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MMT, a High Promising, Cost Effective Micro-carrier for Gene Delivery

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ABSTRACT

As an alternative to the conventional but expensive gold micro carriers regularly employed in micro-projectile bombardment method, this study had utilized, novel, promising but cheaper clay particles-MMT (montmorillonite) as micro-carriers for gene transfer. Gene expression with novel micro carriers was reported through the universally employed GUS expression system at both transient and stable expression levels in comparison to the gold and tungsten.

Results suggest that GUS expression levels are higher with MMT than tungsten carriers but lower to gold carriers. It is interesting to note that the GUS expression zones are larger (>1 mm) with MMT carriers in comparison to tungsten (0.6 mm) and gold (0.4 mm). Molecular assays on the transformed cells suggest proper gene delivery by MMT carriers. The results suggest that novel micro carriers can be a viable alternative to gold for gene transfer with high promise in minimizing the costs without compromising the transformation efficiency.

Keywords: Rice; Transformation; Biolistics; Micro-carriers; Montmorillonite

INTRODUCTION

The biolistic or micro-projectile bombardment method, introduced in the late 1980's (Sanford, 1988), was an efficient method of plant transformation. This method is more versatile and allows transformation of plants and other organisms that are not amenable through other modes of gene transfer like Agrobacterium mediated transformation [1]. The Agrobacterium-mediated transformation, the other popular method, cannot be applied to all plant species/ genotypes due to poor infectivity or other related physiological parameters. Biolistic system has become the method of choice because it alleviates the need for preparing protoplasts, reduces the time needed to regenerate transgenic plants, and results in transgenic plants with higher fertility [2]. Employing this approach, pure linear transgene sequences can be transferred without any vector backbone interference. To achieve higher levels of transformation efficiency, biolistic method is preferable as large number of cells can be treated and transformed at a single time [3]. In addition, particle-mediated gene delivery is the only method reported so far to introduce foreign genes into cell organelles such as chloroplasts [4].

The first generation of this technique for gene transfer into plant cells was developed by plant virologist [5]. Particle bombardment has been widely exploited to produce tissues and plants expressing traits with agronomic value and has a major impact on basic plant science research and biotechnology [6-9]. Transgenic plants generated by this method have been reported for monocotyledonous species such as Kentucky bluegrass [10], jute [11], rice [12,13] and dicotyledonous species including moth bean [14], cowpea [15], sunflower [16] soybean [17] and wheat [18].

Application of biolistic method has demonstrated transient gene expression in plant studies by production of genetically transformed plants and tissues [7,19-22]. The universality of application through cell types, size, shape, presence or absence of cell wall and direct introduction of biological material into the cell has very high delivery efficiency to enhance the transformation rates [23]. This physical method of gene transfer is very often exclusively employed for DNA delivery in transient gene expression studies like analysis of tissue specific promoters [24] or to deliver DNA molecules carrying a marker gene and a chemical that is needed for transgene expression into plant cells simultaneously and release the encapsulated chemical in a controlled manner to trigger the expression of co-delivered transgenes in the cell [24]. Biolistic method is highly adaptable and may be used in other fields as well. For instance, the use of biolistic as a method to administer vaccines to humans is currently in preclinical trials [25]. The use of this technology for human vaccination is not unreasonable, as it is already being used to inoculate plant tissue with viruses for study [26].

Despite of these advantages, biolistic procedure is tedious, cumbersome and expensive. Moreover, introduced DNA

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will be expressed in the plant cell only if it is integrated into plant chromosomal DNA [27]. Hence integration of gene into chromosomal DNA is a chance factor and this is one of the reasons why the transformation efficiencies of biolistic method are comparatively lower than that of Agrobacterium mediated transformation, but the transient expression levels are always higher than that of stable expression as evidenced by GUS expression studies [28], which can be explained by the stabilization process during various steps of selection after bombardment till regeneration. These drawbacks can be avoided by following well established tissue culture protocols and stringent selection methods to come out with positive transgenic plants. In addition to the utilization of effective protocols and selection strategies, improvement of biolistic procedure is quite essential for effective gene transfer. Micro-carriers play an important role in gene delivery to the explants. Depending upon genotype, size of micro-carrier is also an important factor that governs the transformation efficiency of monocots [29]. A very small micro-carrier will have a lower penetration force and a bigger one will increase tissue/cell damage [30].

