Confocal Imaging on Microprojectile-bombarded Pollen of *Gossypium hirsutum* L.

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ABSTRACT

Confocal laser scanning microscopy (CLSM) was introduced to study transgenic molecular cytobiology. Scanning electron microscopy (SEM) was used to observe the transferring traces of accelerated, DNA-coated, metal particles labeled with 4,6-diamidino-2-phenylindole (DAPI) in mature cotton pollen (G. hirsutum L.). The results indicated that pollen exine can be penetrated by the high-velocity microprojectiles, and the microprojectile with fluorescent probe and its transferring trace in the exine-detached pollen can be observed. The optical sectioning imaging analysis indicated that the microprojectile can be introduced into a bombarded pollen grain approximately 20.6 μm deep. In bombarded grains, transient expression of a β-glucuronidase (GUS) gene in pBI121 was certified by histochemical assay.

Introduction

Although *Agrobacterium tumefaciens*-mediated transformation has been successfully applied in cotton, it is cultivar-dependent in that callus tissue of most cultivars is not embryogenic so its use is limited to the few embryogenic cultivars (Bayley *et al.*, 1992). Other transformation systems based on cell and tissue culture *in vitro* are also genotype-dependent (John and Stewart, 1992). Pollen-mediated transformation, first reported by Leede-Plegt *et al.* (1995), is a new procedure to transfer foreign genes into plants. In this method, pollen grains were bombarded by high-velocity microprojectiles and then used for pollination. Although several approaches have been attempted to introduce DNA into mature pollen, microprojectile bombardment is considered the best procedure (Deng *et al.*, 1997). Confocal laser scanning microscopy (CLSM), allowing optical sectioning of the cells, is a new tool for the fine localization of fluorescent molecules, but there are no reports on transgenic molecular behavior using CLSM. In this paper, we report the transfer of foreign DNA into mature cotton pollen grains and the observation of microprojectile-bombarded pollen grains with CLSM.

Material and Methods

Plant materials and plasmid. A total of 24 Chinese cotton (*G. hirsutum L.*) cultivars, were used (Table 1). "TM-1", used for control, is a genetic standard strain while the other 23 cultivars are genotypes collected from 5 ecological regions and derived from seven ancestors selected according to their production acreage and pedigree. Fresh, mature pollen grains were taken as transformation receptors and the plasmid pBI121 was used as the DNA vector.

Reagents and equipment. DAPI was purchased from Sigma (St. Louis, MO). A helium-driven PDS-1000/He system (DuPont Co., DE) and JQ-700 gunpowder particle bombardment device (Academic Sinica, Beijing) were set up for bombardment. Gold (1.0 μm) and tungsten (1.1 μm) microparticles purchased from BIO-RAD were used for the preparation of microprojectiles. Bombarded pollen grains were observed with a S-450 scanning electron microscope (SEM) (Hitachi Limited., Japan). Study on the molecular cytology with an Ultima 212 CLSM (Meridian Co.) was conducted in the Peking Union Medical College (Beijing).

Preparation of microprojectiles. The plasmid pBI121, carrying a GUS gene driven by 35S promoter, was isolated and purified according to the procedure of Sambrook *et al.* (1989). Plasmid DNA (4μg) was precipitated onto 50 μg of gold particles using the procedure of Dunder *et al.* (1995).

Preparation of pollen grains. One sterile Whatman No.1 filter paper and one camera lens paper were placed successively onto the surface of a 60μm Petri plate. On the afternoon prior to blooming, flower buds were tied closed with cotton rope. On the morning of anthesis the flowers were collected, and the pollen grains on the dehiscent anthers were spread over the camera lens paper.

Bombardment. Bombardment by the PDS-1000/He apparatus transferred 6μl (about 0.5 μg plasmid DNA) of the microprojectile suspension to the centre of a

macrocarrier. The size of the rupture disk was rated at 1100psi and the stopping screen/target distance was 8 cm. The JQ-700 device placed 3μL of this suspension on the front hollow surface of a cylindrically shaped polyethylene macroprojectile. The microprojectile launch velocity was 450m/s and the stopping screen/target distance was 5 cm.

