Transformation of tomato using biolistic gun for transient expression of the β-glucuronidase gene

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We report for the first time, conditions for the biolistic transformation of tomato for the introduction of β-glucuronidase gene (gusA) into different explants viz., shoot tips, hypocotyls and cotyledons of genotype IPA-3. The respective per cent plant regeneration in different explants was 95.16, 79.30 and 90.14% on MS media supplemented with BAP (2.0 mg L⁻¹) and Kn (1.0 mg L⁻¹). The influence of physical parameters of particle gun on rates of transient GUS expression have been investigated viz., quantity of DNA, distance between the microcarrier launch assembly and target tissues; and the biological factors associated with the target tissues i.e., effect of osmoticum (mannitol), pre-bombardment culture period and post-bombardment culture period. Maximum GUS expression of 25.00, 33.00 and 22.20% was respectively recorded when 10-d-old shoot tips, hypocotyls and cotyledons were bombarded with 18 µL of DNA suspension. A firing distance of 7.5 cm was found to be most suitable recording 34.12, 36.56 and 22.69% transient GUS expression in shoot tips, hypocotyls and cotyledons, respectively. Addition of osmoticum into the culture medium reduced per cent GUS expression significantly in all the explants even at 1 molar concentration. Pre-culture of explants prior to bombardment also deduced the transformation efficiency, however post bombardment culture period of one day resulted in maximum GUS expression in all the explants.

Keywords: Lycopersicon esculentum, biolistic transformation, GUS assay, cotyledons, shoot tips, hypocotyls, β-glucuronidase gene

Introduction

Tomato (Lycopersicon esculentum Mill. 2n=2x=24) is a major vegetable crop that has gained tremendous popularity over the last century. In India, productivity of tomato is low primarily owing to its vulnerability to various biotic and abiotic stresses. Among the insect-pests, tomato fruit borer (Helicoverpa armigera Hubner) is the most devastating one. The pest has developed resistance to insecticides due to its continuous exposure1. Development of resistant crop varieties is an economical, sustainable and environmental friendly alternative. The wild tomato species, Lycopersicon hirsutum f. glabratum, possesses genetic resistance to the fruit borer2. But the conventional breeding methods are tedious, time-consuming and liable to an inadvertent linkage drag3. Therefore, transgenic approach, where crops are so designed to produce their own insecticides seems to be a viable approach. Whilst there have been several reports of Agrobacterium-mediated gene transfer in tomato4, however, the same is not true for biolistic/particle gun mediated genetic transformation, despite the fact that it has proved a valuable tool for gene expression and stable transformation5,6. Biolistic transformation is a method by which foreign genes are introduced into intact plant cells and tissues via high velocity micro projectiles7,8. This approach is genotype independent, rapid and helps in introduction of multiple genes through co-transformation and causes least disturbance to the recipient genotype. To maximize the number of transformed cells using Bio-Rad Helium-driven PDS-1000/He device9, it is necessary to optimize both the physical parameters of particle-gun and the biological parameters associated with the target tissues10. Since there have been no previously reported attempts to transform tomato shoot tips, hypocotyls and cotyledons by particle gun, we, therefore, conducted a series of experiments to determine factors affecting particle gun mediated genetic transformation in tomato.

Experiments were carried out to standardize the important factors such as firing distance, quantity of DNA, concentration of osmoticum, pre-bombardment...
and post-bombardment culture periods effecting transformation in tomato shoot tips, hypocotyls and cotyledons by using Bio-Rad Helium-driven PDS-1000/He device.

Materials and Methods

Media Preparation and Raising of Plant Material

Seeds of genotype IPA-3 were surface sterilized with commonly used commercial bleach, ‘Ala Bleach’, for 20 min followed by washing with sterile distilled water. The sterilized seeds were cultured on basal medium containing thiamine HCl (0.1 mg L\(^{-1}\)), pyridoxine HCl (0.5 mg L\(^{-1}\)), nicotinic acid (0.5 mg L\(^{-1}\)), myoinositol (100 mg L\(^{-1}\)), sucrose (3.0 g L\(^{-1}\)) and agar (0.8 %). The pH of medium was adjusted to 5.8 by adding 1N NaOH/1N HCl solution dropwise. The *in vitro* germinated seedlings served as an explant source for gene manipulation studies. The explants were cultured on MS medium supplemented with BAP (2.0 mg L\(^{-1}\)) and Kn (1.0 mg L\(^{-1}\)). The medium was first dispensed into glass jars (100 mL/jar) when still hot. These glass jars were properly capped before autoclaving at 1.05 kg cm\(^{-2}\) square pressure and 121\(^\circ\)C temperature for 22 min. To prevent the inoculation of microbes, all decontamination procedures and aseptic manipulations were performed in a laminar flow hood.

