Evaluation of parameters for high efficiency gene transfer via particle bombardment in Indian mulberry

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Particle bombardment is a popular method of direct gene delivery into cell, tissue and organs since it requires minimum pre- and post-bombardment manipulation. In addition, this technique is much easier and fast to perform with intact tissue/organ and reduces the period of in vitro culture. Genetic transformation of mulberry, Morus indica cv. K2 was attempted by particle bombardment using hypocotyl, cotyledon, leaf and leaf callus explants. The effect of various physical and biological parameters during bombardment were studied by the histochemical localization of GUS reporter gene following two days of bombardment and by assessing the number of blue spots per explant. p35SGUSINT was used for optimization of different parameters. The percentage of GUS positive explants was very low with tungsten (20%) as compared to gold particles (36%) indicating tungsten toxicity to the tissue. Maximum GUS activity was observed at 1100 psi helium pressure and 9 cm target distance for hypocotyl, cotyledon and leaf. Double bombardment of explants with 10 μg of DNA loaded on macarriers clearly yielded a better (up to 56%) result as compared to a single bombardment (30%). Amongst the various plasmids tested, pBI221 gave the highest (100%) GUS positive explants in the leaf callus.

Mulberry (Morus sp.) besides being the chief feed for silkworm Bombyx mori, is a multipurpose tree as all plant parts, i.e. root, stem, bark, leaf and fruits are useful in many ways. It is thus rightfully recognized as 'Kalpavrika'. Thus, in this era of genetic engineering, genetic manipulation of mulberry towards an ideal plant type is highly desirable.

Agrobacterium-mediated transformation of mulberry has been attempted by some workers, however, the transformation efficiency was low and no transgenic plant was obtained. With a strong backup of efficient and reproducible regeneration protocols for mulberry established in our lab, we reported high efficiency gene transfer via Agrobacterium tumefaciens and production of transgenic mulberry plants.

When compared to Agrobacterium-mediated transformation, particle delivery is a convenient method since it requires minimum pre- and post-bombardment manipulation, especially reducing the in vitro tissue culture duration. In addition, this technique is much easier and faster to perform especially with organized tissues. Introduction of gus gene into mulberry by particle bombardment was first reported by Machii in suspension cultures, however, the production of whole plants from suspension cultures is not known in mulberry. There after, transient GUS expression was demonstrated in leaf explants via particle bombardment and in calli-derived protoplasts via electroporation, but protoplast to plant protocol is also not established for mulberry. In this paper, we report the optimization of various physical and biological factors for high levels of foreign gene incorporation in hypocotyl, cotyledon, leaf and leaf callus of Morus indica cv. K2 by particle bombardment.

Materials and Methods

Plant material and culture conditions

Seeds of Morus indica cv. K2 were collected from six- to seven-year old, field-grown plants and stored in a desiccator at room temperature. Seeds were surface sterilized with 0.1% mercuric chloride (Qualigens, India) for 8 min, rinsed 5-6 times and imbibed in sterile distilled water for 24 hr prior to culture. Seeds were sown on MS medium supplemented with 0.1 mg/l TDZ and kept in diffuse light. Hypocotyl and cotyledon explants were excised from 10-days old, in vitro raised seedlings. Leaf explants were taken from in vitro maintained axillary bud cultures (on MS + 0.1 mg/l TDZ) and kept in diffuse light. Hypocotyl and cotyledon explants were excised from 10-days old, in vitro raised seedlings. Leaf explants were taken from in vitro maintained axillary bud cultures (on MS + 0.1 mg/l TDZ + 2 mg/l IAA + 2 mg/l AgNO₃ for over 2 months. All explants were precultured for five days on regeneration medium (RM: MS medium + 1.1 mg/l TDZ), prior to
bombardment. The pH of the medium was adjusted to 5.8 prior to autoclaving at 104 kPa at 121°C for 15 min. Cultures were maintained at 25°C ± 1°C under a daily photoperiodic regime of 16 hr light and 8 hr darkness. Four cool white fluorescent tubes (Philips, TL40 W/54) provided a light intensity of 65 μmol m−2 s−1.

