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RESEARCH ARTICLE

ASSESSMENT OF PLANT TRANSFORMATION TECHNOLOGY OF MAIZE

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ABSTRACT

Crop improvement is important criteria in the world. For this, conventional breeding is used to increase food production and create thousands of crop varieties to fulfil the demands of agricultural products. But conventional breeding is failure due to time consuming, unpredictability and lack of relative varieties. So, to overcome such problem, genetic engineering was introduced. It allows gene transfer without conventional breeding, for unrelated genera or species. It helps in increasing genetic resource to improve crop and also introduce known function of genes to achieve the goals of crop improvement by genetic engineering programme which is more predictable. There are no robust transformation technologies available in maize for higher transformation efficiency and quality of products. For this, two methods are developed. They are tissue culture dependent and independent for gene transfer technology. The methods are *Agrobacterium* mediated transformation and particle bombardment. To these methods, results are evaluated and assessed for both *Agrobacterium* and biolistic gene gun/particle bombardment with parameters like transformation efficiency, copy number, expression level of *GUS* gene and its inheritance. The transformation efficiency and copy number is higher in the biolistic gene gun/particle bombardment when compared to *Agrobacterium* mediated transformation but expression levels are low, may be attributed due to gene silencing and suppressing. Whereas in *Agrobacterium* mediated method, transformation efficiency and copy number is low, but expression levels are higher when compared to particle bombardment. The present report indicates that the *Agrobacterium* derived transformants has low copy number, higher expression of the transgene and its stability of gene is more than the biolistic gene gun/particle bombardment.

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INTRODUCTION

To improve crops through genetic engineering, an efficient transformation system is required. Currently, there are several transformation protocols available, but still it need to be optimizing transformation efficiency, reduce cost and improve the quality of products. In 1992, pollen tube pathway was developed to soyabean. But it was failure due to lack of poor repeatability and insufficient molecular evidence for confirmation evaluated (Lin *et al*., 2003).

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For this, two methods are developed. *Agrobacterium* mediated transformation and biolistic gene gun/particle bombardment. *Agrobacterium* transformation was first established (Ishida *et al*., 1996) and also *Agrobacterium* mediated transformation was successfully reported (Gordon-Kamm *et al*., 1997; Mc Cabe *et al*., 1988). These transformations has extended too many years and well established. Later it has extended to monocotyledon plants (Hiei *et al*., 1994; Smith and Hood, 1995; Tingay *et al*., 1997). Meanwhile, biolistic gene gun/particle bombardment was introduced. It is the most effective plant transformation system applied for cereals (Christou *et al*., 1991; Chen *et al*., 1998a; Li *et al*., 1993; Spencer *et al*., 1992; Vasil, 1994). In recent years, protocols for particle bombardment have been well developed, modified and routinely used in various crop (Zhang *et al*., 1996; Zheng

et al., 1996) and *Agrobacterium*-mediated transformation has also been adapted to rice (Park *et al*., 1996; McElroy *et al*., 1990). The quantity of transgene was evaluated by copy number, expression and stability of transgene expression of both *Agrobacterium* mediated and biolistic gene gun/particle bombardment. In present investigation, we focused on assessment of transformation technology in both *Agrobacterium* mediated and biolistic gene gun/particle bombardment of maize.

MATERIALS AND METHODS

Bacterial strain and plasmid

The experiment was carried in Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh, India on 3rdday March 2015 to 20thday August 2015. A disarmed *Agrobacterium tumefaciens* strain LBA 4404 harboring a binary plasmid pCAMBIA-1301 was used (Fig-I). The plasmid contained the *β*-glucuronidase as reporter gene (*GUS*) from *Escherichia coli* with an intron, driven by the cauliflower mosaic virus (*CaMV*) 35S promoter and *nos* poly-A terminator sequences and the selectable marker gene *neomycin phosphotransferase* gene (*nptII*) and *hygromycin phosphotransferase gene (hptII)* for antibiotic resistance to *kanamycin* and *hygromycin* which are under the control of *CaMV* 35S promoter and *CaMV* 35S poly-A terminator.