Observation with SEM. The bombarded pollen grains were placed in a 0.5 ml Eppendorf tube and fixed in a solution of 2.5% glutaradehyde for 10 h to several days at 4°C. The samples were then washed several times with 0.1M phosphate buffer (pH 7.1). When exine-detached pollen was prepared, the sample was transferred into 10% sodium hypochlorite solution for 20 min at 60°C. All samples were dehydrated in a graded ethanol series (20% steps for 10 min each), and incubated in isopentyl acetate solution overnight. For exine-detached pollen preparation, the sample was subjected to a CQ_{50} ultrasonator for 5 min. The pollen was critical point dried in a HCP-2 Hitachi Critical Point Dryer, and mounted on an objective stage with double-sided tape and coated with gold.

Labelling of plasmid DNA. After centrifugation for 2 seconds in a microfuge and removal of the supernatant, the microcarriers were incubated in 20 μL of 2 μg/mL DAPI dissolved in TAN buffer (Nemoto *et al.*, 1988) without disturbing the pellet for 30 min at room temperature. The supernatant was removed and residual water was displaced in a graded ethanol series. For the final step 50 μL of 100% ethanol was added for the preparation of microprojectiles.

Observation procedure with CLSM. The pollen grains bombarded with labeled microprojectiles were fixed, washed and incubated with sodium hypochlorite as described above. Supernatant was removed and displaced in a graded TAN-buffer series. The pollen grains were dissected on glass slides with a surgical needle. Unbombarded pollen was stained for 10 min with 100 μl of 2 μg/mL DAPI. Exine-detached pollen grains were burst by application of slight pressure to a cover slip placed on top of them. The exine characteristics of bombarded pollen could be observed directly.

Under CLSM, the sample was observed with a fluorescence microscope. When the target point was found, the UV laser module was excited with a band length of 351-364 nm. The point-detector was scanned through the specimen forming a complete image. Image analysis of optical sectioning was conducted by image software and confocal assistant software designed for the Ultima 212 CLSM.

GUS histochemical assay. GUS histochemical assay of bombarded pollen was based on the method described in 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, sections were rinsed in 70% ethanol overnight and mount on microscope slides.

Results

Pollen exine can be penetrated by accelerated microprojectiles. Because the cell wall limits DNA permeation, it is essential for gene delivery into pollen that the pollen grain exine should be penetrated by accelerated microprojectiles. In this experiment, mature cotton pollen grains were bombarded by PDS-1000/He or JQ-700 microprojectile delivery devices. It was difficult to confirm the penetration of pollen exine by microprojectiles with ordinary optical microscopy. Under the SEM, many traces struck by the microprojectiles could be observed on the surface of the exine (Plate I, 1,2), while in the unbombarded pollen there were no traces (Plate I, 3). We used CLSM imaging procedures to analyze pollen grains bombarded with labeled microprojectiles. When a series of 0.8μm thick sections were produced from outside to inside of a pollen grain, there were some positions with no fluorescence conformed by scanning imaging in line (Plate II, 1,2). Because the pollen wall, and especially the exine, autofluoresces, it could be concluded that sites with the absence of fluorescence were the holes bombarded by the accelerated microprojectiles. In other words, the pollen grain exine can be penetrated by microprojectile bombardment.

Microprojectiles can be delivered into pollen cytoplasm. Some holes were observed on bombarded exine-detached pollen grains by the SEM (Plate I, 4). A series of 1.0μm thick sections with CLSM were produced from the outside to inside of a bombarded exine-detached pollen grain. There were holes in the sections from the first to the twelfth layer (Plate II, 3). When analyzed together with exine imaging, it could be concluded that a hole 20.6 μm deep was produced by the accelerated microprojectiles.

When pollen grains were bombarded by microprojectiles coated with fluorescence-labeled DNA, a fluorescent glistening dot was found in a series of 100nm thick sections (Plate II, 4,5), and *in vitro* the same fluorescent dot could be observed with CLSM. The fluorescent glistening dot was under 2μm in diameter and much smaller than the pollen cell nucleus labeled with DAPI (Plate II, 6). Therefore, it was assumed to be the bombarded microprojectile. In other words, exogenous DNA can be delivered by microprojectile bombardment.

