Optimization of Microprojectile Bombardment Parameters for Pollen-Mediated Transformation in *Gossypium hirsutum* L.

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ABSTRACT

Mature pollen-mediated transformation in cotton (Gossypium hirsutum L.) by microprojectile bombardment was established under gaseous medium in our laboratories. Bombardment parameters influencing the vitality, in vitro germinability and GUS transient expression of bombarded pollen were studied and optimized by the Gray System Analysis method. Optimum conditions for cotton are summarized as follows: the microprojectiles were 0.8-1.1 μm diameter metal (tungsten or gold) granules; target distance between the stopping screen and the tissue was 5 cm for a JQ-700 gene delivery device, or 8 cm for a PDS-1000/He apparatus; and mature pollen was bombarded one time at a velocity of 450 m/s for the JQ-700 gene delivery device, or under 1100 psi shot pressure for the PDS-1000/He apparatus. Seeds were set after pollinating with the bombarded pollen.

Introduction

It is vitally important for genetic engineering to develop a simple, effective and practical transformation system. The pollen-mediated transformation procedure, first reported by Leed-Plegt *et al.* (1995), is a new method to transform plant species taking advantage of the plant self-embryogenetic mechanism. It is essential that the transformed pollen grains be alive so that they can be used for pollination. Since the pollen grain of many plant species, such as *Gossypium hirsutum* L., will be ruptured in water easily, the bombardment of pollen grains must be conducted under gaseous medium. Although there are many reports on optimization of microprojectile bombardment, there is no report on bombardment-mediated transformation of pollen under gaseous medium. DNA can be delivered into pollen grains through biolistics (Deng *et al.*, 1997). The major purpose of the present study was to optimize the parameters for cotton pollen bombardment-mediated transformation to increase the transformation efficiency.

Material and Methods

Plant materials and plasmid. Eleven Chinese cultivars of *G. hirsutum* were chosen for this test. Texas Marker 1, "TM-1", used as a control, is a genetic standard strain for *G. hirsutum* L. (Kohel *et al.*, 1970). Fresh, mature pollen grains were taken as transformation receptors and the plasmid pBI121 was used as the DNA carrier.

Reagents and equipment. A helium-driven PDS-1000/He system (DuPont Co.) and a JQ-700 gunpowder particle bombardment device (Academic Sinica, Beijing) were used for bombardment. Fluorescein diacetate (FDA) was purchased from Sigma. Gold and tungsten particles from BIO-RAD were used to prepare microprojectiles. Bombarded pollen was observed under a S-450 scanning electron microscope (SEM)(Hitachi Ltd, Japan).

Preparation of microprojectiles. The plasmid pBI121, carrying the β-glucuronidase (GUS) gene driven by 35S promoter (Jefferson, *et al.*, 1987), was isolated and purified according to Sambrook *et al.* (1989). Four μg of plasmid DNA was precipitated onto 50 μg of gold particles (Dunder *et al.*, 1995).

Bombardment. One sterile Whatman No.1 filter paper and one camera lens paper were put onto the surface of a 60 mm Petri dish successively. A steel tube, 15 mm high and 38 mm inner diameter, was laid on the centre of the camera lens paper. A 35 mm diameter central area in the steel tube, divided into an inner circle of 17.5mm diameter and an outer circle was used for sampling. On the afternoon before anthesis, flower buds were tied shut with cotton twine. These alabastra were collected next morning and dehiscing pollen grains were spread over the camera lens paper. When bombardment was with the PDS-1000/He apparatus, 6 μl (about 0.5 μg plasmid DNA) of microprojectile suspension was transferred to the center of a macrocarrier. When the JQ-700 device was used, 3 μl of the above suspension was placed on the front hollow

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surface of a cylindrically shaped polyethylene macroprojectile.

Assessing the viability and germinability of bombarded pollen. The viability of bombarded pollen was determined by FDA staining and observing under fluorescence microscopy with reflected light fluorescence, in which the size of filter cube, excitation filter bandpass, dichromatic mirror and suppression filter was I3, BP340-380, BKP510 and LP515, respectively. Yellow-blue pollen grains were considered as viable.

A modified hang-droplet method (Taylor, 1972) was used to evaluate pollen germinability. The droplet was liquid medium in which the agar was removed and 100 mg/L each of glutamine, lysine, proline and serine were added, and the sucrose was increased from 25% to 40%. Approximately 4 hours after dispersal in the droplet, the number of pollen grains ejecting a tube-like structure was recorded. The pollen grains with an ejected tube equal or longer than the diameter of the pollen grain was considered as germinated and those with a shorter ejected tube as germinating. After staining with aniline blue, the pollen could also be observed by fluorescence microscopy with the above parameters set at A, BP340-380, RKP400 and LP425, respectively.