**Plasmid isolation and purification**

The 13.2 kb p35SGUSINT was employed for the optimization of various physical and biological parameters. It has the reporter gene gus, interrupted by a plant intron that restricts β-glucuronidase expression to plant cells and nptII as the selectable marker for kanamycin resistance. The GUS gene is controlled by the CaMV 35S promoter and polyadenylation signal. While the nptII gene is under nos promoter. Other plasmids used were pBI1101:Act1 (Act1INT–gus and nos–nptII construct), pBI221 (35S-gus construct), pCAMBIA1301 (35S-gusINT and 35S-hpt construct) and pCAMBIA2301 (35S-gusINT and 35S-nptII construct).

The plasmids p35SGUSINT, pBI221 and pBI1101:Act1 were amplified in A. tumefaciens cultures grown in YEB medium supplemented with suitable antibiotics at 28°C in dark, shaking at 240 rpm for 20-24 hr while the CAMBIA plasmids were isolated from the E. coli cultures grown in YEP supplemented with suitable antibiotics at 37°C in dark, shaking at 240 rpm for 20-24 hr. Isolation of the plasmids was performed by alkali lysis method of Brinboim and Doly. The closed circular plasmid DNA was purified by equilibrium centrifugation in cesium chloride ethidium bromide gradient.

**Preparation and coating of particles**

Both gold and tungsten particles were employed for transformation. 30 mg of the gold particles (0.6 μm) were taken in a 1.5 MCT (Micro centrifuge tube) and 1 ml of 70% ethanol (v/v) was added to it. MCT was vortexed vigorously for 3-5 min and particles were allowed to remain soaked for 15 min. The tungsten particles (1.1 μm) were suspended in 1 ml absolute ethanol and after vortexing for 3-5 min, it was heated at 95°C in a waterbath for 90 min. By spinning at 10,000 rpm for 5 sec both gold and tungsten microparticles were pelleted and supernatant removed and discarded. Pellet was resuspended in 1 ml sterile water, vortexed vigorously for 1 min and particles allowed to settle for 1 min. Microparticles were centrifuged briefly at 10,000 rpm for 5 sec and water was replaced by fresh 1 ml sterile water. After washing thrice with water, the particles were suspended in 500 μl of 50% glycerol and stored at -20°C at a final concentration of 60 mg/ml.

This particle suspension was thawed when required and vortexed vigorously to resuspend the particles. 50 μl of microcarriers were taken in a 1.5 ml MCT and while vortexing continuously (for uniform DNA precipitation onto microcarrier) following were added sequentially, 5 μl DNA (2 μg/μl), 50 μl 2.5M CaCl2, 20 μl 0.1M spermidine. Contents were vortexed for 5-6 min, microcarrier allowed to settle for 1 min, pelleted by spinning for 2 sec at 10,000 rpm and liquid removed and replaced by 140 μl of 70% ethanol. The wash with 70% ethanol was followed by 100% ethanol and finally particles were resuspended in 48 μl of 100% ethanol. These coated particles were kept at 4°C and used within 1 hour of preparation. 6 μl of the coated particle suspension was loaded on the macrocarrier membrane and allowed to dry for 10 min prior to use.

**Particle bombardment**

The bombardment was performed according to Sanford et al., using Biolistic™ PDS-1000/He particle delivery system (Bio-Rad Laboratories, California, USA) which uses pressurized helium to accelerate sub-cellular size microprojectiles coated with DNA (or other biological material) over a range of velocities necessary to optimally transform many different cell types. Thus, various physical and biological factors i.e. microcarrier particles, helium pressure, distance between macrocarrier assembly and target plate, quantity of DNA, number of bombardments per plate, type of plasmid and plant material employed, were optimized using p35SGUSINT coated gold particles in Morus indica cv. K2. The explants bombarded without plasmid DNA were taken as control. The transformation efficiency was calculated based on the results of three replicate experiments with thirty explants each.

**GUS histochemical assay**

After two days of bombardment with plasmid coated microprojectiles, the intact explants were incubated overnight at 37°C in the histochemical buffer [50 mM NaHPO4, pH 7; 10 mM EDTA, pH 7; 0.5 mM K3Fe(CN)6; 0.5 mM K4Fe(CN)6; 0.1% Triton X-100; 1 mM X-Gluc]. The transient expression of the β-glucuronidase was detected with the synthetic substrate (X-gluc), which upon cleavage formed a blue
precipitate visually detectable within transformed cells. Explants showing blue spots were scored as GUS positive.