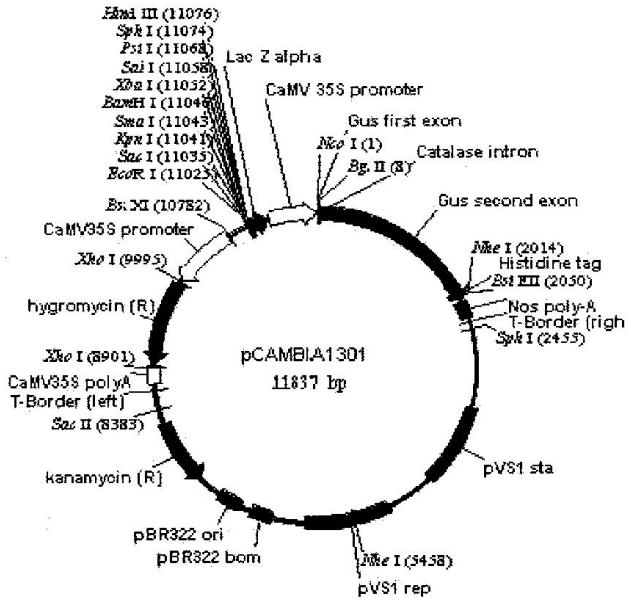


Fig.1. Restriction map of the plasmid pCAMBIA 1301

Agrobacterium mediated transformation

Agrobacterium is cultured in Luria Bertani medium containing 5µL *Rifamcin* and 50mg L^{-1} *kanamycin* at room temperature for 3 days. *Agrobacterium* culture was cultured, collected and resuspended in ½ MS medium with 100µM acetosyringone, with an optical density (OD) of 0.5-1.0 at 600nm. The maize calli are transferred to bacterial suspension for 10-15 min. After briefly draining the calli on sterilized paper filter, the calli are transferred to co-culture medium. The co-culture medium contains MS salts, 3% sucrose, 250 mg L^{-1} *cefotaxime* and 0.4% agar-agar. The *cefotaxime* is used to repress the overgrowth of *Agrobacterium.* The p^H was adjusted to 5.8. After 7 days, the calli are transferred to selection medium contain *kanamycin* 50 mg L^{-1}.

Biolistic gene gun/Particle bombardment transformation

Biolistic gene gun/Particle bombardment was carried out on the same batch of calli that were raised along with the calli used for *Agrobacterium* mediated transformation. Transgenic plants are produced as described and also with some modified protocol.

Gus assay

By Gus assay, the expression levels (Jefferson, R.A., 1987) in transgenic plants give very useful information in optimizing the transformation protocol. Transfer the explants (calli from immature embryos) into ½ MS medium; incubate the explant in *Agrobacterium* culture harboring pCAMBIA 1301 gene construct in dark for 3 days. Transfer the co-cultivated explants in MS medium supplemented with *cefotaxime* (5mg L^{-1}) for about a week so as to suppress the growth of *Agrobacterium*. After 7 days of incubation, immerse explants in 0.5mL of buffer P in a micro centrifuge tube and incubate at 37°C for 1hr. Remove the buffer and add 0.3mL of buffer x place under mild vacuum for 5 min. Then incubate the setup for 18hrs at 37°C and examine explants colour change under stereomicroscope.

Reagent setup

100Mm X-gluc: Dissolve 5mg X-gluc (sigma B_{6650}) in 1mL of ethylene glycol monomethyl ether (sigma E_{5378}).Store in dark at -20°C.

50mM NaH_2PO_4: Dissolve 7.8g $NaH_2PO_4.2H_2O$ in 900 mL of distilled water and makeup to 1000mL.

50mM Na_2HPO_4: Dissolve 17.91g $Na_2HPO_4.12H_2O$ in 100mL of distilled water and makeup to 1000mL.

Buffer P: Add 50mM Na_2HPO_4 (about 500mL) to 1000mL of 50mM NaH_2PO_4 until the P^H reaches to 6.8. Sterilize using a 0.22µm cellulose acetate filter and store at room temperature. Mix 9.9mL of this buffer and 0.1ml of triton X-100 before use.

Buffer X: Mix 8mL of buffer P, 0.1mL of 100mM X-gluc and 2mL methanol just before use.

Southern blotting

Genomic DNA was extracted from leaf sample as per protocol reported by Dellaporta *et al*., 1983. For each sample, 10 µg of genomic DNA was digested by restriction enzyme *Xba1* to restrict the genomic DNA which cuts at a single restriction site within the plasmid DNA. Then southern blots are carried out as described.

