Pollen-mediated Transformation by Microprojectile Bombardment is Genotype Class Independent in *Gossypium hirsutum* L.

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ABSTRACT

The biological characteristics of bombarded pollen were presented by the optimized delivery mode for TM-1, a genetic standard, and 23 Chinese G. hirsutum cultivars, which were collected from 5 ecological regions and derived from 7 ancestors. The results showed that, compared with unbombarded pollen, the average percentage of GUS transient expression of bombarded pollen grains is about 61.12%, the percentage of viable and germinated pollen grains is decreased by 14.34% and 25.83%, respectively. There are remarkable differences as compared with the control by statistical test. Although the biological effects of bombardment on pollen differ among 24 cultivars and the cultivars could be divided into four groups by Star-Site Cluster analysis, differences estimated by mutual information analysis were not related to their ecological type or ancestry. Therefore, pollen-mediated transformation method was independent of genotype. This is not only a new approach to studying the relationship between a plant transformation system and its receptor genotype, but also a first report that the transformation system is genotype-independent. Stigmata sprayed with the screening liquid were pollinated with bombarded pollen grains and seeds were set in 11 cotton cultivars. On average, 2.87 seeds could be set pollinated with one dish bombarded pollen grains. To screen for transgenic plants, the embryonic roots from the resulting seeds were immersed in Hogland solution with 100 mg/L kanamycin. Many plants have survived, and for cotton cultivar "Ji 938" 3.0 % of the plants survived.

Introduction

Transformation of plants has achieved great success, and now over 100 transgenic plant species have been developed. However, most transformation systems are limited by genotype specificity, such as the Coker lines (John *et al.*, 1992) in *Agrobacterium*-mediated transformation of cotton. Inserted gene(s), such as the Bt gene, have to be transferred by backcross to popular cultivars to be grown by farmers. Exogenous DNA can not be delivered easily into every desired variety (Songstad *et al.*, 1995). Pollen-mediated transformation, first reported by Leede-Plegt *et al.* (1995), is a new procedure to genetically transform plant species in which the pollen, as a natural transformation vector, is bombarded by biolistics. The bombarded pollen is then used for pollination and seeds are produced directly. This transformation system utilizes the reproductive mechanism of the plant. The pollen mediated-transformation technique has been established in cotton in our laboratories and its bombardment parameters have been optimized (Deng *et al.*, 1998a,b). The major purpose of this paper was to study the relationship between *Gossypium hirsutum* L. genotypes and their mature pollen-mediated transformation by microprojectile bombardment to determine if there is genotype dependence in pollen-mediated transformation.

Material and Methods

Plant materials and plasmid. Twenty-four Chinese upland cotton (*G. hirsutum* L.) cultivars were chosen (Table 1) as test lines. Texas marker 1, "TM-1", used for control, is a genetic standard line for *G. hirsutum* L. (Kohel *et al.*, 1970), and the others are representative genotypes derived from seven ancestors (Huang, 1996) and collected from 5 cotton ecological regions in which their acreage exceeded 0.15 million hectare in China. Fresh, mature pollen grains were taken as transformation receptors and the plasmid pBI121 was the DNA vector.

Reagents and equipment. Helium-Driven PDS-1000/He system (DuPont Co.) and JQ-700 gunpowder particle bombardment device (Academic Sinica, Beijing) were used for bombardment. Fluorescein diacetate (FDA) was purchased from Sigma. Gold and tungsten microparticles, purchased from BIO-RAD, were used for the preparation of microprojectiles.

Preparation of microprojectiles. The plasmid pBI121, carrying GUS gene driven by 35S promoter, was isolated and purified according to Sambrook *et al.* (1989). Four μg of plasmid DNA was precipitated onto 50 μg of 1.0 μm gold or 1.1 μm tungsten particles (Dunder *et al.*, 1995).

Preparation of pollen grains. A sterile Whatman No.1 filter paper and then a camera lens paper were put onto the surface of a 60 μm Petri plate. One day pre-anthesis flower buds were tied with cotton string. The next morning (anthesis) pollen from dehiscing anthers of one previously tied flower was spread over the camera lens paper of one 60 μm Petri plate.

Bombardment. Bombardment was conducted the optimized delivery mode (Deng *et al.*, 1998b). When the bombardment was performed by the PDS-1000/He apparatus, 6 μl (about 0.5 μg plasmid DNA) of the microprojectile suspension was transferred to the centre of a macrocarrier. The size of rupture disk was 1100 psi and the stopping screen/target distance was 8 cm. When the JQ-700 device was used, 3 μl of the microprojectile suspension was placed on the front hollow surface of a cylindrically shaped polyethylene macroprojectile. The launch velocity of microprojectile was 450 m/s and the stopping screen/target distance was 5 cm.

