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Gene delivery into cultured plant cells by DNA-coated gold particles accelerated by a pneumatic particle gun

A. Iida *, M. Seki, M. Kamada, Y. Yamada and H. Morikawa **

Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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Summary. Transient expression of the β-glucuronidase (GUS) gene in suspension-cultured cells of tobacco was obtained after gene delivery with a pneumatic particle gun device driven by compressed air. Both the pneumatic pressure used to accelerate the particles as well as the distance between the point of release of the particles from the device and the location of the target cells could be varied. Efficiency of gene delivery was tested by accelerating pressure from 63 to 200 kg/cm^2 and distances from 5 to 15 cm. At 150 kg/cm^2 and 10 cm, 618 ± 49 blue spots of GUS-expressing cells were evenly distributed over the surface of the cell layer (3.5 cm diameter). The frequency of transient gene expression was estimated to be more than 1.2×10^{-3}, which is comparable to, or even higher than, that obtained by the gunpowder-driven device.

Key words: β-Glucuronidase – Compressed air – Pneumatic particle gun – Tobacco cells – Transient gene expression

Introduction

Since the pioneering studies by Klein et al. (1987) and Christou et al. (1988), biolistic gene delivery has become a useful method for delivering foreign genes into intact plant cells and tissues. Stable transformants obtained by this method have been reported with maize (Klein et al. 1989), soybean (Christou et al. 1988; McCabe et al. 1988), and tobacco (Klein et al. 1988 b; Morikawa et al 1989; Iida et al. 1990).

We have been studying biolistic gene delivery into plant cells and tissues using a gas-pressure-driven device that does not use gunpowder or arc-discharge explosion. We previously reported both successful transient expression of foreign genes in plant cells and tissues and stable transformation of tobacco cells, using a simple device driven by nitrogen gas pressure (Morikawa et al. 1989; Iida et al. 1990).

We report here on pneumatic particle gun device that is driven by the pressure of compressed air, and the successful transient expression of foreign genes in plant cells obtained by this method. This device has an accelerating pressure of up to more than 220 kg/cm^2, which is almost ten times higher than that of our previous device. In addition, it retains the advantages of the previously reported gas-pressure-driven device: controllable accelerating pressure, lack of explosion heat, and avoidance of cell damage caused by expanding gas due to a "self-sealing effect" of the projectile (Morikawa et al. 1989).

Materials and methods

Plant materials

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L. cv 'Bright Yellow-2,' provided by Dr. T. Matsumoto, Japan Tobacco Inc., Tokyo) were subcultured weekly as reported previously (Morikawa et al. 1989).

Device

The design of our pneumatic particle acceleration device is shown in Fig. 1. This device has a barrel to which an air chamber is connected at the bottom end. The air chamber is equipped with a plunger pump (Tokyo Rifle Co., Tokyo, model 800) at one side and an exhaust valve (Tokyo Rifle Co., Tokyo, model 320) at the other. The maximum pressure of this device was

* *Present address*: Sumitomo Chemical Co., Ltd., Biotechnology Laboratory, Takarazuka Research Center, 4-2-1 Takatsukasa, Takarazuka, Hyogo 665, Japan
** *Present address*: Department of Biological Sciences, Faculty of Science, Hiroshima University, Hiroshima 730, Japan

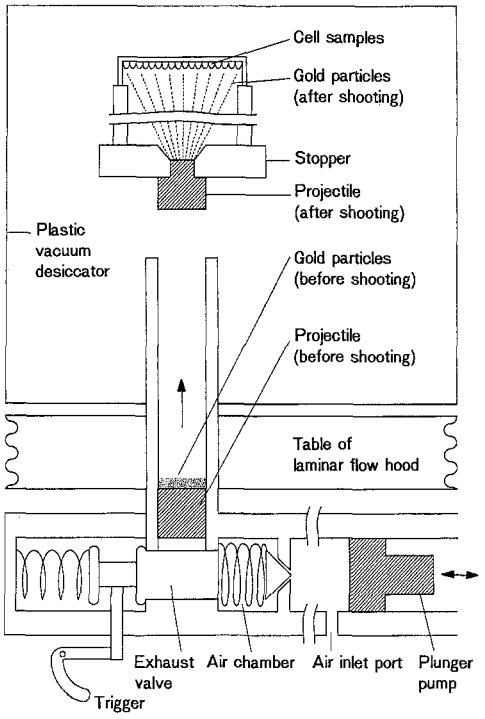

Fig. 1. Design of the pneumatic particle acceleration device. See text for details