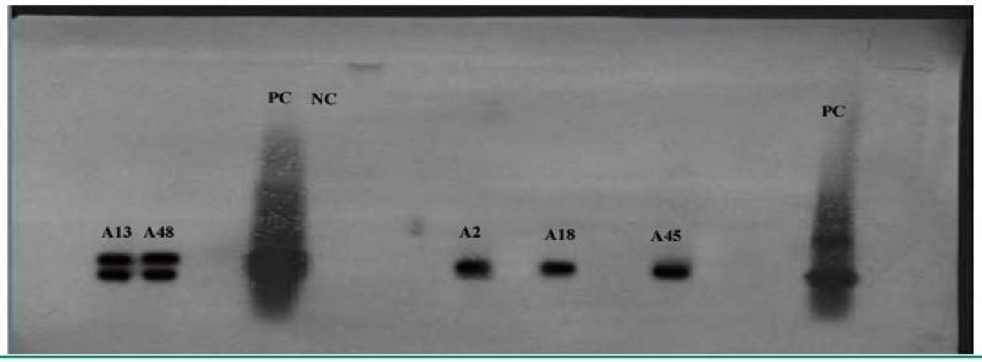
A probe for *nptII* gene, a 0.8 kb fragment within the *kan*^{r} coding region was cut from pCAMBIA using *Xho I* restriction enzyme or PCR amplified using the following primers Forward primer: 5'-CGT TAT GTT TAT CGG CAC TTTG-3'; Reverse primer: 5'-GGG GCG TCG GTT TCC ACT ATCG-3' respectively. Apart from *nptII* gene as a probe, *GUS* gene probe of around 0.6 kb was also used in southern blotting experiment to determine the gene copy number in the transgenic plants. It is shown in Fig-II A & Fig-II B

If leaves become yellow, they are non-resistant and if leaves are green and healthy, they are resistant. These are selected as putative transgenic plants.

RESULTS AND DISCUSSION

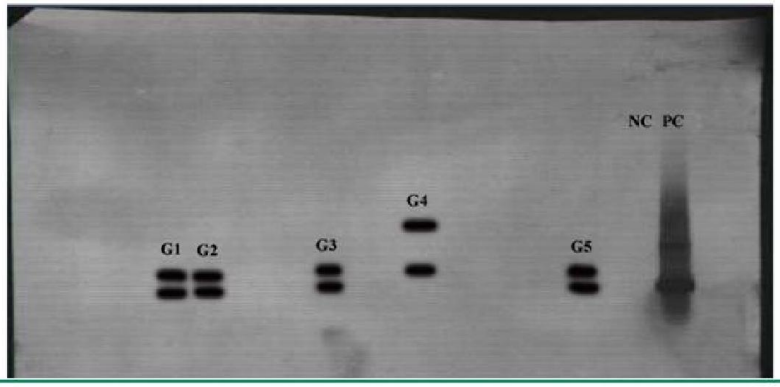
To control other factors that affect development of transgenic plant, we used a plasmid called pCAMBIA 1301 for both *Agrobacterium* mediated and biolistic gene gun/particle

Fig.II A Southern hybridization blot developed via *Agrobacterium*-mediated transformatin



PC : Positive control (pCAMBIA-1301 digested with restriction enzyme Xba1)
NC: Negative control (genomic DNA from control untransformed plant)
Lanes A -2, A-13, A-18, A-45, A-48: *Agrobacterium* mediated transformation events

Fig.II B Southern hybridization blot developed via particle gun mediated transformatin



PC : Positive control (pCAMBIA-1301 digested with restriction enzyme Xba1)
NC: Negative control (genomic DNA from control untransformed plant)
Lanes G-1, G-2, G-3, G-4, G-5: Particle gun mediated transformation events

Analysis of *kanamycin* in T_0 progeny

Seeds of T_0 Plants are sterilized and germinated on MS medium for 10-15 days. Leaves are collected from the seedlings and *GUS* histochemical assay was performed. Some of the seedlings are subjected to *kanamycin* selection (50 mg L^{-1}).

bombardment transformation with selection medium (*kanamycin*) is same for both. The difference among the *Agrobacterium* mediated and biolistic gene gun/particle bombardment are, transformation efficiency and antibiotic used (Table-I & Fig-III). The transformation efficiency is determined by the number of plants resistant to *kanamycin* and

Table 1. Efficiency of transformation among *Agrobacterium* and Biolistic gene gun/particle bombardment of maize

