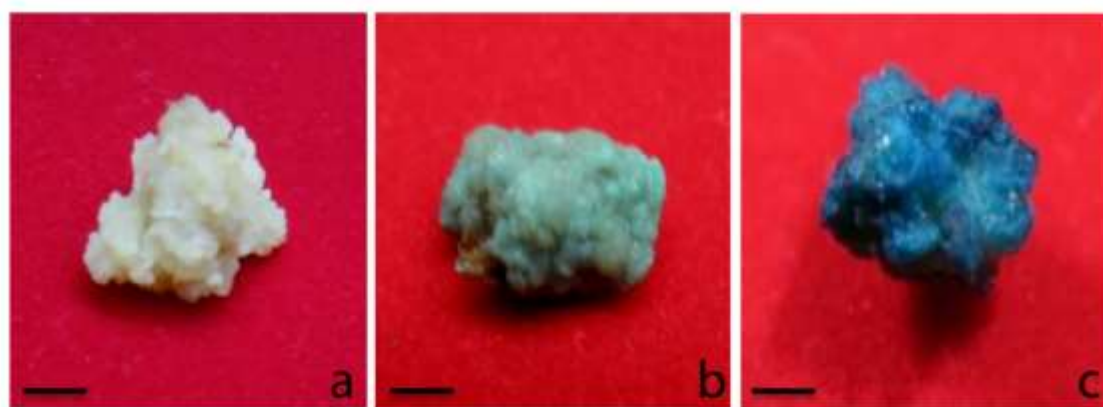


Fig. 6: Transient GUS expression (%) in embryogenic callus of two cultivars of *Oryza sativa* spp. *indica* of Monoharsali and Ketokijoha after 3-days of co-cultivation. X axis showing rice cultivar used in transformation and Y axis showing percentage of GUS expression in embryogenic calli.

Calluses derived from mature seeds have been effectively used as target for *Agrobacterium*-mediated transformation and regeneration of transgenic plants in a number of *indica* rice varieties (Hiei *et al.*, 1994, Ignacimuthu and Arockiasamy 2006, Kumar *et al.*, 2005, Ozawa *et al.*, 2009, Tyagi *et al.*, 2007).

Conclusion

Production of embryogenic callus with high regeneration capacity is a prerequisite for highly efficient genetic transformation in rice. Our findings established an efficient protocol for *in vitro* plant regeneration from mature seed derived callus in two of the most popular *indica* rice varieties of Northeast India, Ketokijoha and Monoharsali. Competency of plant tissues in *Agrobacterium* infection is an important factor in *Agrobacterium*-mediated transformation. Our results demonstrated that regeneration competent scutellum-derived callus of both the *indica* varieties are amenable to *Agrobacterium* mediated transformation and combined with their shoot proliferation ability could lead to regeneration of stable transgenic plants. The established method may find immense application as a practical tool for genetic improvement for valuable traits in two of these popular *indica* rice varieties of Northeast India.

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