

## Simple and efficient biolistic procedure for the plant transfection with cDNA clones of RNA viruses

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**Summary.** – Simple, fast, low-cost, and efficient procedure for DNA delivery to the cell nuclei of whole plants was developed. The procedure was optimized for the Plum pox virus (PPV) and its host *Nicotiana benthamiana*. It is based on the leaf bombardment with tungsten microparticles with bound DNA using common air gun. The procedure did not require special equipment and source of driving gas. The transfection efficiency obtained by the newly developed procedure was close to 100%, but this level dropped with the age of bombarded plants.

**Keywords:** biolistics; air gun; infectious clones

Biolistics (particle bombardment) is an effective technique for the delivery of nucleic acids into various cell types. DNA or RNA coupled with inert metal particles (gold or tungsten) is shot under the pressure of driving gas (helium or nitrogen) into the target cell, tissue, or whole organism (Johnston and DeVit, 1996). Two types of commercial biolistic instruments are available from Biorad, the box-shaped PDS-1000/He and pistol-shaped Helios Gene Gun. The shooting by the first of them is performed in vacuum chamber to enhance the effective velocity of microprojectiles. The target size, however, is limited by the chamber dimensions (about 10 x 10 x 12 cm). The pistol-shaped type is better suited for the work with plants. It is easily movable and larger plants may serve as target. The main advantage of biolistics is the preservation of cell viability in spite of hitting the nucleus. Therefore, this technique is useful for the transfection of plants by cDNA clones of RNA viruses. However, the disadvantage of this procedure represents relatively high initial cost that makes the routine application of biolistics quite demanding for small laboratories. Consequently, several authors developed protocols for various microparticle delivery systems that were constructed by them (Gray *et al.*, 1994; Sikorskaite *et al.*, 2010). In this work, we describe an

alternative technique of biolistic transfection of plants using two types of sport air gun.

We used PPV infectious cDNA clone pIC-PPV obtained kindly from Prof. J.A. García (CSIC Madrid) and its chimera with the *Nib-CP* genomic region of the Slovakian PPV isolate BOR-3 constructed in our laboratory (López-Moya and García, 2000; Šubr *et al.*, 2010). *Nicotiana benthamiana* was used as target plant species, where PPV multiplies and systemically spreads very well. The plants were grown in climatic chamber at 20°C and 14 hrs/10 hrs of light/dark period.

Tungsten microprojectiles M-10 with the average size of 0.7 µ (Biorad) were applied for the DNA delivery. The microprojectiles were washed by 70% ethanol followed by distilled water and resuspended in 50% glycerol (60 mg/ml). The binding of DNA was performed as follows: 5 µl of DNA (10 µg/µl), 30 µl of suspension of microprojectiles (thoroughly homogenized by vortexing prior the addition), 1.5 µl of 1 mol/l spermidin and 32 µl of 2.5 mol/l CaCl<sub>2</sub> were mixed in pre-cooled microtube and kept for 10–20 mins at 0°C with occasional gentle mixing. Next, the tungsten particles were sedimented by short centrifugation (15 secs, 16,000 g). The supernatant was discarded and sediment was washed five times by ice-cold ethanol. Finally, the microprojectiles with bound DNA were resuspended in 20 µl of ethanol. The suspension in volume of 10 µl was dropped on celluloid membrane (a triangle piece of the common slide cover) with surface of about 1 cm<sup>2</sup> and allowed to dry for 5 mins

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**Abbreviations:** PPV = Plum pox virus; dpi = days post infection