more than 220 kg/cm^2, at which the initial velocity of the projectile was more than 440 m/s as determined by an electronic velocity meter. A metal stopper designed to stop the plastic projectile is connected at the top end of the barrel. The barrel is fixed vertically in a plastic vaccuum desiccator that is placed in a laminar flow hood (NKS, Osaka, HS1-M; see Fig. 1).

Five milliliters of suspension culture taken from tobacco cells 4 days after subculture (containing about 100 mg fresh cells or 5×10^5 cells) was placed on a filter paper (ADVANTEC TOYO No. 2, 3.5 cm diameter) on a funnel with sintered glass (Shibata Co., Tokyo, Microfilter 618–4711, and medium was removed by vacuum filtration. The funnel with the cells was turned upside down and placed 5–15 cm over the stopper.

A polyethylene projectile with the top surface covered with DNA-coated, or noncoated, gold particles was introduced from the top and placed at the bottom end of the barrel. Air was compressed (to 63–200 kg/cm^2) with a plunger pump and accumulated in the chamber. After the pressure in the desiccator had been reduced to 60 mm Hg, the compressed air was released instantaneously from the chamber to the barrel by triggering the exhaust valve. The projectile accelerated in the barrel and collided with the stopper, "stabbing" into it and sealing off the aperture. The gold particles continued their trajectory through the aperture of the stopper. The self-sealing by the projectile was important because, otherwise, expanding air reaching the aperture after the projectile would dislodge the target cells (Morikawa et al. 1989). The diameter of the distribution pattern of the gold particles was more than 5 cm when the accelerating pressure was 63–200 kg/cm^2, while the distance between target cells and the stopper stayed at 5 cm. Throughout this study a single shot was given to a cell sample.

Plasmid DNA and coating gold particles with DNA

Chimeric plasmid DNA, pBI221 (Clontech, Palo Alto/CA), which has the β-glucuronidase (GUS) gene under the control of the cauliflower mosaic virus 35S promoter and nopaline synthetase (NOS) polyadenylation signal, was used. The gold particles (1–3 μm in diameter, Alfa Chemical Co., Danvers/MA) were coated with the plasmid DNA (4 μg DNA/mg particle) by coprecipitation in ethanol, as reported previously (Morikawa et al. 1989). The closed circular form of plasmid DNA was used unless otherwise stated. For experiments with linear DNA, DNA was linearized by digestion with AatII at a unique site 465 bp downstream from the NOS polyadenylation signal. After digestion, the reaction mixture was deproteinized by phenol extraction. Linearization was confirmed by agarose gel electrophoresis. Ethanol suspension of the DNA-coated gold particles was placed on the surface of the projectile so that the concentration of the gold particles was 0.2 mg/projectile, after which it was dried and used for bombardment.

Assay of GUS expression

After being shot, the filter paper with the cells was transferred onto a plastic mat in a plastic container (Toyobo Co., Osaka, Plantex ccp-102) containing 17 ml of culture medium, kept for 24 h in the dark at 26 °C, and assayed for transient GUS expression. The GUS activity assay was carried out essentially according to Klein et al. (1988a): the filter paper was transferred to a sterilized plastic petri dish onto which 400 μl of filter-sterilized GUS substrate mixture had been previously placed. The substrate mixture consisted of 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 1.9 m*M* 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, a chromogenic substrate of GUS; Sigma Chemicals Co.), and 0.3% (v/v) Triton X-100. The cells were incubated for 24 h at 26 °C prior to observation under a binocular microscope (× 6.6, Nikon, SMZ-10). Gus-expressing cells were detected as blue-colored spots. The size of the blue spots varied from 400 to 1,000 μm, but each spot, regardless of its size, was considered as one GUS-expression unit.