GUS histochemical assay. GUS activity in bombarded pollen was based on the method described by Jefferson *et al.* (1987). More than one hour after bombardment, the pollen grains were incubated in 20 µl X-Gluc buffer for 4 to 12h at 37°C. After staining, the pollen was rinsed in 70% ethanol overnight and then mounted on microscope slides for observation.

Statistical methods. Microprojectile bombardment parameters were optimized by the Gray System Analysis introduced by Deng *et al.* (1993) in which the determination of weighting coefficient vector, $\{w_j\}$, on biological characteristics of bombarded pollen was based on Statty's multiple-scale method introduced by Zhao *et al.* (1986):

$$\{w_j\} \geq 0,\ j=1,2,0,0,,p,\quad ,\ \sum_{j=1}^{p} w_j = 1$$

Results

Pollen grain migration during bombardment. Before bombardment, the pollen from dehiscing anthers was spread over camera lens paper. Although there are spines on cotton pollen grains, the grains on the paper should be put down by tweezers, otherwise, the pollen grains will migrate during bombardment. As can be seen from Table 1, the higher the microprojectile delivery velocity at a fixed number of bombardment times, and the more bombardment times at a fixed velocity, the higher the percentage of migrated pollen grains, especially for inner circle pollen. However, compared to the first bombardment, the percentage of migrated pollen grains is not as high for the second or third bombardment. Therefore, it can be considered that the bombarded pollen grains were fixed in their original positions. SEM revealed many microprojectiles on the bombarded pollen grains (Fig. 1a) but none on unbombarded grains (Fig. 1b).

Microprojectile bombardment modes affecting pollen viability and GUS transient expression. When the microprojectiles were 1.0 µm gold particles and pollen of "Ji938" was bombarded by the PDS-1000/He device with a 1100psi rupture disk, the percentages of *in vitro* germinated and GUS positive pollen grains decreased with the number of bombardments. (Table 2)

Regardless of the diameter or delivery velocity of the microparticle, some bombarded pollen grains of "TM-1" were alive (Table 3, Fig. 2a,b). However, the higher the velocity and the greater the particle diameter, the lower the viability and germination ratio for bombarded pollen (Fig. 3a,b). The highest percentage of GUS transient expression for bombarded pollen was obtained at a delivery velocity of 450 m/s, with microprojectiles prepared with 0.8 µm or 1.1 µm tungsten particles (Fig. 4a,b).

Optimization of microprojectile bombardment. Following different modes of bombardment (Table 3), the percentages of GUS positive, germinated, viable, ruptured and germinating pollen grains differed. Weighting coefficient vectors on these biological characteristics were (0.408, 0.263, 0.178, 0.112, 0.040), respectively, produced by the multiple scale method. The approximate maximum value of the characteristics, but 100 times reciprocal for the percentage of ruptured pollen, was taken as the reference series of ideal pollen biological characteristics, i.e. 60.00, 45.00, 90.00, 4.50, 45.00, respectively. Based on the above, the Gray System analysis for the seven microprojectile bombardment parameters shown in Table 3 was conducted, and the degree of weighting relevancy for the parameters from top to bottom in Table 3 was 0.7558, 0.7577, 0.6957, 0.7578, 0.5996, 0.6248 and 0.5259, respectively. Therefore, the optimum bombardment parameter for mature pollen transformation can be summarized as follows: the microprojectiles were prepared with 0.8 µm or 1.1 µm diameter metal granules, bombarded one time with a delivery velocity of 450 m/s. Seeds could be set following pollination with bombarded pollen by the optimized mode in 11 cotton cultivars, with an average of 2.87 fertile seeds produced per bombardment.

Discussion

Microprojectile bombardment invented by Sanford *et al.* (1987) has been extensively used for producing transgenic plants. There are many factors affecting transformation efficiency of microprojectile bombardment. Scientists focused on the preparation of

microprojectile, bombardment parameters and biological factors (William, 1992). The quality of microprojectile was influenced by the diameter of metal granules and DNA precipitation procedure and method. Owing to the DNA precipitation standardization and popularization, it is essential to choose suitable diameter metal granules for transformation of pollen by bombardment. Transformation will be influenced by the cell type, density, thickness, physiological state, processing method and other factors. Although plant cell walls have pores with a diameter of ca 100 Å (Carpita *et al.*, 1979) they do not allow DNA permeation. Bombardment (Klein *et al.*, 1987) is a way for high speed metal particles carrying exogenous DNA to pass through cell walls and membranes and enter into the cell. Cotton and other plant pollen grains are covered with a thick, rigid exine, so exogenous DNA is not easy to be transferred into cotton pollen except by bombardment. Pollen- mediated transformation was established *via* mature pollen bombarded under gaseous medium in our laboratory. Thus, it differs in suitable microprojectile bombardment parameters from liquid medium as reported by Leede-Plegt *et al.* (1995).