Results and Discussion

The ability to accelerate DNA-coated particles (microprojectiles) directly into intact tissue by particle bombardment technique has expanded the range of organisms that can be genetically transformed. But till date there are only a few tree species namely papaya,[19-20], Populus,[1] yellow poplar,[22-23], Pinus[24] and silver birch[25] that have been successfully transformed. At least five key factors interact to affect the transformation efficiency of the cells in bombarded tissue i.e., size and composition of microprojectiles, DNA attachment to the microprojectiles prior to bombardment, impact velocity of the microprojectile/DNA complex, degree of tissue damage suffered on bombardment and genetic construct and tissue type. In our study, optimization of various physical and biological parameters for successful gene delivery via particle bombardment was performed with p35SGUSINT coated gold particles.

Potential of various explants/tissues

The gus histochemical assay was performed after 48 hours of bombardment of the hypocotyl, cotyledon, leaf and callus explants with p35SGUSINT coated gold particles.

Conditioning the explants

The hypocotyls, cotyledons and leaves were excised from the seedling/axillary bud and cultured on 1.1 mg/l TDZ for 5 days prior to bombardment. Pretreatment is shown to enhance the transformation efficiency of the cells in bombarded tissue. The percentage GUS positive explants obtained was 38% hypocotyl, 56% cotyledon, 55% leaf and 39% leaf callus (Table 1). In earlier experiments on transformation of mulberry, blue spots were obtained only in the suspension cultures and none in the immature leaf and callus[26], regardless of the type of plasmid used. In terms of the number of blue spots per plant and intensity of the blue spots, we observed that the cotyledon (Fig. 1A) and leaf (Fig. 1B) explants scored higher than hypocotyl (Fig. 1C) and leaf callus (Fig. 1D). This could be attributed to the larger surface area of the cotyledon and leaf explants to receive the bombarded particles than the hypocotyl. The control explants did not show any blue coloration. It is known that the GUS gene expression depends on the type of explant.

Table 1 — Optimization of parameters for transformation of Morus indica cv. K2 by bombardment*

<table>
<thead>
<tr>
<th>Explants</th>
<th>Hypocotyl (%GUS+ve)</th>
<th>Cotyledon (%GUS+ve)</th>
<th>Leaf (%GUS+ve)</th>
<th>Leaf Callus (%GUS+ve)</th>
</tr>
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<tbody>
<tr>
<td>(a) Particle</td>
<td></td>
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<tr>
<td>Gold, 1550 psi</td>
<td>28.0±5.0</td>
<td>44.5±5.0</td>
<td>35.5±1.9</td>
<td>37.5±3.5</td>
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<tr>
<td>Tungsten, 1550 psi</td>
<td>10.0±4.9</td>
<td>15.0±3.4</td>
<td>20.0±1.6</td>
<td>15.0±3.5</td>
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<tr>
<td>(b) Pressure (psi)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>900</td>
<td>24.5±6.9</td>
<td>31.0±3.8</td>
<td>27.0±3.3</td>
<td>30.0±7.0</td>
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<tr>
<td>1100</td>
<td>37.0±5.0</td>
<td>55.0±3.8</td>
<td>47.0±5.0</td>
<td>32.5±3.5</td>
</tr>
<tr>
<td>1550</td>
<td>28.0±5.0</td>
<td>44.5±5.0</td>
<td>35.5±4.9</td>
<td>37.5±3.5</td>
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<tr>
<td>(c) Distance (cm)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>13.0±3.3</td>
<td>17.0±3.3</td>
<td>12.0±1.9</td>
<td>12.5±3.5</td>
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<td>6</td>
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<td>55.0±3.0</td>
<td>47.0±4.9</td>
<td>35.5±7.0</td>
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<tr>
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<td>35.5±1.9</td>
<td>56.5±4.8</td>
<td>55.0±4.7</td>
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<td>12</td>
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<td>22.0±5.4</td>
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<td>12.5±3.5</td>
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<tr>
<td>(d) DNA quantity (μg)</td>
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<td></td>
<td></td>
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<tr>
<td>5</td>
<td>29.0±1.9</td>
<td>31.0±7.7</td>
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<tr>
<td>10</td>
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<tr>
<td>(e) Shots/plate</td>
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<td>38.0±3.4</td>
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</tr>
<tr>
<td>2</td>
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<td>55.0±5.0</td>
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</tr>
</tbody>
</table>

*Where not specified, the bombardment was made with gold particles, at 1100 psi, 6 cm target distance with p35SGUSINT construct with single bombardment of 10 μg DNA.
efficiency in mulberry, though the exact physiological reasons for the favorable effect of pretreatment on the receptivity of the target tissue are unknown. Application of TDZ promotes meristematic development and differentiation and the actively dividing cells are shown to be the most receptive targets for transformation. Osmotic pretreatment of the explants is known to enhance bombardment efficiency in Citrus and Pinus.