Exp date	Embryos infected	Callus events expressing gus	Events regenerated	Transformation efficiency%
Agrobacterium				
Day third	21	2	2	9
Day seventh	82	8	13	10
Day eleven	63	4	18	6
Day seventeen	126	10	45	6
Day twenty	155	15	48	9
Particle bombardment/Biolistic gene gun				
Day third	21	4	1	19
Day seventh	82	12	2	15
Day eleven	63	7	3	11
Day seventeen	126	22	4	17
Day twenty	155	35	5	23

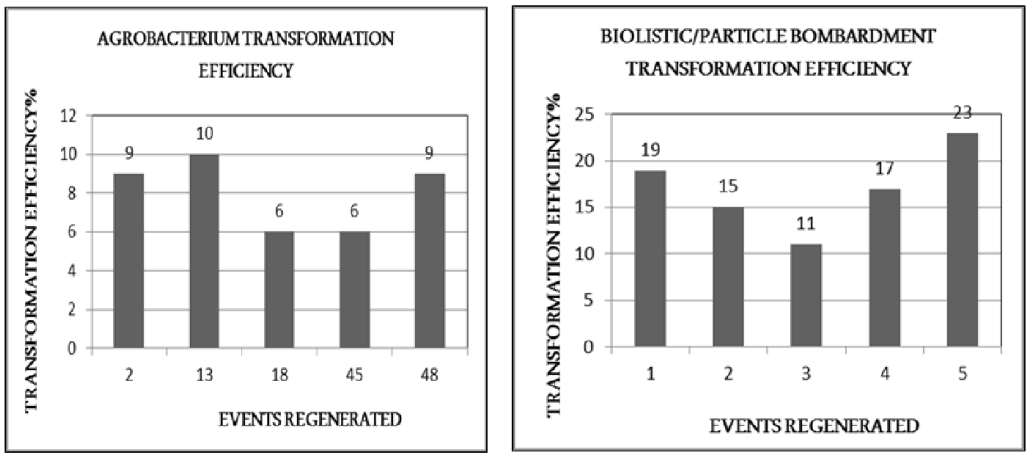


Fig. III. Graphical representation of transformation efficiency of Agrobacterium and Biolistic gene gun/particle bombardment of maize

Table 2. Estimation of Transgene copy number in maize via both *Agrobacterium*-mediated and Biolistic gene gun/particle gun bombardment

Events of *Agrobacterium*Transformation	Copy number	Events of particle bombardment	Copy number
A_2	1	G_1	16
A_{13}	2	G_2	28
A_{18}	1	G_3	35
A_{45}	1	G_4	29
A_{48}	2	G_5	42

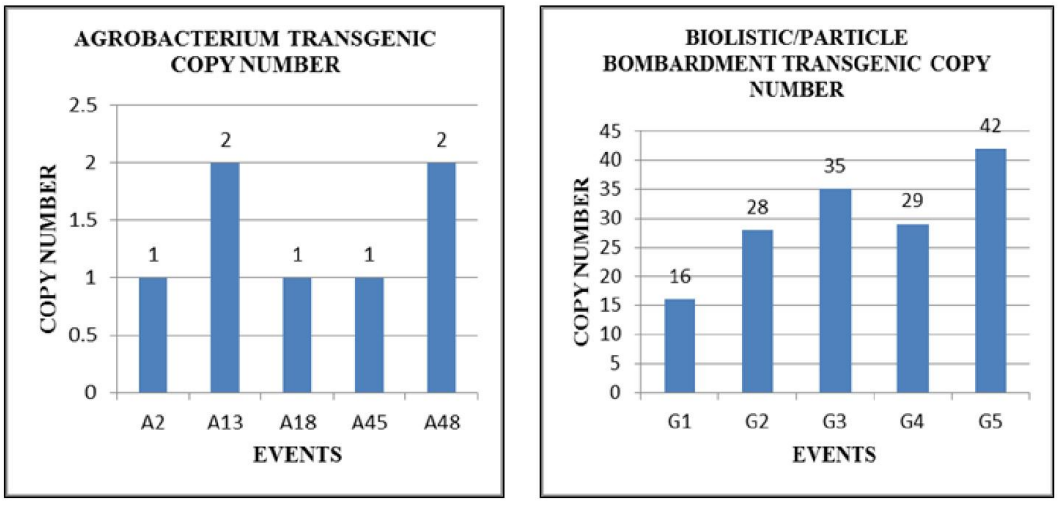


Fig. 4. Graphical representation of transgene copy number of maize

number of calli used. The transgenic plants are transferred to green house for further analysis.