Several researchers used different types of micro-carriers for gene delivery viz. gold, tungsten, platinum, glass etc. Platinum and gold are expensive, while tungsten and glass have limitations in their usage. Gold particles are also preferred due to their high density, low toxicity and lack of chemical reactivity [31-33]. Moreover, gold particles, which are homogenous in size, biologically inert, non-toxic and do not degrade DNA bonds, are often preferred for particle bombardment. Tungsten particles are highly heterogeneous in size and shape, toxic, affects growth of calli and regeneration, can also acidify solutions and catalyse plasmid DNA degradation and moreover they are non-biocompatible [34-36]. There is evidence that tungsten toxicity can reduce the recovery of stable transformants in some plant species [37]. Low density, biocompatibility and particle impaction are important parameters to be considered in this area of research because cell damage may occur through particle impaction [38,21]. Hence there is search for alternate micro-carriers. MMT carriers have prominent advantage as they have low density, and particle impaction. Moreover clay particles are more biocompatible, making them a good carrier material for gene transfer. In this study, the potential of MMT as a micro carrier vis-a-vis gold and tungsten was reported. The assumption that clay minerals played an important role in the prebiotic formations of biomolecules that are basic to life, has paved the way for a series of studies on the adsorption of various organic molecules, including nucleic acids [39-41], DNA molecules have a persistent ability to transform competent cells when bound to clay minerals and other particles [42-44]. Much research has used 2:1 type layer phyllosilicates (e.g., montmorillonite) as adsorptive particles to understand DNA adsorption by soils. The phosphate group at the end of the DNA molecule binds directly to the OH groups present on the edges of phyllosilicates such as montmorillonite (Figure 1) [45,46].

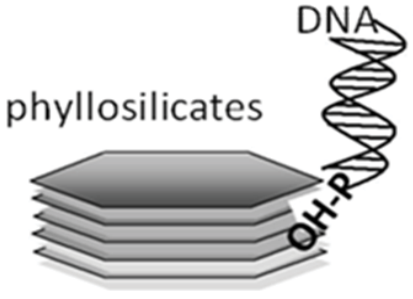


Figure 1: Conceptual figure of DNA adsorption by Phyllosilicates (MMT).

DNA retention can occur on negatively charged phyllosilicates through the formation of bridges by cations. Ca^{2+} binds to the SiOH groups of minerals such as silica and depresses the negative charges on the mineral surfaces, resulting in increased DNA adsorption [47]. This can be co-related to one of the important steps during preparation of micro-carriers where $Cacl_2$, when added, enable DNA to bind onto the micro-carriers. As it is evident that clay particles like montmorillonite could adsorb DNA, the present study reports an assessment of MMT with gold and tungsten as controls for delivery of DNA in biolistic mode of transformation.

MATERIALS AND METHODS

Genotypes

The rice genotypes selected for the study were three elite indica rice cultivars Swarna, Gayatri and Samba Mahsuri. Swarna, a widely grown indica rice variety in eleven states of India, is highly popular with a yield potential of 8.0 t/ha [48]. It is also being extensively grown in Bangladesh and Myanmar suggesting its wide adaptability [49]. Gayatri, a high yielding cultivar released from NRRI, is widely grown in shallow and medium low land ecology in Eastern India. Samba Mahsuri (BPT 5204) is one of the India's most popular and highly prized rice varieties because of its high yield 4.5 to 5.0 t/ha and excellent cooking quality [50].

Callus induction and proliferation

Mature dehusked grains of the three cultivars were washed with sterile distilled water and were surface sterilized successively with, 70% ethanol for two min, sodium hypochlorite (contains 4% (v/v) active chlorine) for 15 min and with 0.1% (w/v) aqueous mercuric chloride solution for 5 min with intermittent repeated washings with sterile distilled water [51]. The kernels were inoculated in culture tubes containing semisolid callus induction (CI) medium [MS medium supplemented with 2, 4-dichlorophenoxy acetic acid (2, 4-D) (2 mgl^{-1}), maltose (30 gl^{-1}) and solidified with gel-rite (2.6 gl^{-1})] [52] and the cultures were incubated in dark at 25 ± 1°C for three weeks. The scutellum-derived calli were excised and sub cultured on the same CI medium for another four days and clusters of highly embryogenic compact calli (3-5 mm in diameter) were selected and arranged (80-100 no/dish) side up in the center of petridishes (90 X 15 mm) containing 20 ml of semi-solid modified MS medium (MS salts and vitamins, maltose (30 gl^{-1}) L-proline (500 mg l^{-1}), casein hydrolysate (300 mg l^{-1}) and 2,4-D (2.0 mgl^{-1}), Myo Inositol (100 mgl^{-1}), Mannitol (36.4 gl^{-1}), Sorbitol, (20 g l^{-1}) Gelrite, (2.6 g l^{-1}) pH 5.8) ready for bombardment.

