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**SHORT COMMUNICATION**

# OPTIMIZATION OF GENE GUN "GENE PRO HE-2000" FOR MICROPROJECTILE GENOMIC TRANSFORMATION IN PAPAYA (*Carica papaya* L.)

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## INTRODUCTION

Pathogen Derived Resistance (PDR) has proved to be an effective tool in combating plant viruses. PDR has been found to be effective, whereby the transgenic plants expressing virus genome sequence resist the attack of corresponding viruses. Coat protein (*cp*) mediated resistance has been applied for a large number of transgenic lines, such as tomato, *citrus*, papaya, potato, peanut, squash and sugar beet. In papaya, Papaya Ring Spot Virus (PRSV) resistant transgenic cultivars, called SunUp and Rainbow have been developed by cloning *cp* gene of PRSV HA 5-1 and already commercialized in Hawaii.

Microprojectile transformation method is one of the most effective vector-less direct gene transformation method which has been used worldwide to transform papaya (Cai *et al.*, 1999; Fitch *et al.*, 1992; Tennant *et al.*, 1994). In that, gene gun is the instrument used to transfer the gene of interest into particular tissue. Gene Pro-HE-2000 (Endevour Enterprize, Hyderabad, India) is a new model of gene gun that delivers microprojectile suspension directly in to the tissue.

The Gene Pro-HE-2000 is equipped to change the distance from microholder to explant and the pressure inside the chamber for effective utilization of the microprojectile system. It is crucial to optimize pressure (He) and distance (microholder to explant) for effective utilization of the microprojectile system depending on the plant. Therefore the objective of this study was to optimize the Gene Gun "Gene Pro HE-2000" for papaya, var. Pusa Delicious.

## MATERIALS AND METHODS

The present study was carried out at Biotechnology Laboratory, Central Institute for Subtropical Horticulture, Lucknow (UP), India from August 2009 to June 2010. Somatic embryos developed from immature zygotes of papaya cultivar Pusa Delicious were utilized for this study.

**Culture medium**

Half strength basal Murashige and Skoog (1962) medium supplemented with different hormones, sucrose etc. was used as the culture media as follows;

(i) Induction medium
After bombardment, the somatic embryos were further proliferated on half strength MS basal medium fortified with 400 mg/l glutamine, 2-4, D 10 $mgl^{-1}$, sucrose 60 $gl^{-1}$and 100 $mgl^{-1}$ kanamycin (pH-5.8).

(ii) Maturation medium
In order to mature the somatic embryos, regeneration medium (devoid of Benzyl Amino Purine (BAP) and Naphthalene Acetic Acid (NAA) was fortified with poly ethylene glycol (PEG) under the selection pressure of kanamycin (50 $mgl^{-1}$). The pH was kept at 5.8.

(iii) Regeneration medium
Matured and germinated plantlets were further sub-cultured on regeneration medium for growth. Half strength MS medium was fortified with 0.2 mg/l BAP + 0.1 mg/l sucrose 60 $gl^{-1}$NAA along with 100 mg/l kanamycin (pH 5.8).

**Genetic transformation of papaya**

(i) The gene construct
Coat protein (*cp*) gene of PRSV cloned in pBI121 vector, which is driven by 35S promoter, *nos* terminator, *gus* reporter gene and *npt*-II selection marker gene were utilized for the transformation work. Plasmid from *E.coli* containing *cp* gene was isolated and utilized for microprojectile transformation studies. The construct used in the study has been developed by R.K. Jain, Plant Pathology Division, IARI, New Delhi, India.

(ii) Preparing tissue for bombardment
Young somatic embryos were chosen for bombardment. Around 100 embryos were kept in a petri plate and utilized for microprojectile transformation.

(iii) Preparation of Microprojectile suspension
Microprojectile suspension containing 50 mg of tungsten or gold particles, 50 µl of plasmid DNA (1 µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl of filter sterilized 0.1M spermidine was prepared.

(iv) Gene gun operation
Gene Gun "Gene Pro HE-2000", (Hyderabad, India) was utilized for bombardment of embryos. The He pressure of the gene gun was adjusted according to the treatments of the study (8, 10, 12 $kg/cm^2$). Three µl of

plasmid suspensions was taken from the centrifuge tube and transferred to the sample holder. First petri plate with pre-treated material was taken and placed in the center of sliding tray just below the filter holder of Gene Gun. Varying distance between holder and petri plate were 6, 7, 8 and 9 cm were maintained. A vacuum of 500 mm/Hg was maintained. The micro projectile was delivered into the target tissue with a powerful blasting.