Results and discussion

Figure 2 shows typical results of the GUS enzyme assay of the tobacco cells that were shot with the DNA-coated or noncoated gold particles and incubated with the substrate mixture, as described in "Materials and methods". Many blue spots or clumps of GUS-expressing cells were observed among the cells shot with DNA-coated gold particles (Fig. 2B). These blue spots became visible under the microscope approximately 1 h after addition of the substrate mixture. No blue spots were detected in the control cells that were shot with noncoated gold particles (Fig. 2A).

Interestingly, the blue spots were spread over the whole surface of the cell sample on the filter paper (Fig. 2B), indicating that successful delivery of the plasmid DNA occurred evenly over the whole surface of the cell layer with our device. In addition, there was not significant disruption of the cells at the center of the target by the expanding air. This is due to the fact that the projectile sealed off the stopper, preventing the expanding gas from blowing directly on the target cells (see "Materials and methods").

One of the advantages of the pneumatic particle gun developed in this study is that the accelerating pressure

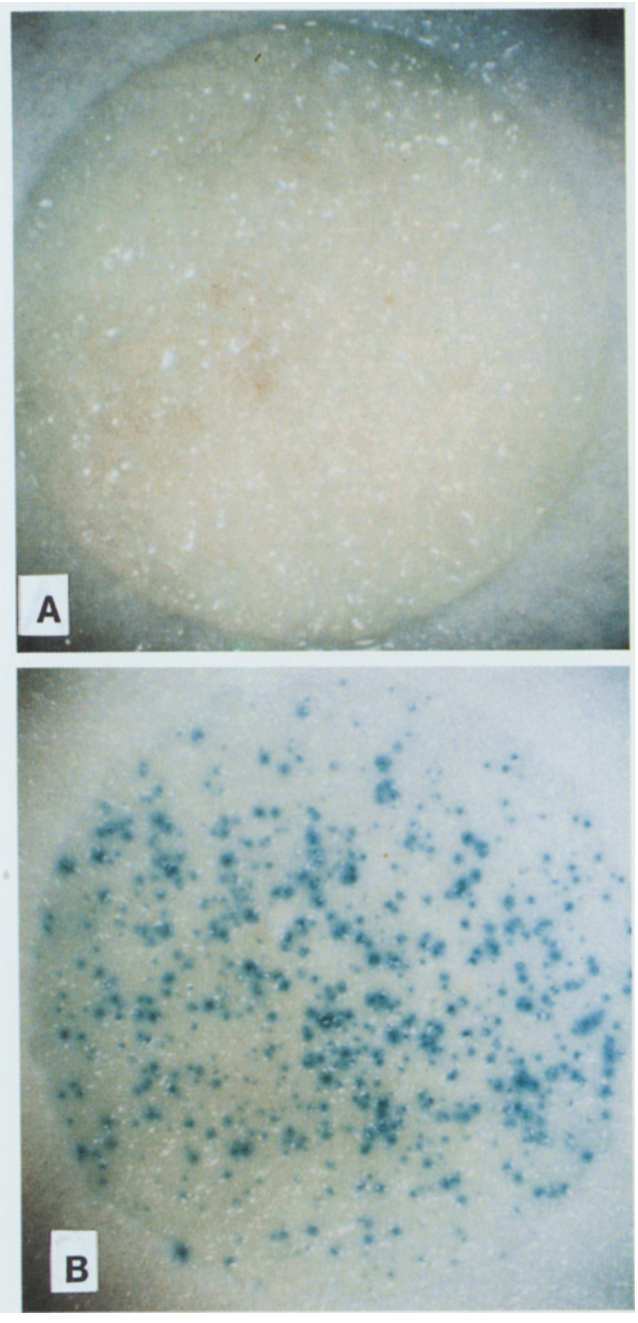

Fig. 2 A and B. Microphotographs of tobacco cells that were shot with DNA-coated (**B**) and noncoated (**A**) gold particles and assayed for the GUS expression. A number of blue spots of the GUS-expressing cells were observed in **B**, while no blue spots were seen in **A**. The diameter of the cell samples was 3.5 cm

can easily be adjusted. When the accelerating pressure was varied between 63 and 200 kg/cm^2 and the distance between the target cells and the stopper was varied betweeen 5 and 15 cm, the highest number of GUS-expression units (618 ± 49 per 5×10^5 cells on a 3.5-cm filter paper) was observed at an accelerating pressure of 150 kg/cm^2 and a distance of 10 cm, as shown in Table 1.