Plasmid preparation

A single colony of *E.coli* strain *DH5α/pCAMBIA*^{1301} carrying a GUS reporter gene and hygromycin selectable marker gene, under control of 35S promoter was picked from a freshly streaked selection plate and inoculated into 5 ml of LB medium [53] supplemented with the appropriate selective antibiotic to initiate a starter culture (Figure 2). The starter culture was incubated for approximately 8 h at 37°C with vigorous shaking (~220rpm). The starter culture was diluted (100 µl of starter culture was added to 100 ml LB medium supplemented with selective antibiotics) and the cells are grown at 37°C for 12-16 h with vigorous shaking (~ 200 rpm). The bacterial

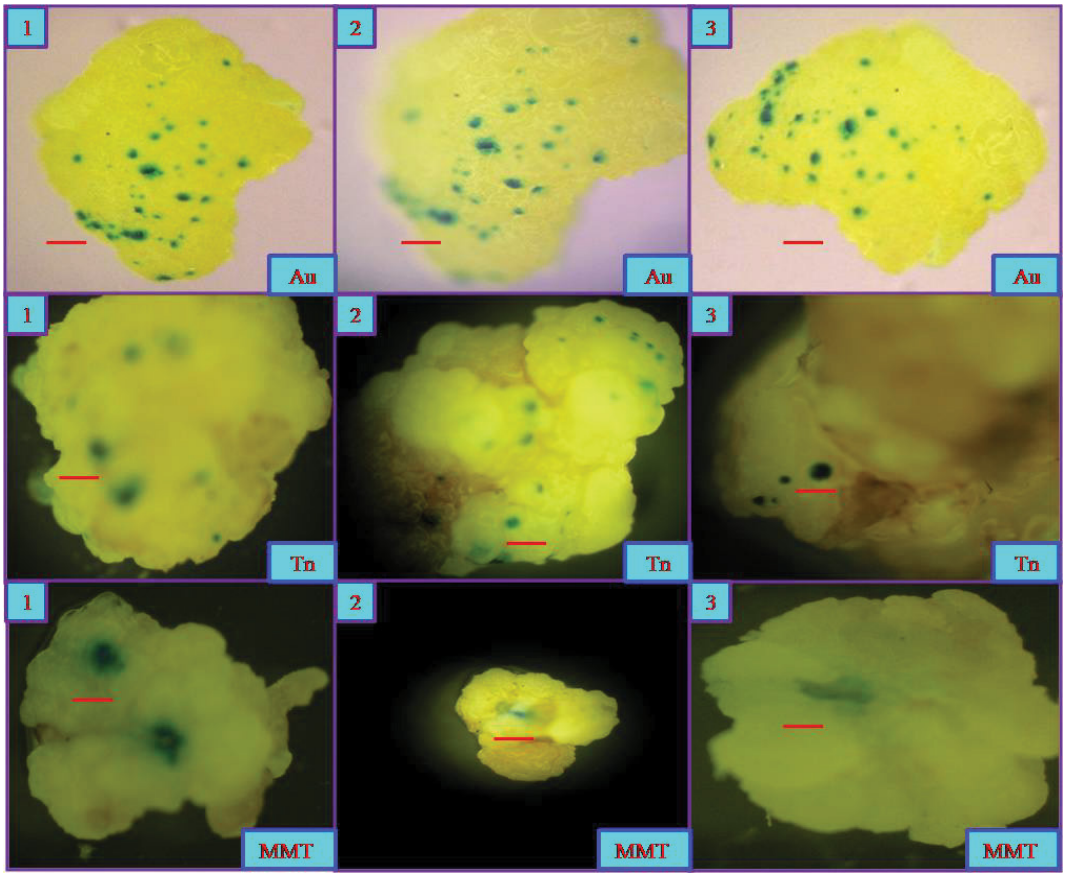


Figure 2: GUS expression recorded with three different micro carriers.

cells were harvested by centrifugation at 6000 g for 15 min at 4°C. Further plasmid extraction steps were followed using Quiagen Plasmid extraction kit as per manufacturer's instructions.