Influence of microcarrier

Influence of two types of microcarrier i.e., gold and tungsten particles was investigated. The tungsten particles come in a wide range of sizes (0.5-2 μm), are relatively inexpensive and easy to coat with DNA but are often subjected to surface oxidation that can alter DNA binding and catalytically degrades the DNA bound to it over time. On the other hand, the gold particles though being expensive, are uniform in size and biologically inert. The explants were placed 6 cm away from the macrocarrier assembly and bombarded at 1550 psi. The efficiency of transformation was nearly three folds with gold particles as compared with tungsten (Table 1a). The lower percentage of GUS positive explants in transformation attempts with tungsten may be due to its toxic effect on the tissue.

Fig. 1 — Transformation of Indian mulberry via particle bombardment. GUS positive explants, two days after bombardment with p35SGUSINT coated gold particles. [A. Cotyledon, B. Leaf, C. Hypocotyl and D. Leaf callus]
Pressure of helium gas
Changes in pressure were found to dramatically affect the level of transient expression. The percentage GUS positive explants increased by nearly one and half fold with an increase of pressure from 900 to 1100 psi with 37% hypocotyl, 55% cotyledon and 47% leaf explants (Table 1b). At higher pressure (1550 psi) the leaf callus explants gave the best response (39%), however, cell damage resulted in decreased transformation of other plant materials.

Target distance
The distance between the petriplate containing the explants to be bombarded and the macrocarrier assembly showed a direct relationship with the pressure of the helium gas. At short distances (3 and 6 cm) low pressure (900) was sufficient. The best response was obtained at 9 cm target distance with 1100 psi where 36% hypocotyl, 56% cotyledon and 55% leaf explants tested positive for gus gene. 35% of the leaf callus explants tested positive for gus gene from the bombardment at 6 cm with 1100 psi (Table 1c). At longer distances (12 cm) there was always a low transient activity regardless of the pressure, indicating the dispersion of the particles away from the center of plate where the explants were placed.

DNA quantity
The quantity of DNA with respect to the amount of microcarrier determined the quality of coating and thus influenced the delivery efficiency. For maximum transformation, the optimum DNA quantity was 10 μg per 3mg of particle. Further increase in DNA amount resulted in improper coating and hence decrease in number of GUS positive explants (Table 1d). In our experiments, we have used the CsCl-EtBr density gradient purified circular plasmid DNA, linearisation of DNA prior to transformation has been shown to improve transient expression in Pinus embryonic cultures.

Number of bombardments
In all the experiments double bombardment of the same tissue increased the number of transformed explants (Table 1e).

Suitability of plasmids
The expression of a gene within minutes or hours following gene delivery into cells is referred to as transient gene expression. It does not necessitate the integration of foreign DNA into the genome for expression, and thus is not influenced by host sequences. Here it was used to study the effect of five plasmids having gus and nptII/hpt genes driven by different promoters. In hypocotyl, cotyledon and leaf explants, maximum GUS expression was observed in transformation at 1550 psi and 6 cm target distance with the p35SGUSINT plasmid (Fig. 2) which has CaMV35S promoter driven gus gene and nos driven nptII gene. The pBI101 with Act1 promoter proved
better than the CAMBIA plasmid pCAMBIA2301 that has both gus and nptII under the CaMV 35S promoter. Interestingly, in case of leaf calli, bombardment at a further distance (9 cm) gave a better response (Fig. 2) and with pBI221 all the calli tested positive for gus at 1100 psi and 6-9 cm target distance (Fig. 2). However, in the absence of any selection trait in this plasmid, selection and screening of putative transformants was not possible and despite higher gene delivery frequencies, this plasmid could not be exploited for raising transgenic mulberry. Nonetheless, our studies show that it is possible to transfer genes to different tissue (hypocotyl, cotyledon, leaf and leaf callus) in mulberry by particle bombardment. In terms of the quantity and quality (number of spots/explant) of gene expression, the leaf explant is undoubtedly the material of choice. Towards achieving stable transformation of mulberry, double bombardment of leaf tissue by plasmid p35SGUSINT or leaf callus by pCAMBIA1301 coated gold particles at 1100 psi helium pressure and 6-9 cm target distance will prove beneficial.

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References