Particle Gun Bombardment

‘Bio-Rad Gun’ (PDS-1000/He systems) was used for the bombardment of shoot tips, hypocotyls and cotyledons of genotype IPA-3. Various accessories of particle-gun are microcarriers or tungsten particles (0.6 µm), rupture discs (1100 psi), rupture disc holders, macrocarriers (51 µm), macrocarrier launch assembly, shelf for placing target tissues and helium gas cylinder to provide the required pressure. Plasmid pGSFRI DNA carrying *gusA* and *hpt* under the control of maize ubiquitin promoter was used to optimize transformation of different explants. *gusA* is a reporter gene and *hpt* is a selectable marker gene, which imparts resistance to the antibiotic hygromycin B. The plasmid DNA was isolated by a standard procedure using Qiagen kit. Prior to carrying out transformation, a suspension of tungsten particles was prepared and then DNA carrying *gusA* and *hpt* genes were coated on this suspension.

Standardization of Physical Factors of Particle Gun

A macro carrier travel distance of 3 mm was kept and the chamber vacuum of particle gun was maintained at 28 mm Hg during every bombardment. Explants were incubated for 24 h after the bombardments and the number of transient transformation events was recorded by histochemical GUS assay.

Effect of Target Distance on Transformation Efficiency

Shoot tip, hypocotyl, and cotyledon explants of tomato were placed in the centre of target plate in a circle of about 1 cm diameter containing MS+BAP (2.0 mg L\(^{-1}\)) + Kn (1.0 mg L\(^{-1}\)). The bombardments were made at varying distances (2.5, 5.0, 7.5 and 10.0 cm) from the micro carrier launch assembly, with 18 µL of plasmid DNA suspension. Two bombardments were made at an interval of 4 h. A fixed gap distance (distance between rupture disc and macro carrier) of 2 cm was used. A single Petri plate containing 30-40 explants was bombarded for each target distance with three replications.

Standardization of Quantity of DNA

Similar to previous experiment, the explants were arranged in the centre of the target plate in a diameter of 1 cm. Four different amounts of plasmid DNA (*gusA* and *hpt*) coated tungsten particles, i.e. 3, 6, 9 and 12 µL were loaded on macro carriers and used for bombardment of shoot tips, hypocotyls and cotyledons of tomato genotype IPA-3. Two bombardments were made into the explants after an interval of 4 h, which meant that explants were transformed with 6, 12, 18 and 24 µL DNA suspension. Two bombardments were made to allow for misfire from faulty and poorly set rupture discs and hence increase the frequency of transformation. The explants were fired at 1100 psi with a fixed gap distance of 2 cm and a target distance of 7.5 cm. For each concentration of plasmid DNA, two Petri plates were bombarded and replicated three times.

Optimization of Biological Parameters

Explants were arranged in the centre of the target plate in 1 cm circle. Biolistic parameters used for each experiment were 18 µL of DNA suspension fired at 1100 psi with a gap distance of 2 cm and a target distance of 7.5 cm. Explants were incubated on MS supplemented with BAP (2.0 mg L\(^{-1}\)) and Kn (1.0 mg L\(^{-1}\)) for 24 h after the bombardments and the number of transient transformation events was recorded by histochemical GUS assay.
BAP (2.0 mg L\textsuperscript{-1}), Kn (1.0 mg L\textsuperscript{-1}) and mannitol at either 0.0, 0.1, 0.2 and 0.3 M L\textsuperscript{-1} for 4 h before bombardments. For each explant and osmoticum treatment, a single Petri plate was bombarded and replicated thrice.