Selection procedure

Just after the bombardment, the tissues were transferred to MS medium and kept for one week and thereafter transferred to the induction medium supplemented with 50 mg/l kanamycin. Four weeks after, the embryos were transferred to maturation medium with PEG and 50/mg/l kanamycin and kept for two weeks. Thereafter it was transferred to the regeneration medium. The survived plantlets in kanamycin medium were selected and utilized for further investigations.

## Molecular analysis

PCR was performed on total DNA extracted from leaves of putative transformants, by CTAB method. Transformed plants were identified using forward and reverse primers (Table 1) of *cp*, *nptII* and *gus* of *cp*-antisense gene construct. Thermocycler device 'My Cycler PCR system' (Bio-Rad) was used for amplification. The agarose gel electrophoresis was carried out using a submarine horizontal agarose slab gel as described by Sambrook *et al.* (1989).

**Table 1. List of primers**

| | *Primer name* | *Sequence (5'-3'), length of Primer* | *Annealing temperature ($^0$C)* | *Product size base pairs* |
|---|---|---|---|---|
| 1. | *cp* | (F) 5'-TCC AAR AAT GAA GCT GTG GAT GCT -3'<br>(R) 5'- GTT GCG CAT ACC CAG GAG AG -3' | 60 | 861 bp |
| 2. | *nptII* | (F) 5'- TCT CAC CTT GCT CCT GCC -3'<br>(R) 5'- AGG CGA TAG AAG GCG ATG -3' | 54 | 480 bp |
| 3. | *gus* | (F) 5'- AGC ATC TCT TCA GCG TAA GG -3'<br>(R) 5'- TAG ACA ACG AAC TGA ACT GG -3' | 55 | 400 bp |

### *gus*-assay

To screen the expression of ß-glucuronidase (*gus*) activity in genetically modified plants the method of Jefferson *et al.* (1987) was carried.

## RESULTS AND DISCUSSION

Somatic embryos and leaves of the transformants survived in kanamycin selection medium were tested for expression of *gus* activity, which cause the leaves to turn blue in the presence of the substrate *X-Gluc*. PCR amplification and genomic DNA analysis were carried out in order to test the presence of *gus*, *npt-II* and PRSV *cp* genes. DNA analysis is also a useful method to analyze the nature of the integration event (such as re-arrangement or copy number). Since integration of genes by gene gun process is a random event, the relevant *cp* and marker genes of the transformation vector did not always co-integrate, thus kanamycin-resistance is not always correlated with *gus* activity or presence of the *cp* gene.

### *gus* assay

Intense blue foci were observed under stereoscopic microscope on all putative transformants transformed with *cp* gene in antisense orientation in pBI121. Almost all embryonic tissues were stained with *gus* solution during transient expression. However, distinct staining of meristematic dome was observed during stable transformation. Our result was similar with those of Fitch *et al.* (1990) who mentioned that the nature of *gus* staining differed considerably in tissues from different transformation events. Some embryos appeared uniformly dark blue, while others were pale, as if only epidermis had been transformed. From a single event, globular somatic embryos showed an intense blue *gus* response, while cotyledons from the same isolate showed only pale blue *gus* expression.

### PCR analysis

*cp* gene from PRSV was cloned in the binary vector pBI121, which is driven by *35S* promoter, *nos* terminator and *npt*-II selection marker gene. Genomic DNA isolated from transformants were subjected to gene specific primers, which amplified 861 bp of amplicon of *cp* gene, 400 bp of *gus* gene and 480 bp of *npt-II*. DNA analysis showed the successful transformation of *cp, npt-II* and *gus* genes into the transformants.

### Effect of pressure and distance on transformation

Embryos were bombarded on four different pressures (12, 13, 14 and 15 kg/cm$^2$) and from three distances (9, 10.5 and 12.5 cm) (Table 2) and

subsequently shifted to medium containing 100 mg-1 kanamycin to observe kanamycin resistant embryonic clumps. Treatments (pressures), sub-treatments (distance from microholder to explants) and their interactions were significant at 5% probability level (Table 2). The highest number of embryo survival was observed at 12 kg/cm$^2$ pressure level with 9 cm distance (18.6%). Increase of He pressure causes rupturing of the tissues leading to death of cells. When pressure is less, the microprojectiles could not penetrate the tissues properly.