Assessing the bombarded pollen viability and germinability. Viability of bombarded pollen was determined by staining with FDA, and observation by 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. Under the microscope, a yellow-blue pollen grain stained by FDA was considered as viable.

A modified hanging-droplet method (Taylor 1972) was used to evaluate pollen germinability. Taylor's medium was modified by removing the agar, adding 100mg/L each of glutamine, lysine, proline and serine, and increasing the sucrose from 25% to 40%. After being dispersed on the droplets for approximately four hours, the number of pollen grains ejecting a tube-like structure was recorded. A pollen grain with an ejected tube equal to or longer than its diameter was considered as germinated, and shorter than that as germinating. After staining with aniline blue, the pollen was also observed by fluorescence microscopy with the viewing parameters described above set at A, BP340-380, RKP400 and LP425, respectively.

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

Pollinating bombarded pollen and producing seeds. After stripping off the androecia of one day pre-anthesis flower buds, a lucite tube, 5 cm in long and 0.4 cm in diameter, was immediately slipped over the stigma and style. The next day (anthesis) between 1100-1200, the lucite tubes were removed, the stigmata sprayed with screening liquid (2×10^{-4} H_3BO_3, 2.5×10^{-4} kanamycin, 20mM EDTA, 40% sucrose) and bombarded pollen grains placed on the stigmata. The Lucite tube was replaced and the flower labeled. One and four days after pollination 20 ppm gibberellin in water was spread on the carpels of each ovary. Seeds in the ovary were harvested when the cotton boll opened.

After removal of the fiber from harvested seeds, they were put on a germinating advice and cultured at 30°C. When the radicle of a germinating seed exceeded 3.5 cm, the seed was placed on a pore of a perforated plate, so that the radicle was immersed in Hoagland solution with kanamycin (100mg/L). After 96 hours in the screening solution the seedling was planted in a nutrilite trough.

Statistical methods. The determination of weighting coefficient vector on the biological characteristics of bombarded pollen, $\{w_j\}$, was based on Statty's multiple-scale method introduced by Zhao *et al.* (1986):

The weighted cluster analysis for 24 genotypes on biological characteristics of bombarded pollen was conducted using the Star Site Graph Cluster presented by Wakimoto (1977). The coordinate of genotype "a" in the star site graph is

$$\left(\sum_{j=1}^{1} w_j \cos\xi_{nj}, \sum_{j=1}^{1} w_j \sin\xi_{nj}\right); 1=1,2,\ldots, p$$

For two kinds of cluster for N samples, cluster I for m groups which contains n_1, n_2, …, n_m samples, respectively, and cluster II for t groups which contains s_1, s_2, …, s_t samples, respectively, the interdependent assessment was produced through mutual information (Orloci, 1975). The mutual information, "I", between cluster I and cluster II was determined by the following formulation:

When two times **I** (cluster I; cluster II) was more than χ^2_{α}, [(m-1) x (t-1)], interdependency between cluster I and cluster II was considered indicated.

Results

Bombarded pollen viability. The viability of mature pollen bombarded by the optimized delivery system is shown in Table 2 for 24 *G. hirsutum* genotypes. The average percentage of viable unbombarded pollen grains (control) was 71.2%, while the viability of bombarded pollen grains was 56.9%. There is a significant difference between them by the t test. Based on percentage of viable pollen, the cultivars could be

$$I(clusterI, clusterII) =$$

$$N \ln N - \sum_{i=1}^{m} n_i \ln n_i - \sum_{j=1}^{i} s_j \ln s_j + \sum_{i=1}^{m} \sum_{j=1}^{i} n_{ij} \ln n_{ij}$$

divided into three groups, i.e., changeable type of pollen viability (>40%), intermediate type (10% to 40%), and stable type (<10%). Because two times mutual information between this classification and clusters according to ecological type was 12.259 and less than $X_{20.05}$, it can be concluded that there is no relationship between pollen grain viability after bombardment and cultivars from different ecological regions. By the same method, it was proven that there is also no relationship with their ancestors.

In vitro germinability of bombarded pollen. For the 24 genotypes, the average germination percentage of unbombarded pollen grains was 45.2%, whereas that of the bombarded pollen was only 19.4% (Table 2). The difference was significant by the T-test. There was no relationship between pollen germinability *in vitro* after bombardment and *G. hirsutum* cultivars from different ecological regions or ancestors, although there was a higher germination percentage for cultivar Ji 938 (Plate I).