Transient expression of GUS in the bombarded pollen. The pollen grains were bombarded with pBI121-coated microprojectiles. One hour later, the bombarded grains were incubated in X-Gluc buffer. After 4 hours of incubation, blue spots could be observed on the pollen grains. If decolorized in 70% ethanol and the exine detached from the bombarded pollen, blue dots remained in pollen grains incubated with X-Gluc for 12h. It is clear that transient

expression of GUS occurred in bombarded pollen. Furthermore, transient expression of GUS in bombarded pollen grains was present in all 24 upland cotton cultivars incubated with X-Gluc (Table 1). The percentage of pollen grains with transient GUS expression for the 24 cultivars ranged from 27.4% for "Liaomian 1" to 88.7% for "Jimian 1", and the average percentage was 61.1%.

Discussion

The intracelluar location of exogenous genes with the experimental biological method is important for understanding the mechanism of pollen-mediated transformation. Observations on gold particle delivery into lily pollen grains were made by Yamashita *et al.* (1991), who found that gold particles in nuclei, vacuoles and cytoplasm could be clearly observed under optical microscope when the bombarded cells were fixed and stained with orcein. Because there is no label in the gold particles, the result is hardly approved in the paper. In the present report, the transferring traces of accelerated microprojectiles labeled with DAPI in cotton pollen grains were observed with CLSM. Our observations on the intracelluar location of exogenous genes and imaging of the transformed cell are novel. Because of autofluorescence of the pollen wall, a fluorochrome-labeled substance in the pollen grain is difficult to detect directly. Although research on exine-detached pollen is in progress (Xia *et al.*, 1995), there are no reports on the preparation and use of exine-detached pollen for CLSM. Intact exine-detached pollen grains have been made for SEM and CLSM in the present paper. Pollen grains are covered with a thick, rigid exine except for the area of the pollen tube pore that has a less thick wall. Morikawa *et al.* (1994) suggested that DNA-coated metal particles are delivered into pollen cells through the pollen tube pore only, however, they can be delivered into a pollen grain anywhere by SEM.

The pollen cell is one of the most intriguing subjects in cytology, and it has many advantages as a transforming acceptor. (1) The pollen cell is large, so, not only is the frequency of the pollen grains accepting exogenous genes higher, but also the percentage of bombarded cells is higher and the statistical figures are more exact. The reason is that a single layer of cells can be prepared on a plate easily with the larger pollen grains. (2) Owing to great diversity in male gametophytes, a suitable cell type could be found for this kind of transformation system. (3) Pollen grains for plant species are vigorous *in vitro*, thus, pollen transformation can be conducted in the gaseous medium. When DNA-coated gold particles are bombarded at an acceleration pressure of 115kg/cm$_2$ to the layers of 1% agar plate, introduced particles can penetrate ca. 60 and 150μm (Morikawa *et al.*, 1994). This suggests that the presence of a water layer on the surface of sample cells interferes with the entry of bombarded gold particles. (4) Pollen cells are, in general, rich in cytoplasm and practically lack a vacuole. Thus, they seemed to tolerate the damage from multiple introduction of metal particles by bombardment. (5) Pollen cells have natural ability for reproductive growth, so genetically transformed progeny can be produced by pollination with mature pollen bombarded with DNA-coated particles, i.e., pollen-mediated transformation. It avoids time-consuming tissue culture steps that are known to create undesirable somaclonal variations and the development of chimeras, thus making it broadly applicable for the technology and plant genotypes. (6) A pollen grain is a haploid cell. Transgenic haploid plants would be produced through direct embryogenesis of immature pollen transformed by the microprojectile bombardment or electroporation. Transgenic haploid plants are useful materials for basic genetic studies and breeding, and also should prove useful for gene-tagging and mutation studies. (7) The pollen grain is a useful cell type for research on exogenous gene participation in cell movement because of the advantage of detecting effects on microtubular cytoskeleton during development of the pollen and zygote, and pollen tube participation in cell fusion, and so on. It can, therefore, be concluded that the pollen cell, a large cell with special functions, can be used as an experimental cell system for not only promoter and transformed vector analysis but also transgenic molecular cytobiology research. Microscopic observations on bombarded pollen of *G. hirsutum* have not only laid a foundation for studying this entire cell system but also has given a definite answer on whether the exogenous gene delivery into pollen cell occurs or not. It is essential information for the development of pollen-mediated transformation.