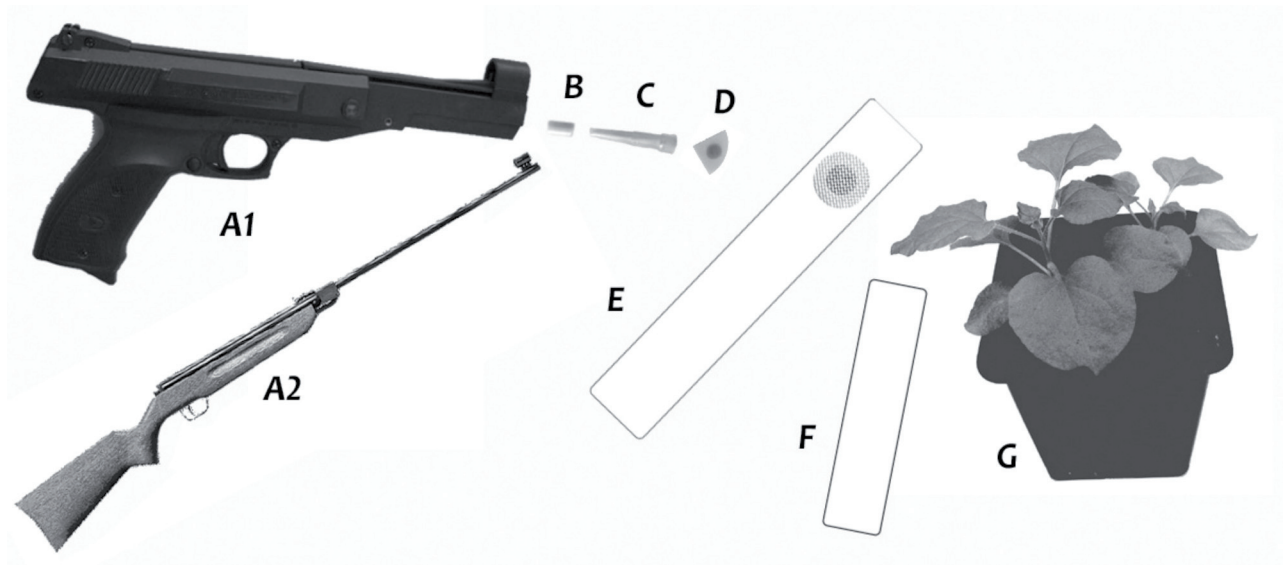


Fig. 1

#### Scheme of the biolistic technique of plant transfection

A1 – air gun P-800, A2 – air gun Slavia, B – sealing tube, C – shortened pipette tip, D – celluloid membrane with sample, E – holder with the stopping grid, F – underlying glass slide, G – target plant.

at 37°C. After drying the membrane was gently rolled and positioned into slightly shortened 200 µl yellow pipette tip (Sarstedt). The tip was connected with the gun-barrel using a short piece of silicone tube. Individual leaves of plants were shot through a metal grid (stopping screen for PDS-1000/He, Biorad) positioned in simple plastic holder fixed to vertically adjustable platform. During the shooting, distance between the tip aperture and leaf surface was about 2–5 mm and the leaf was secured by glass slide. The scheme of the procedure is shown in Fig. 1.

We used two types of air guns a long one (Slavia 631, caliber 5.5 mm, Czech Arsenal) and short one (P-800, caliber 4.5 mm, Gamo). The transfection efficiency was evaluated according to the visible symptoms recorded at 10–14 days post infection (dpi) and by immunoblotting of the crude plant sap using polyclonal antibody against PPV (Šubr and Matisová, 1999).

PPV genome represents single-stranded RNA of positive polarity (Salvador *et al.*, 2006) that replicates exclusively in the cytoplasm of infected cells without any DNA intermediate. In the applied constructs, the cDNA of PPV genome was cloned under the control of doubled Cauliflower mosaic virus 35S promoter (López-Moya and García, 2000), which enabled its primary transcription after delivery into the host cell nucleus. After transport to the cytoplasm the generated RNA multiplied autonomously mimicking common viral infection. Therefore, the symptoms development and presence of viral proteins reflected the efficiency of primary DNA introduction into the nucleus.

A few minutes after the bombardment, the intensive shock reaction from mechanical injury of the leaf was observed. Later on, small necrosis developed in the place of injury (Fig. 2). The hit leaves were often deformed, but they usually survived, die-back was observed only when the target leaves were too small during shooting.

At 7–10 dpi, the plants showed typical symptoms of the systemic PPV infection including vein clearing, leaf rolling, mosaic, and deformations (Fig. 2). In the bombarded plants, the presence of PPV capsid protein was demonstrated. The results were consistent with the ones obtained from plants infected with the corresponding PPV isolate in virion form (Fig. 3). The transfection efficiency was close to 100 with the both guns used (counted as ratio of infected/bombarded plants). No difference was observed, when celluloid membrane or commercial material (cut macrocarriers for PDS-1000/He, Biorad) was used as supporting material for microprojectiles.

Furthermore, we tested the technique efficiency depending on the plant age. Two–three-week-old *N. benthamiana* plants at the stage of 4–6 fully developed leaves were usually used for the PPV multiplication. As expected, the efficiency of the transfection decreased with the plant age along with the increasing period of symptom appearance (Table 1). Roughly 50% efficiency was still recorded with 8-week-old plants, but the first symptoms of infection occurred significantly later (at 14 dpi). The transfection of 10-week-old plants failed.