When DNA-coated gold particles are bombarded at an accelerating pressure of 115 kg/cm$_2$ onto layers of 1% agar, the microprojectiles penetrate ca. 60 to 150 μm on average with 1.1 and 2.0 μm particles (Morikawa *et al.*, 1994). This suggests that the presence of a water layer of ca.100 μm on the surface of sample cells interferes with the entry of bombarded gold particles. Thus, removal of the water layer from the surface of the acceptor or bombardment under gaseous medium is essential for successful gene delivery into these cells. However, there is no report on this aspect. Pollen grains are favorable for high frequency transformation because they provide a single cell layer and exposed surface. Transformation of mature pollen, bombardment by optimized parameters, and maintaining bombarded pollen viability, are all essential for making pollen-mediated transformation applicable.

Several technical barriers on cotton experimental and reproductive biology (Hu, 1982) have been resolved in this program. The percentage of *in vitro* pollen germination was increased to 45.24% using our *in vitro* culturing method, compared to approximately 30% by the method of Taylor (1972).

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Table 1. Migrated pollen on the surface of Petri dish after bombardment as a mean percentage of the unbombarded control (three replications).

"One", "Two" and "Three" indicates the number of bombardments.

	Outer lane			Inner circle			Mean		
	One	Two	Three	One	Two	Three	One	Two	Three
300m/s	6.45	10.87	23.26	5.25	8.54	9.20	5.84	9.71	16.23
350m/s	11.40	20.20	22.31	31.81	44.76	35.47	22.71	32.48	28.89
440m/s	36.23	36.84	45.26	39.10	56.24	54.91	37.75	46.54	50.09
470m/s	50.45	51.00	61.79	40.77	57.89	59.67	45.57	54.45	60.73

Table 2. Biological effect of repeated bombardment on pollen. Values are the mean of two replications.

Number of bombardments	GUS positive pollen (%)	Vigorous Pollen (%)	Pollen Culture in vitro Percentage		
			Germinating Pollen	Germinated Pollen	Ruptured Pollen
1	76.76	80.00	21.69	42.33	29.10
2	60.11	69.45	4.52	35.03	37.35
3	54.27	62.45	11.35	29.79	28.37
control	0.00	88.94	9.82	64.29	20.09

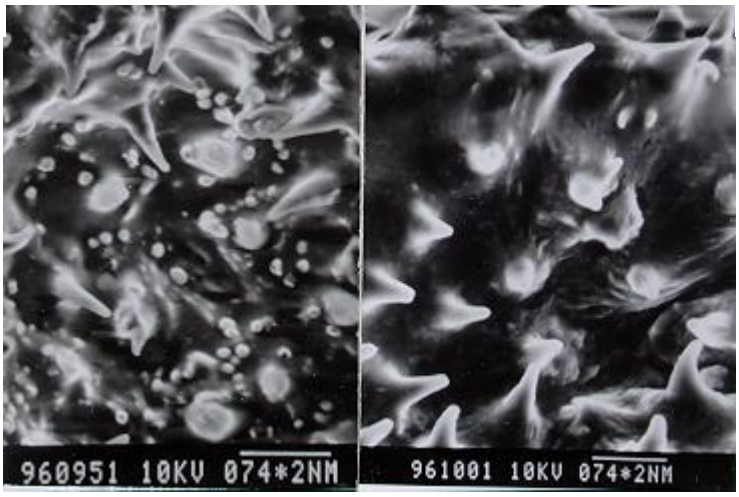
Table 3. Biological effect of bombarded parameters on pollen. Values are the mean of three replications.

Modes	Vigorous Pollen (%)	GUS Positive Pollen (%)	Bombarded Pollen Culture in vitro Percentage		
			Germinating Pollen	Germinated Pollen	Ruptured Pollen
0.8μm/300m/s#	80.39	39.73	28.11	39.41	25.11
0.8μm/450m/s	74.75	57.59	27.50	28.33	36.63
1.1μm/300m/s	80.77	42.46	44.87	26.21	23.51
1.1μm/450m/s	79.47	52.31	23.83	33.36	30.29
1.1μm/600m/s	74.76	46.65	22.71	12.92	38.80
1.4μm/450m/s	77,99	44.58	37.00	6.23	24.52
1.4μm/450m/s	65.12	35.39	7.05	1.90	25.28
Control	92.96	0.00	27.78	48.61	26.39

#Microprojectiles were 0.8 μm diameter tungsten particles coated with pBI 121 accelerated with a velocity of 300 m/s.