Transformation

Micro-carriers were prepared as per standard protocol using gold (1 µ), tungsten (>1 µ) and MMT (<1 µ) particles. Embryogenic calli were bombarded at 1100 psi helium pressure with the transformation vector pCAMBIA1301 (contaning both GUS and Hpt genes) coated on three different micro carriers in three individual experiments using the particle gun PDC-1000/He system (BIORAD) following manufacturer's instructions.

Selection

After the bombardment, the calli were plated on media supplemented hygromycin (30 mgl^{-1} for Swarna and 40 mgl^{-1} for Gayatri and Samba Mahsuri) and the selection was continued for four selection cycles and GUS expression was studied in the first (transient) and last (stable) selection cycles.

DNA Extraction and PCR assay

Actively growing calli on the selection media were selected and from a half portion of the callus, DNA was extracted following the mini prep method [54] while, GUS histochemical assay was conducted on the second half of the callus as per [55]. The presence of GUS gene was examined by polymerase chain reaction (PCR) with the help of specific primers to give an amplification product of ~ 1.2-kb size. The plasmid DNA (pCAMBIA1301) was used as the positive control and non-transformed callus DNA is taken as negative control. The PCR mix contained 1 µl of plant DNA (20 ng), 0.8 µl of 2.5 mM dNTPs (Fermentas), 1.0 µl of 10X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, and 15 mM $MgCl_2$; Sigma), 0.2 µl of Taq DNA Polymerase (5 U/µl Sigma), 1 µl each of both forward and reverse primers (5 pico moles/µl Sigma) and 5 µl of autoclaved sterile distilled water in a total volume of 10 µl. The amplification was done in a thermal cycler (Eppendorf Vapo protect) under following conditions: An initial denaturation of template DNA at 94°C for 2 min followed by 36 cycles of amplification i.e., 30 sec denaturation at 94°C, 30 sec primer annealing at 55°C, 1 min primer extension at 72°C and 10 min final primer extension at 72°C. Isolated DNA was also examined for the presence of Hpt gene which was used as the antibiotic selectable marker as per the conditions similar to that of GUS gene amplification mentioned above but with the different PCR program as per the following conditions: an initial denaturation of template DNA at 94°C for 5 min followed by 40 cycles of amplification i.e., 1 min denaturation at 94°C, 1 min primer annealing at 58°C, 1 min primer extension at 72°C and 5 min final primer extension at 72°C. PCR products were separated in 1.2% agarose gel (in 1X TBE electrophoresis buffer) containing 0.5 g/ml ethidium bromide. Separated PCR products were visualized under UV light and photographed by gel documentation system (Alpha innotech) to examine the size of the product.

GUS assay

The GUS gene expression analysis was performed on calli within 2-4 days after bombardment before subjecting the tissues for antibiotic selection [56] and also at the end of the 4th selection cycle. For the histo chemical detection, segments (5 mm in length) of rice tissues were incubated in a reaction mixture of 50 mM phosphate buffer (pH 6.8), 1% Triton X-100, 20% methanol and 1 mM 5-bromo-4-

chloro-3-indolyl-β-D-gluronide (X gluc). The reaction was initiated under a mild vacuum for few min and carried out overnight at 37°C. The frequency of transient transformation is expressed as the ratio between the number of calli showing GUS expression and the total number of calli kept for staining. For qualitative assay, the area of GUS expression and intensity of the blue colour at each spot were given due weightage so as to give a holistic picture of the effects of the various micro-carriers used. Through visual observations, distinction could be made between small spots (<0.5 mm in diameter) representing one or few GUS expressing cells, and large spots (>1 mm in diameter), representing a complete cell cluster expressing the GUS gene [57].