**Effect of Pre-bombardment Culture Period on Transformation Efficiency**

The 10-d-old explants were pre-cultured on the MS medium supplemented with BAP (2.0 mg L\textsuperscript{-1}) and Kn (1.0 mg L\textsuperscript{-1}) prior to bombardment for varied durations of 0, 1, 2 and 3 d. For each pre-culture period, a single Petri plate containing 30-40 explants was bombarded and the experiment was replicated three times.

**Effect of Post-bombardment Culture Period on Transformation Efficiency**

After making the bombardments, explants were incubated on the MS medium supplemented with BAP (2.0 mg L\textsuperscript{-1}) and Kn (1.0 mg L\textsuperscript{-1}) for 1, 2, 4 and 6 d prior to histochemical GUS assay. For each bombardment and culture period, a single Petri plate containing 30-40 explants was bombarded with three replications.

**GUS Assay**

The bombarded explants were analyzed histochemically for transient GUS activity, using X-gluc (5-bromo-4-chloro-3-indolyl-\(\beta\)-D-glucuronide)\textsuperscript{15}. The bombarded explants viz., shoot tips, hypocotyls and cotyledons were put into 1 mL filter sterilized GUS substrate (Table 1). Explants were incubated in GUS substrate for 24 h at 35ºC. The GUS substrate was then decanted off and replaced with 1 mL of 70% (v/v) ethanol to stop the reaction, maintain aseptic conditions and partially clear the tissues. Cells expressing GUS activity were found as blue under the inverted microscope. Each blue explant was scored as one transformation event.

**Statistical Analysis**

For each treatment, total number of GUS positive explants per target plate was counted. Per cent GUS expression was calculated as the number of transformation events over total number of bombarded explants per target plate. Statistical analysis was done according to the CPCS-I package using factorial CRD design. CD values at 5% level of significance were calculated and the interpretations were made accordingly.

**Results and Discussion**

Establishment of a tissue culture base line is a prerequisite for the genetic transformation studies. Therefore, per cent plant regeneration in different explants (shoot tips, hypocotyls & cotyledons) was evaluated. The respective per cent plant regeneration in different explants was 95.16, 79.30 and 90.14% on MS media supplemented with BAP (2.0 mg L\textsuperscript{-1}) and Kn (1.0 mg L\textsuperscript{-1}).

The analysis of variance presented in Table 2 for the experimental design revealed the significance of mean squares due to explants and different parameters of particle gun viz., target distance, osmoticum, pre-bombardment and post-bombardment culture periods. The mean square values were non-significant between different explants for the variable DNA quantity. The interaction effects were also significant between explants and different parameters of particle gun. This indicated that per cent GUS expression recorded in different explants varied significantly in different explants as well as all the parameters of particle gun studied. Figure 1 shows the GUS expression in different explants.

**Table 1: Composition of X-gluc solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Solvent</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>X-gluc (5-bromo-4-chloro-3-indolyl-(\beta)-D-glucuronide)</td>
<td>Ethanol/DMSO</td>
<td>88.9 mg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1N Na OH</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} (1M)</td>
<td>Autoclaved distilled water</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Triton-X (10%)</td>
<td>-</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Methanol (20%)</td>
<td>-</td>
<td>20.0 mL</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>7.0</td>
</tr>
</tbody>
</table>

**Table 2: Analyses of variance for per cent GUS expression as affected by different parameters of particle gun**

<table>
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<th>Source</th>
<th>df</th>
<th>MS\textsubscript{1}</th>
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<th>MS\textsubscript{3}</th>
<th>MS\textsubscript{4}</th>
<th>MS\textsubscript{5}</th>
</tr>
</thead>
<tbody>
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<td>Explant</td>
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<td>210.71*</td>
<td>9.66</td>
<td>215.61*</td>
<td>45.29*</td>
<td>8.24*</td>
</tr>
<tr>
<td>Parameters</td>
<td>3</td>
<td>1457.75*</td>
<td>1508.98*</td>
<td>2556.14*</td>
<td>772.85*</td>
<td>548.94*</td>
</tr>
<tr>
<td>Explants (\times) parameters</td>
<td>6</td>
<td>37.12*</td>
<td>52.72*</td>
<td>41.27*</td>
<td>1207.1*</td>
<td>3.21</td>
</tr>
<tr>
<td>Error</td>
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<td>7.04</td>
<td>14.78</td>
<td>3.57</td>
<td>12.25</td>
<td>26.42</td>
</tr>
</tbody>
</table>