**Table 2. Effect of pressure and distance on transformation in Papaya (*Carica papaya* L.)**

| Pressure (kg/cm$^2$) | Distance (cm) | Number embryo clumps survival in 100 mg/l kanamycin (16 weeks) |
|---|---|---|
| 12 | 9 | 18.6 |
| | 10.5 | 15.4 |
| | 12.5 | 11.2 |
| 13 | 9 | 13.2 |
| | 10.5 | 5.6 |
| | 12.5 | 3.6 |
| 14 | 9 | 5.2 |
| | 10.5 | 7.6 |
| | 12.5 | 10.6 |
| 15 | 9 | 0.0 |
| | 10.5 | 0.0 |
| | 12.5 | 1.1 |
| CV % | 12.3 | |
| $LSD_{(p=0.05)}$ (Pressure) | 0.81 | |
| $LSD_{(p=0.05)}$ (distance) | 0.70 | |

## Effect of types of particles on transformation efficiency

**Table 3. Effect of particle types on transformation efficiency**

| *Particle* | *Mean number of plants survived in kanamycin (4 weeks)* |
|---|---|
| Tungsten | 12.30[a] |
| Gold | 03.40[b] |
| $LSD_{(p=0.05)}$ | 3.24 |

Means followed by the same letter in the column is not significantly different (p = 0.05)

Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells. The concept has been described in detail by Sanford (1988). Klein *et al.* (1987) observed that, tungsten particles could be used to introduce macromolecules such as RNA

and DNA into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds. Christou *et al.* (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells and result in the recovery of stable transformants.

To optimize type of particle for carrying plasmid DNA into host tissues, two types of particles *viz.*, tungsten (0.6 μ Sigma, USA) and gold dust (0.1 μ Sigma, USA) were tried. Number of putative transformants was significantly high with tungsten as compared to gold particles (Table 3) and therefore, tungsten is better suited for biolistic transformation in papaya. Furthermore, tungsten particles are cheaper over gold dust, moreover, it facilitates clear visual observations. Many authors have reported the successful use of tungusten (Cai *et al.*, 1999; Fitch *et al.*, 1990) to deliver gene of interest into the target tissues.

## CONCLUSION

Microprojectile genomic transformation of papaya was successfully done using the gene gun "Gene Pro-He 2000" (Endevour Enterprises, Hyderabad). In order to optimize the biolistic gun for proper gene delivery, pressure (He) and distance (from microholder to the explant) were optimized. The highest percentage of embryo survival in kanamycin was observed at 12 $kg/cm^2$ pressure level with 9 cm distance. In order to optimize the type of particle for carrying plasmid DNA into host tissues, tungsten was better suited to that of gold. Bombarded embryos were analyzed in the presence of *gus* spot for transient and stable transformation. Intense blue foci were observed under stereoscopic microscope and it proved the presence of *gus* marker gene in putative transformants. DNA analysis also showed successful transformation of *cp* gene, along with *npt-II* and *gus* genes into the putative transformants.

## REFERENCES

Cai, W.Q., C. Gonsalves, P. Tennant, G. Fermin, M. Souza, N. Sarindu, F.J. Jan, Y. Zhu, and D. Gonsalves. 1999. A protocol for efficient transformation and regeneration of *Carica papaya* L. *In-Vitro* Cellular and Developmental Biology-Plant. 35: 61-69.

Christou, P. 1992. Genetic transformation of crop plants using microprojectile bombardment. The Plant Journal. 2(3):275-281

Fitch, M.M.M., R. M. Manshardt, D. Gonsalves, J.L. Slightom, and J.C. Sanford, 1990. Stable transformation of papaya via microprojectile bombardment. Plant Cell Reports. 9:189-194.

Jefferson, R.A., T.A.Kavangh. and M.W. Bevan, 1987. GUS Fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal. 6.3901-3907.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth of and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15:473-497.

Sambrook, J., E.F. Fritsch, and T. Manitis, 1989. Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbour, N.Y.

Tennant, P. F., C. Gonsalves, K. S. Ling, M.Fitch, R. Manshardt, J. L. Slightom, , and D. Gonsalves. 1994. Differential protection against papaya ringspot virus isolates in coat protein gene transgenic papaya and classically cross-protected papaya. Phytopathology. 84 (11):1359-1366.