RESULTS AND DISCUSSION

The results suggest that gold carriers were ranked first both in transient (8.75%) and stable (20%) assays and MMT, was ranked 2nd in transient (7.84%) but 3rd in stable assays (16.6%) while tungsten was ranked last in transient expression (7.36%) but 2nd (20%) in stable expression assays (Tables 1 and 2). From the results it is evident that gene transfer is feasible with MMT carriers at reasonable frequency in all the genotypes tested.

When the GUS expression zones were measured, the area (diameter) of the zones varied for different types of micro-carriers. It is interesting to note that the diameter of the zone with MMT carriers is more (>1 mm) compared to that of both tungsten (0.6 mm) and gold (0.4 mm) carriers though for the number of GUS expression zones category, gold is ranked first followed by tungsten and MMT (Table 3 and Figure 3). Though gold carriers can induce higher number of expression zones, it is interesting to note that area of GUS expression zone is large in the case of MMT carriers followed by tungsten and gold.

The results on the amplification of GUS and Hpt gene sequences through PCR assays suggest that the presence of both GUS and Hpt genes was detected with all the three micro-carriers with varying frequencies. While 12 positives were recorded with gold, 9 positives were recorded with tungsten and 4 positives were recorded with MMT for both the genes where as amplification was not observed in the wild types (Figure 4a-c). The stable integration of the GUS gene was corroborated by molecular analysis and the same is case with Hpt gene. It is clear from the results that with all the three micro-carriers, stable transformation is possible.

However, one of the important factors to be taken into consideration is the presence of regions with dark brownish colour in the calli in case of both tungsten and MMT. However, these dark regions are in higher frequency in tungsten than in MMT and in case of tungsten, the origin of these regions is attributed to toxicity of the tungsten to the cells [37]. In addition, from this study, it is evident that genotypes vary in the transformation rates and influenced by the type of carriers employed. These results are in accordance with our earlier report on the variation in genotypes for transformation rates [28].

As in the other transformation experiments, the transient gene expression rates are more in this study also. Since the transient gene expression is temporary and it occurs almost immediately after gene transfer, its rate was higher but this require stable integration of the transgene. Transient gene expression is a rapid and useful method for analysing the function of gene of interest [58] and the transient expression frequency provides the most convenient measure of the frequency of introduction of DNA into explants during the optimization of bombardment conditions [59]. In contrast, although the stable expression occurs with the lower frequency, the expression was maintained for long term as the DNA gets incorporated into the chromosome of the recipient cell [60,61].

Introduction and expression of an exogenous gene into cells does not always involve stable integration of the gene into the genome of the recipient cell. This may be because of the tissue culture conditions and the regeneration capability of the genotypes used

Table 1: Transient GUS expression observed with three different micro carriers.

Variety	Particle	No. of calli tested	No of GUS positive calli	Transformation Efficiency (%)	Rank
Swarna	Gold	190	26	13.68	1
Gayatri	Gold	80	7	8.75	2
Swarna	MMT	102	8	7.84	3
Samba Mahsuri	Tungsten	190	14	7.36	4

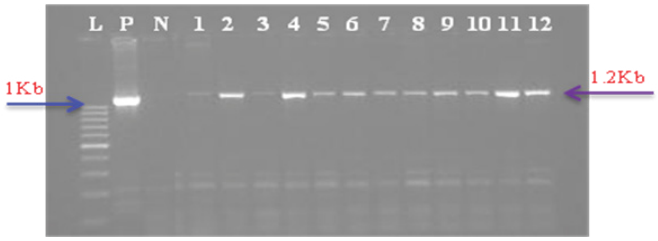
Table 2: Stable GUS expression observed with three different micro carriers.

Variety	Particle	No. of calli tested	No of GUS positive calli	Transformation Efficiency (%)	Rank
Gayatri	Gold	5	1	20.0	1
Samba Mahsuri	Tungsten	20	4	20.0	2
Swarna	MMT	18	3	16.6	3
Swarna	Gold	18	2	11.1	4

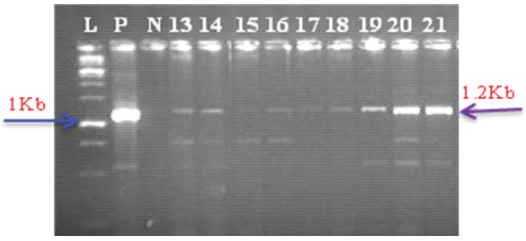
Table 3: GUS expression zones (in dia) observed with the three micro carriers.