Gene copy number

The copy number of foreign genes within T-DNA region was analysed by southern blotting. The average copy number of the *GUS* gene was 1.8 and 3.5% among *Agrobacterium* and biolistic gene gun/particle bombardment respectively (Fig-IV). The average copy number of genes per line was similar for most of the plants obtained by *Agrobacterium*-mediated transformation. In contrast, the number of gene copies was more variable for lines of biolistic gene gun/particle bombardment (Table-II). The *GUS* expression level and *GUS* stability and activity are more in *Agrobacterium* mediated when compared with biolistic gene gun/particle bombardment. In case of segregation pattern, the *GUS* activity and resistance of *kanamycin* was analysed. It showed that in *Agrobacterium* mediated transformation segregation pattern, followed the Mendelian pattern of inheritance i.e one homozygous and two heterozygous. The pattern of segregation of *GUS* and kan^r gene was also investigated. They showed 1:1 segregation pattern, as genes are co-segregated. In most of transgenic plants derived showed, *Agrobacterium* mediated lines has co-integrated in generated lines whereas biolistic gene gun/particle bombardment showed abnormal segregation pattern reported (Chen *et al*., 1998b; Hade *et al*., 1996). In case of fertility, most of transgenic plants derived by *Agrobacterium* mediated are fertile than biolistic gene gun/particle bombardment.

In the present investigation, we reported transformation efficiency, copy number, gene expression and fertility of transgenic line. The transformation efficiency was low in *Agrobacterium*-mediated when compared with biolistic gene gun/particle bombardment. *Agrobacterium* mediated transformation is genotype dependent method as reported earlier (Rashid *et al*., 1996) which influence a copy number on level of gene expression. The present reports too agree well with the earlier reports. Generally increase in copy number should increase gene expression but it leads to decrease in expression, because may be due to co-suppression and gene silencing (Vancheret *et al*., 1998). In some cases, simple gene copy number also undergoes silencing as reported by Elmayan and Vancheret, (1996). Generally, low copy number decrease the co-suppression and gene silencing. Hence increase in gene expression takes place (Hobbs *et al*., 1993). In present investigation, *Agrobacterium* mediated transformation has low copy number; hence it has high expression of gene, whereas in biolistic gene gun/particle bombardment, multiple copies takes place. So expression will be low. The transgenic co-integration and co-segregation phenomenon in transgenic plants developed from direct DNA delivery transformation systems makes the job easy to genetically select pure lines from progenies. It also useful for map-based breeding program as reported earlier (Kohli *et al*., 1998). The integrity of the foreign DNA is different for two types of transformants. May be T-DNA and vector integrates to plant chromosomes or partial integration of T-DNA takes place (Kononov *et al*., 1997). In this case, *Agrobacterium* mediated transformation has better intact to plant chromosome than biolistic gene gun/particle bombardment.

From copy number, it has showed that variability in case of integrity in biolistic gene gun/particle bombardment. The re-arrangement and multimerization of transgenes resulted in direct and an indirect repeat of co-integration, as well as integration of partial transgenes was explained by Pawlowshi and Somers, (1996). May be re-arrangement of transgenes will improve in more expression in their function (Van Eck *et al*., 1995). Fertility of transgenic plants is very important for either using transgenic plants as genetic material for a breeding program or using transgenic plants for basic research. The fertility was better in *Agrobacterium* mediated, as gene stability is within integration of chromosome. So, it has shown Mendelian segregation, whereas biolistic gene gun/particle bombardment has low fertility due to multigenes present in single locus and gene fragment are inserted in random. Multiple genes can be transformed by biolistic gene gun/particle bombardment and arrangement of large fragment was explained. Co-transformation, co-segregation provides simple way to apply transgenic material to breeding programmes. Gene fragment insertion and re-arrangement of gene sequence still need to be improved.

Conclusion

Agrobacterium mediated transformation is well established in dicotyledons plants and also co-transformation method may provide an easy way to produce selection marker free transgenic plant. The disadvantage of *Agrobacterium* are low transformation efficiency and still to be improved in monocotyledons. However, it has its own advantages and disadvantages. The goal of present investigation is to report the assessment of transformation technology by *Agrobacterium* mediated and biolistic gene gun/particle bombardment. The present investigation results shows and supports that *Agrobacterium* mediated transformation has more advantages than biolistic gene gun/particle bombardment.

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