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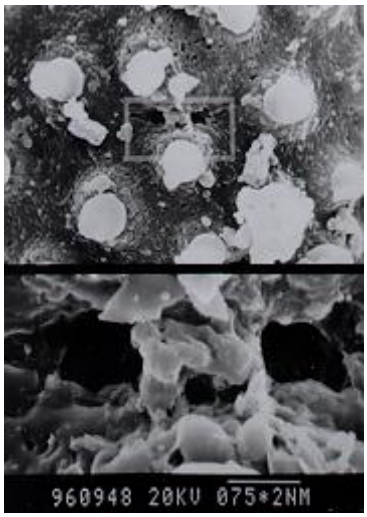
Table 1. GUS transient expression of bombarded pollen in Chinese cotton (*Gossypium hirsutum L.*) cultivars.

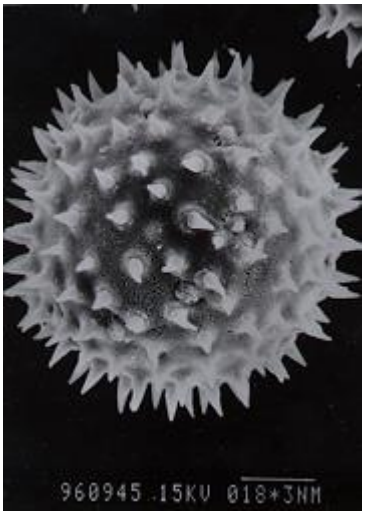
Cultivars	Ecologic types	Ancestors	GUS positive pollen (%)	Cultivars	Ecologic types	Ancestors	GUS positive pollen (%)
Zhongmiansuo12	Huanghe River valley	Uganda	54.29	Xinmian 3	Huanghe River valley	Acala	80.45
Huamian 7	Yangtse River valley	Trice	61.28	Jizhi 82-1	Huanghe River valley	Acala	88.26
Liaomian 7	North earliest cotton region	King	27.37	Zhongmiansuo13	Huanghe River valley	King	85.86
Xinlu 201	East land-locked region	Foster	39.75	Shanmian 7	Huanghe River valley	Foster	86.20
Songzidaling	Yangtse River valley	Lone star	38.46	Jimian 1	Huanghe River valley	Lone star	88.70
Yuelu 1	South region	Deltapine	29.86	Sumian 2	Huanghe River valley	Uganda	29.03
Guimian 3	South region	Deltapine	35.83	Liaomian 10	North earliest cotton region	Foster	75.15
Guimian 2	South region	Deltapine	36.15	Jinmian 2	North earliest cotton region	King	59.98
Nantong1 2	Yangtse River valley	Trice	73.27	Liao 2152	North earliest cotton region	Lone star	53.34
Chuan73-27	Yangtse River valley	Deltapine	86.17	Xinku 80432	East land-locked region	Lone star	64.93
Baoshandaling	Yangtse River valley	Deltapine	82.71	Ji 938	Huanghe River valley	Acala	79.72
Xiangmian 13	Yangtse River valley	Uganda	50.74	TM-1	U.S.A.		59.04

Plate I Photomicrograph of bombarded pollen exine by SEM

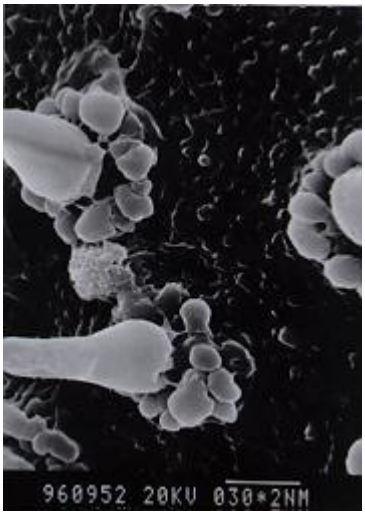
(1) There are two bigger bombarded pores in the exine;



(2) The morphological character of unbombarded pollen exine;



(3) There is one bombarded pore in the exine;



(4) Pores in the exine-detached pollen bombarded by PDS-1000/He.