GUS transient expression in the bombarded pollen. GUS transient expression in the bombarded pollen for 24 cotton cultivars is presented in Table 2. The percentage of GUS positive pollen grains ranged from 27.4% to 88.7% and the average percentage was 61.1%. Among the ecological types, the percentage of GUS positive pollen grains appeared to be higher for the cultivars from the Yellow River valley and lower for those from the South region. Similarly, the percentage of GUS positive pollen grains of cultivars derived from Acala was higher. The 24 cultivars could be divided into three groups according to the percentage value of GUS positive pollen grains, high (>70%), intermediate (50% to 70%), and low (<50%). However, the mutual information analysis indicated that there was no relationship between the status of GUS transient expression in bombarded pollen grains and genotypes from different ecological regions or their ancestors.

Star Site Cluster analysis on biological characteristics of bombarded pollen. Table 2 gives three biological characteristics of bombarded pollen, percentage of GUS positive grains, *in vitro* germinated grains, and viability. The weighting coefficient vectors, (0.54, 0.30, 0.16), were produced by the multiple scale method for Star Site Cluster analysis. As can be seen from Figure 1, four star site groups were produced for the 24 cultivars. The mean of biological characteristics for the four groups ranged monotonously (Table 3). The percentage of GUS positive bombarded pollen grains decreased stepwise from group I to group IV, while the difference in germinated pollen percentage between bombarded and unbombarded pollen increased stepwise from group I to group IV. The difference in the viable pollen percentage of both bombarded pollen and control increased from group I to group III, but in group IV, it decreased. The relationship between the results of Star Site Cluster and their ecological types or ancestors was evaluated by mutual information analysis. Two times mutual information was less than $X_{20.05}$, thus it can be concluded that no relation existed between the biological characteristics of bombarded mature pollen of the cotton cultivars and their ecological origin in China or their ancestry.

Transgenic seeds were obtained and screened. Stigmata of eleven of the cultivars were pollinated with bombarded pollen grains. One dish of bombarded pollen grains was pollinated into approximately 22.5 stigmata, equivalent to 4.45% of amount of pollen used in conventional cross-breeding. Conversely, approximately 100.3 pollen grains were placed on one stigma, commensurate with 2 times the number of ovules in one ovary. The number of seeds set following placement of bombarded pollen on stigmata sprayed with the screening liquid (Plate II) differed for the 11 cotton cultivars (Table 4). On average, 2.87 seeds were obtained from one dish of bombarded pollen grains, or, inversely, 0.34 dish of bombarded pollen grains were pollinated onto 6.20 stigmata to produce one seed. When unbombarded pollen was placed on stigmata sprayed the screening liquid, no seeds set, and if either the unbombarded or bombarded pollen was pollinated onto stigmata sprayed with the above liquid without kanamycin, many seeds were set.

Embryonic roots are very susceptible to kanamycin. When an untransformed embryonic root was immersed in the Hoagland solution with 50-80mg/L kanamycin, seedling growth was depressed after one day, the colour of the root changed to brown after two days, and the root became putrid after three to four days. To screen for transgenic plants, the embryonic roots of the germinated seeds were immersed in the Hoagland solution with 100 mg/L kanamycin (Plate III). Many plants survived (Plate IV), and 3.0 % of the “Ji 938” plants lived.

Discussion

So far, many plant transformation systems require a protoplast, cell or tissue *in vitro* culturing. However, there are serious problems, especially genotype dependence. Therefore, it is important to set up a plant transformation system that avoids the need for complicated regeneration and that is easily applicable to every desired variety (Bayley *et al.*, 1992).

There has been no scientific analysis and evaluation of genotype dependence of a transformation system. The statistical method for mutual information (Orloci, 1975) is a good scientific evaluation mode. The 24 cultivars in this paper, based on biological characteristics of bombarded pollen such as viability, germinability and/or GUS transient expression, are

divided into several groups. Each group represents different biological characteristics or degree of difficulty of transformation, but the cultivars within a group do not belong to the genetic background or ecotype. For the cultivars, there are natural classes for ecological type and ancestry, and the relationship between the two classes can be assessed through mutual information analysis. Our results clearly show no relationship between *G. hirsutum* genotypes in China and the biological characteristics of bombarded mature pollen grains. This is not only a new approach for studying the relationship between a plant transformation system and receptor genotypes, but it is also the first report that the transformation system is genotype-independent.