*significant at 5% level of significance

MS\textsubscript{1}=Mean square values for target distance; MS\textsubscript{2}=Mean square values for DNA quantity; MS\textsubscript{3}=Mean square values for mannitol; MS\textsubscript{4}=Mean square values for pre-bombardment culture period; & MS\textsubscript{5}=Mean square values for post-bombardment culture.
Effect of Target Distance on Transformation Efficiency

The target distances (2.5, 5.0, 7.5 and 10.0 cm) traveled by the micro projectiles from the micro carrier launch assembly to the target tissues significantly affected the rate of transient GUS expression in different explants of tomato. A maximum of 34.12, 36.56, 22.69% transient GUS expression was observed in shoot tips, hypocotyls and cotyledons, respectively, when micro projectiles traveled a distance of 7.5 cm, and the expression decreased to nil at 2.5 cm target distance in all the explants (Fig. 2). As the firing distance was increased to 10 cm, again a significant decline was recorded in the per cent GUS expression. Analysis of GUS expression in the bombarded explants 24 h after particle delivery produced blue colour visible to the naked eye in shoot tips and hypocotyls but in cotyledons GUS expression was recorded after 48 h of bombardment. No GUS expression was ever observed in tissues bombarded with tungsten particles alone not coated with DNA. However, in sugarcane maximum GUS expression was recorded at 5.00 cm distance after 13.30 h of incubation in X-gluc. Highest transient GUS expression in broccoli was recorded when cotyledon explants were placed at 6 cm distance from micro carrier launch assembly. Similar results were reported in cauliflower stem explants. When embryogenic axis of Vigna anguicularata were bombarded with gusA and bar genes, best transformation rates were obtained at a target distance of 6 cm.

Standardization of Quantity of DNA and Tungsten Particles

When different concentrations of plasmid DNA were bombarded to the target tissues, a significantly variable transient GUS expression was recorded in different explants. The 8 µL of DNA suspension contained 0.83 µg of DNA coated on 500 µg of tungsten particles. Maximum transient GUS expression was recorded when explants were bombarded with 18 µL of DNA suspension i.e., 31.11, 33.23 and 30.20% in shoot tip, hypocotyl and cotyledon explants, respectively (Fig. 3). The 18 µL of DNA suspension contained 1.89 µg of DNA coated on 1125 µg of tungsten particles. Lower as well as higher quantity of DNA (12 and 24 µL) suspension reduced the per cent GUS expression to 20% and 10.3% in shoot tips, 9.39% and 17.17% in hypocotyls and 8.59% and 15.81% in cotyledons, respectively. The rate of transformation is determined both by the number of micro projectiles that penetrate the cells of the target explants and degree of injury into the cells. Transformation efficiency has been reported to vary with different quantities of plasmid DNA bombarded to the target tissues. Lower quantities of plasmid DNA reduced the rates of transformation whereas higher quantities promoted the efficiency of
transformation. However, in some systems, low quantities of DNA (0.2 µg DNA) have produced consistently better results. Higher quantities of DNA or impurities of plasmid DNA can lead to agglutination of micro projectiles, which results in pronounced tissue damage following bombardment. Similar findings were reported in broccoli.

To study the biological factors associated with the target tissues, following optimized biolistic conditions were used as standard. The explants were arranged (30-40) with in 1 cm central zone of the Petri plates were bombarded with tungsten micro projectiles coated with plasmid DNA adjusted to an initial concentration of 18 µL using an acceleration force of 1100 psi with a gap distance of 2 cm and target distance of 7.5 cm.