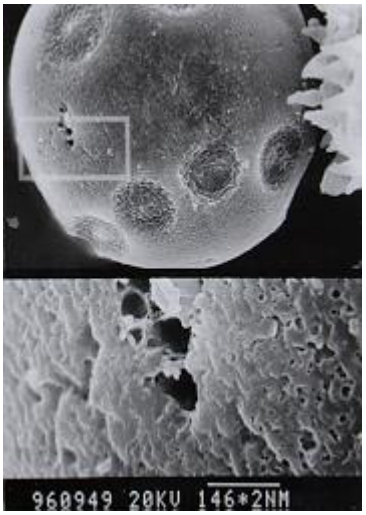
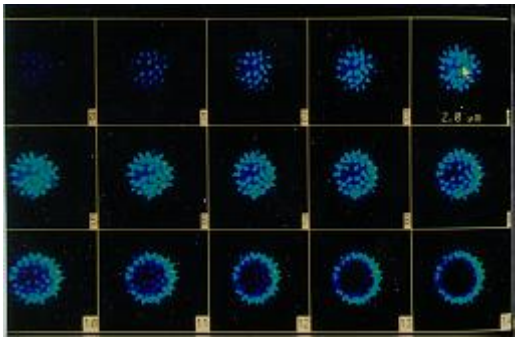
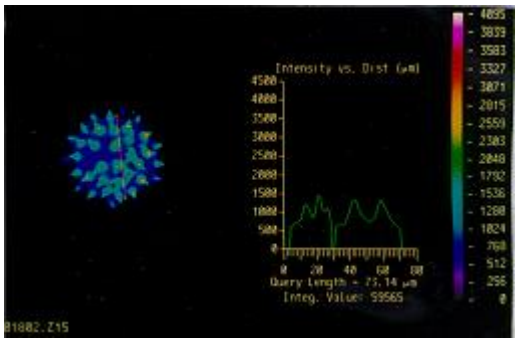


Plate II Confocal imaging on the bombarded pollen

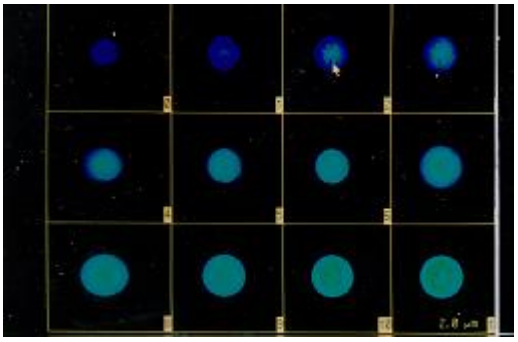
(1) Serial optical sectionings of the exine of bombarded pollen, 0.8 μm thick per step. There are two pores indicated between sections No.0 to No. 11.



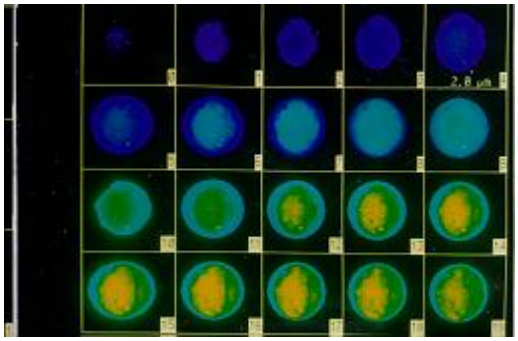
(2) There is minimum fluorescent value in the fourth optical sectioning after scanning in line.



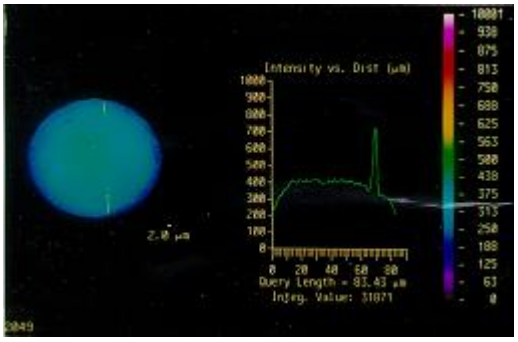
(3) Serial optical sectionings for the exine-detached pollen bombarded by PDS-1000/He, 1.0 μm per step, by CLSM. There are pores in the sections between No.0 to No.10.



(4) Serial optical sectioning for the exine-detached pollen bombarded by PDS-1000/He, 0.1 μm thick per step. There is a microprojectile labeled by DAPI between No.2 to No.13.



(5) In line scan of the tagged position in No.7.



(6) Recombined serial opitical section of the exine-detached pollen in two dimensional space. The generative nucleus and vegetative nucleus labeled by DAPI can be seen.