Table 1. Effects of accelerating pressure and distance on transient expression of the GUS gene[a]

Distance (cm)	Accelerating pressure (kg/cm^2)				
	63	84	115	150	200
5	276 ± 120[b]	314 ± 63	125 ± 12	175 ± 18	54 ± 19
10	247 ± 34	462 ± 64	467 ± 112	618 ± 49	360 ± 79
15	33 ± 21	95 ± 16	275 ± 134	305 ± 106	321 ± 67

[a] The layer of tobacco cells (35 mm diameter) was bombarded with plasmid DNA-coated gold particles. After incubation with the substrate mixture, the number of GUS-expression units (blue spots) was counted

[b] Values are the average number of GUS-expression units of two experiments $\pm$ deviation

This result clearly indicates that not only the distance but also the accelerating pressure is an important factor that determines the efficiency of the biolistic gene delivery. The accelerating pressure appeared to influence the velocity of the projectile (hence that of the gold particles) as well as the distribution pattern of gold particles (hence the density of the gold particles) on the cell layer. Optimal accelerating pressure differed for different types of cells and tissues (unpublished results).

When observed at higher magnification, each blue spot of GUS-expressing cells could be seen to be comprised of a densely stained blue central core, with a less densely stained surrounding region radially arranged away from the core. The central core consisted of a single cell (50–100 μm) or of several cells. The less densely stained surrounding region had a diameter of 400–1,000 μm. The exact number of the cells in a blue spot that received the DNA-coated gold particle(s) and expressed the gene could not be determined. On the basis of the difference in the density of the blue color, we think that only the core cells received the gold particle(s) and that the GUS enzyme or its reaction products (indigo dye) diffused from the core cells to the surrounding cells. It is also possible that the plasmid DNA may diffuse from the core cells to the surrounding cells. Some cells that were stained blue were observed to have gold particles in them, but the exact location of gold particles (inside or outside of the cells) was not clearly determined by optical microscopic observation.

If we assume that each blue spot was composed of a single GUS-expressing cell, the transient gene expression efficiency was calculated to be 1.2×10^{-3} at the optimal bombardment conditions described above. This value is comparable to, or even higher than, the values obtained by the gunpowder-driven device with tobacco (Klein et al. 1988 b), maize, rice and wheat (Klein et al. 1988 a; Wang et al. 1988), and barley (Mendel et al. 1989), where 100–700 blue spots were detected on the cell layer of 5.5 cm in diameter.

Table 2. Effects of conformation of plasmid DNA on transient expression of the GUS gene

Conformation of plasmid DNA	No. of GUS-expression units		
	Exp. 1	Exp. 2	Exp. 3
Closed circular	336 ± 6[a]	164 ± 35	528 ± 118
Linear	485 ± 15	124 ± 11	498 ± 151

[a] Values are the average of two experiments ± deviation

Linearization of plasmid DNA did not appreciably influence the transient gene expression of the GUS gene, as shown in Table 2. Klein et al. (1989) also reported that linearization of a plasmid harboring the neomycin phosphotransferase II (NPT II) gene did not increase the frequency of biolistic transformation of maize cells. We obtained similar results in transformation of tobacco cells with the NPT II gene using our pneumatic particle gun (unpublished results).

We have shown efficient gene transfer with the pneumatic particle gun device driven by compressed air. Further optimization of other factors that may influence the efficiency of gene delivery may be possible. Even at the present efficiency, however, this should be a useful tool for gene transfer into intact cells and tissues.

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