Recently, the biolistic delivery of nucleic acids into the living cells using either commercial equipment or home-made

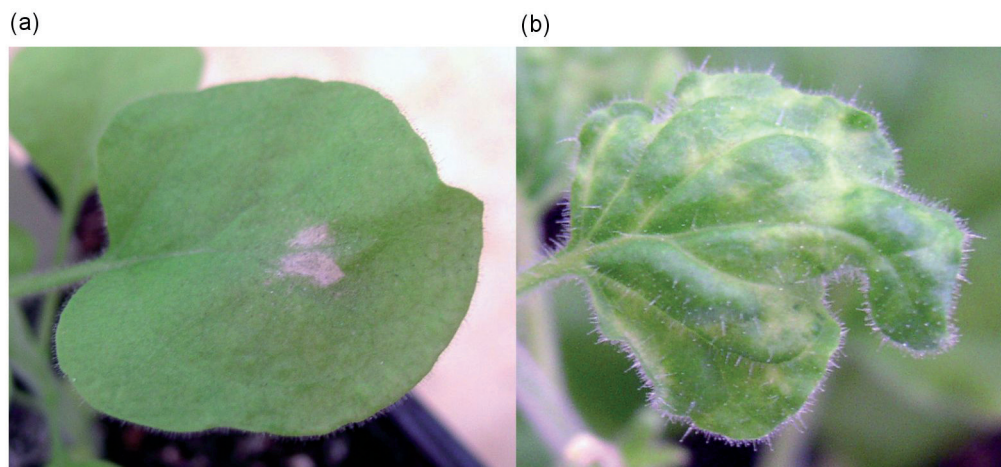


Fig. 2

*N. benthamiana* plants after the biolistic transfection

(a) necrosis developed few days after the biolistic application, (b) systemic infection developed three weeks after the biolistic transfection.

devices has become more common (Sikorskaite *et al.*, 2010). The work with the whole plants requires handling flexibility and therefore, the pistol-shaped devices are more convenient. The main advantage of our technique was rapid decrease of costs for experimental work. The technique does not require special equipment or an external source of driving gas. The purchase price of the air gun P-800 was about 70 €. We used some consumables for PDS-1000/He, namely the microprojectiles M-10 and stopping screens that could be used repeatedly after ethanol sterilization and autoclaving. The protocol of this technique is simple and the procedure itself is fast. Preparation of the microprojectiles (DNA binding and washing) takes approximately 30 mins. According to our experience with the parallel sample handling, one person is able to prepare up to 20 samples in no more than one hr. The biolistics itself takes few minutes. One person is able to manage the whole experiment using short gun. However, co-operation of two persons is essential for the work with long-barrel gun (one person for the gun operation

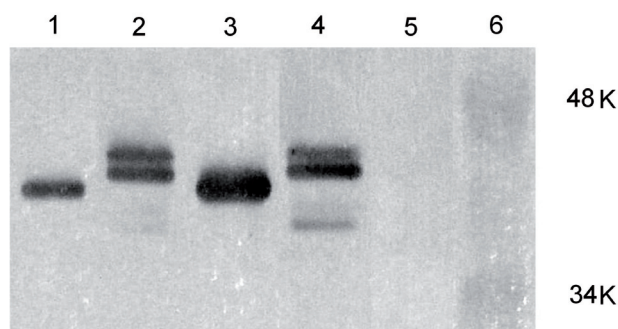


Fig. 3

Immunoblotting of PPV capsid protein in the sap from *N. benthamiana*

Biolistic transfection by pIC-PPV (lane 1), pIC-BOR3 chimera (lane 2) and regular infection with corresponding purified PPV isolates Rankovic (lane 3) and BOR-3 (lane 4), respectively, healthy plant (lane 5). Size markers (lane 6) are indicated on the right.

Table 1. Relationship between transfection efficiency and the age of *N. benthamiana* plants

Plant age (weeks)	Transfection efficiency (%)	Symptom appearance (dpi)
2	88	7
3	100	8
4	100	9
5	75	9
6	63	10
8	50	14
10	0	-

and one for the handling plants). The use of exchangeable stopping grids and consumables (tips, celluloid holders) excludes potential contamination of the samples handled one after another.

The presented biolistic technique enables fast, low-cost, and effective transfection of the herbaceous plants. It may be applied in the basic research for plant-virus interactions or may be adapted for routine preparative transient expression of heterologous genes in plants.

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