Figure 1. Photomicrograph of microprojectiles on the bombarded pollen by SEM (a) Microprojectiles on the bombarded pollen; (b) Control.

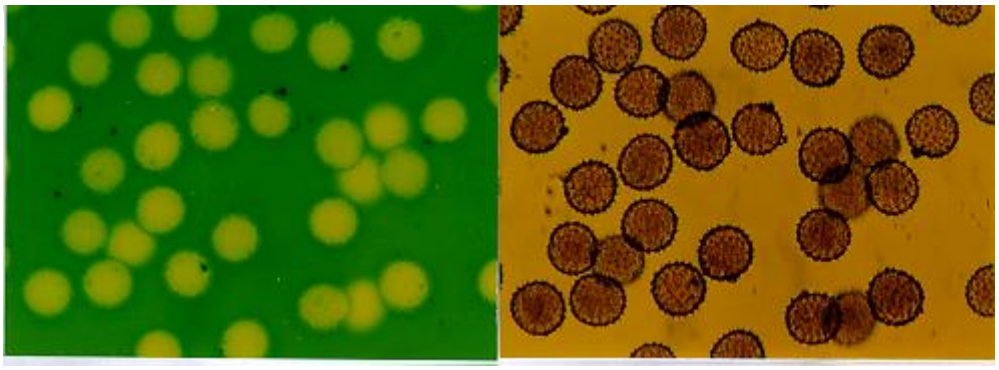
a



b

Figure 2. Photomicrograph on activity of the bombarded pollen by fluorescence microscopy
a)Vigor bombarded pollen, excited on I3 (BP450~490)
b) Vigor bombarded pollen, excited on the visible light.

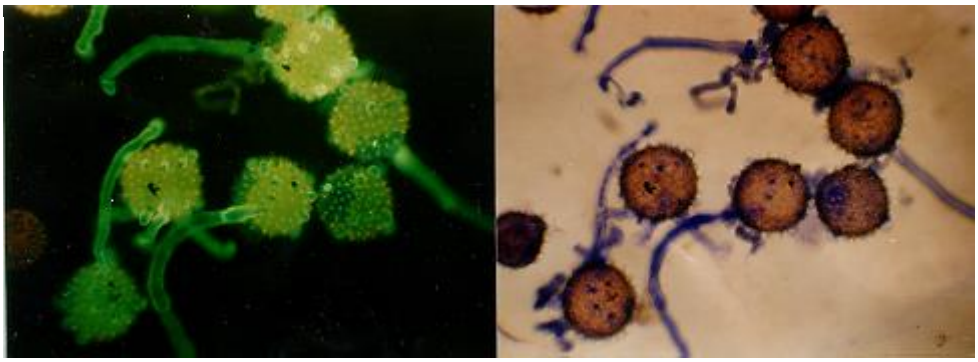
a



b

Figure 3. Photomicrogiaph of in vitro germination of the bombarded pollen by fluorescence microscopy
(a) Germinating pollen bombarded, excited on A (BP340~380)
(b) Germinating pollen bombarded, excited on the visible light.

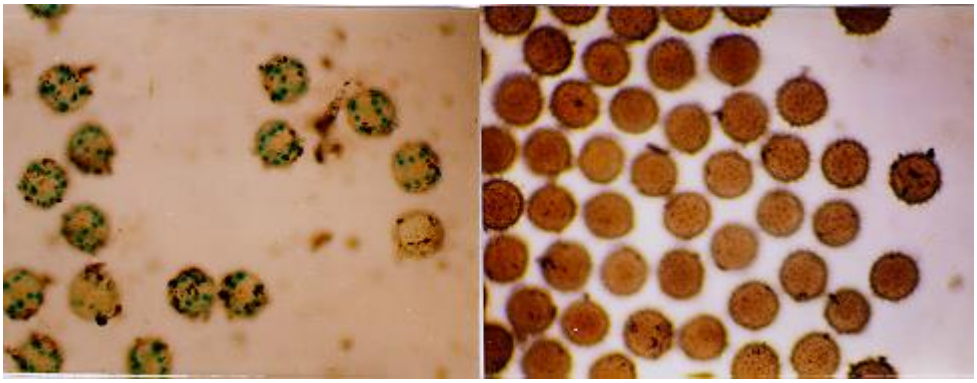
a



b

Figure 4. Transient expression of GUS in bombarded pollen incubated by X-GLUC
(a) The blue spots on the bombarded pollen;
(b) Control;

a



b