**Effect of Osmoticum on Transformation Efficiency**

Pre-incubation of explants on medium supplemented with an osmoticum (mannitol) has enhanced both transient and stable transformation in several species and explants. But in tomato shoot tips, hypocotyls and cotyledons incorporation of osmoticum in the culture medium proved detrimental to the transformation efficiency. The data presented in Fig. 4 pertaining to per cent transient GUS expression indicated that rates of transient GUS expression declined significantly with increasing concentration of osmoticum. It was highest in control lacking mannitol in all the explants viz., 38.79, 41.48 and 36.56% in shoot tips, hypocotyls and cotyledons, respectively. GUS expression was nil in all the explants when the concentration of osmoticum was 0.3 M. Pre-incubation of sugarcane callus cultures on medium supplemented with 0.2 M sorbitol and 0.2 M mannitol for 4 h improved transformation efficiencies. Beneficial effects of osmotic pre-treatments were also reported for bombardments of embryonic cultures of several species including peach and *Pinus radiata*. Decrease in the transformation efficiency has been reported for osmoticum when used at high concentration. It is believed that these osmotic treatments reduce cytoplasmic leakage and cell rupture by lowering the turger pressure in the target cells and stabilizing cell membranes to enhance better repair of lesions caused by particle penetration.

**Effect of Pre-bombardment Culture Period on Transformation Efficiency**

The data given in Fig. 5 exhibited that incubation of explants (shoot tips and hypocotyls) for 0, 1, 2, and 3 d on MS media supplemented with BAP (2.0 mg L⁻¹) and Kn (1.0 mg L⁻¹) prior to bombardment resulted in significantly reduced rates of GUS expression which was reverse in cotyledons. In shoot tips and hypocotyls per cent GUS expression was maximum with no pre-culture period i.e., 40.65% and 42.96%, respectively, which was reduced to 10.65% and 14.02% at 1-d pre-incubation period. Further, increase in pre-culture period reduced the per cent GUS expression by 100% in both the explants. But there was a dramatic increase in per cent transient expression in cotyledons with the increase in pre-culture period i.e., 8.33, 19.44 and 38.89% at 1, 2 and 3 d, respectively. Explants with juvenile and actively dividing cells are generally considered to be the best target tissues for biolistic transformation. Previous

Fig. 4—Effect of manitol on per cent GUS expression in shoot tips, hypocotyls and cotyledons of tomato.

Fig. 5—Effect of pre-bombardment culture period on per cent GUS expression in shoot tips, hypocotyls and cotyledons of tomato.
studies with leaf explants of sugarcane\textsuperscript{24} and \textit{Arabidopsis thaliana}\textsuperscript{25} have shown that pre-culture on callus induction media promoted cell division and improved the efficiency of transformation. Pre-culturing of the explants prior to bombardment will alter the development and physiological condition of the tissue, which are known to be important determinants of the transformation efficiency\textsuperscript{9}. The increase in tissue damage observed in pre-cultured explants of broccoli indicated that this caused the softening of the explant tissues such that conditions of bombardment became too harsh\textsuperscript{17}. The wounding altered other cellular mechanisms and pre-culturing of explants tissues and these metabolic changes may have also contributed to the changes in the efficiency of transformation.

Effect of Post-bombardment Culture Period on Transformation Efficiency

The per cent transient GUS expression in different explants of tomato presented in Fig. 6 showed significant differences with the increase in post-bombardment culture period. Post-bombardment culturing of explants on MS media supplemented with BAP (2.0 mg L\textsuperscript{-1}) and Kn (1.0 mg L\textsuperscript{-1}) produced a significant decline in the rates of transient transformation. The highest number of transient transformation events was obtained from explants cultured for one day after bombardment i.e., 36.36\%, 38.13\% and 36.05\% in shoot tips, hypocotyls and cotyledons, respectively.

Biolistic transformation of tomato hypocotyls, shoot tips and cotyledons have not been previously reported. Our results showed that efficiency of transient transformation of tomato greatly varied with the concentration of plasmid DNA, target distance, addition of osmoticum, pre-bombardment and post-bombardment culture periods, which was revealed by the GUS assay of the bombarded explants. Earlier work on direct regeneration protocols of tomato from cotyledons, shoot tips and hypocotyls are well established\textsuperscript{26,27} and transient GUS expression signifies the potential of these explants to successfully exploit for the particle mediated genetic transformation.

References