Type of Micro-carrier	No of GUS expression zones per callus			Area of GUS expression (dia)
	Swarna	Gayatri	Samba mahsuri	
Gold(Au)	28	25	26	0.4 mm
Tungsten(Tn)	6	12	4	0.6 mm
Montmorillonite (MMT)	2	1	1	1.0 mm

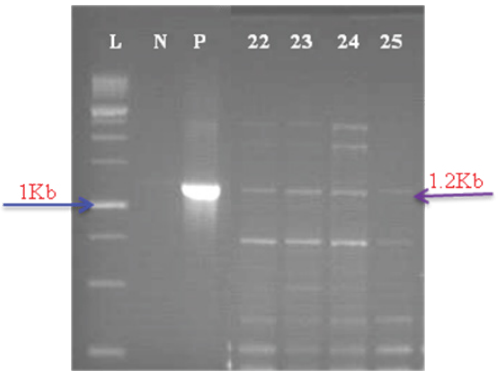
a: Gold micro carriers



b: Tungsten micro carriers

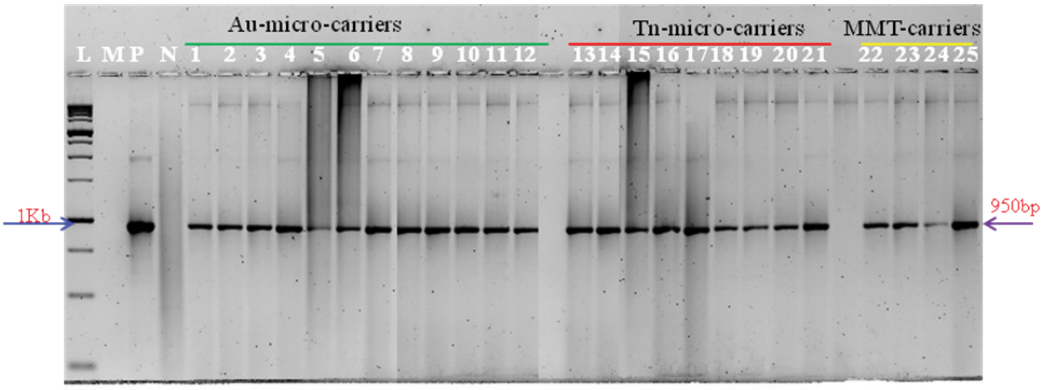


c: MMT micro carriers



L-0.1kb ladder, P- positive control (plasmid pCAMBIA1301),
N-Negative control (wild type), Nos (1-12) (13-21) (22-25) – samples

Figure 3: PCR amplification of GUS gene with three different micro carriers.



L-1kb ladder, P-positive control (plasmid pCAMBIA1301),
N-Negative control (wild type) Nos (1-25) – samples

Figure 4: PCR amplification of *Hpt* gene.

for transformation. It is evident in our previous experiments that the cultivars with good callus induction abilities may not have good regeneration potential and vice versa [28] and also due to stabilization in gene integration process. Frequently, plasmid DNA may be introduced into host cells and may express in a transient fashion. This activity declines over time and eventually disappears. Even though measuring levels of such transient activity may be useful in specific cases, when it comes to the creation of stable transgenic phenotypes, only transformation events leading to integration of the foreign gene into the genome of the host cell are useful [62-65].

Though more experiments are needed to standardize the protocols and to improve its efficiency in gene delivery, the present results suggest that MMT can be viewed as a viable alternative to the highly popular but expensive gold micro-carriers [66,67]. Since MMT constitutes only clay particles, major toxicity concerns associated with the metal micro carriers may not be issue with MMT and being inexpensive, the experimental costs to develop new Transgenics can be minimized and more number of Transgenics can be generated.

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AUTHOR'S CONTRIBUTION STATEMENT

Idea to utilize MMT micro-carriers was the brain child of GJN Rao, He had supervised the entire work, corrected the manuscript and is the driving force for the success of this work. Sai Krishna Repalli had done the bombardment experiments, generated putative transgenic and compiled the manuscript. Chaitanya Kumar Geda had done the GUS expression analysis.

COMPLIANCE WITH ETHICAL STANDARDS

This manuscript is in compliance with ethical standards.

CONSENT FOR PUBLICATION

That is applicable for this work.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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