In crop science, sample selection is important for studies on the biological characteristics of genotypes. Care should be taken to select cultivars in a specially designated population. For these experiments, cultivars were selected from all ecological regions and from nearly all of the ancestors in Chinese *G. hirsutum* to ensure reasonableness and representativeness of the test samples. "TM-1", generally recognized as the genetic standard in *G. hirsutum* L. (Kohel *et al.*, 1970), was also included in this research so that comparative studies can be done easily in this aspect in other nations. Cotton transformation with *Agrobacterium* generally is limited to Coker lines (John *et al.*, 1992) which were derived from hybrid progeny between Foster and Lone Star cotton ancestors (Huang, 1996). However, we have shown that not only can *G. hirsutum* cultivars derived from Foster or Lone Star, but also those from other ancestors such as Deltapine, can be transformed by pollen-mediated transformation.

A clear and natural graph is presented by the Star Site Cluster analysis (Fig. 1). It is attractive and convenient, but to our knowledge, no other paper has reported its use in crop genetic research except for Deng *et al.* (1993). Other technical barriers on experimental and reproductive biology for *G. hirsutum* (Hu, 1982) have been resolved. The percentage of *in vitro* pollen germination was increased to 45.2% with this *in vitro* culturing method, compared to Taylor's 30.0% (Taylor, 1972).

Acknowledgements

We thank Mr. Shanlin Yao, China Academy of Sciences, for technical assistance, Prof. Shiyi Hu, Peking University and Prof. Hongyuan Yang, Wuhan University, for valuable discussions, Mr. Xiongming Du, Cotton Research Institute, China Academy of Agricultural Sciences and Associate Prof. Liufang Min, Nanjing Agricultural University, for supplying cotton cultivars. This project was supported in part by a grant from the Agrobiotechnical Program, the Ministry of Agriculture, P. R. of China.

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Table 1. Chinese cotton (*G. hirsutum L.*) cultivars used in the experiment.

Ecological types Ancestors	Sout regio	Yangtse River valley	Huanghe River valley	North earliest cotton region	East land- Locked region	Total
Acala			Xinmian3 Jizhi 82-1 Ji 938			3
Deltapine	Yuelu 1 Guimian 2 Guimian 3	Chuan 73-27 Baoshandaling				5
Foster			Shanmian 7	Liaomian10	Xinlu 201	3
King			Zhongmiansuo13	Liaomian7 Jinmian 2		3
Lone star		Songzidaling	Jimian 1	Liao2152	Xinku80432	4
Trice		Nantong12 Huamian7				2
Uganda		Xiangmian13	Zhongmiansuo12 Sumian2			3
Total	**3**	**6**	**8**	**4**	**2**	**23**

Table 2. Biological effect of bombardment on Chinese cotton (*G. hirsutum* L.) pollen.

Cultivars	No	Control/ Bombarded	Pollen No.	Vigorous pollen (%)	GUS Positive Pollen (%)	Pollen culture *in vitro* Percentage		
						Germinating	Germinated	Ruptured
Zhongmiansuo 12	1	Control	172	53.39	0.00	22.22	44.44	31.49
		Bombed	565	50.20	54.29	43.06	16.20	22.92
Huamian 7	2	Control	151	96.12	0.00	39.58	43.75	14.58
		Bombed	531	57.19	61.68	45.11	10.45	13.63
Liaomian 7	3	Control	155	95.24	0.00	0.00	56.00	36.00
		Bombed	631	79.99	27.37	32.73	29.09	25.46

Xinlu 201	4	Control	167	85.34	0.00	44.23	36.54	13.46
		Bombed	524	83.21	39.75	34.61	16.42	34.90
Songzidaling	5	Control	159	50.49	0.00	26.79	33.93	26.79
		Bombed	562	45.27	38.46	32.69	21.90	26.40
Yuelu 1	6	Control	159	51.89	0.00	13.21	49.06	28.30
		Bombed	551	50.24	29.86	34.62	12.28	14.85
Guimian 3	7	Control	150	57.14	0.00	46.15	34.62	19.23
		Bombed	505	49.56	35.83	28.86	5.06	9.61
Guimian 2	8	Control	165	88.33	0.00	6.67	82.22	6.67
		Bombed	492	81.54	36.15	27.60	15.51	31.43
Nantong 12	9	Control	145	69.81	0.00	17.95	43.59	35.90
		Bombed	489	66.30	73.27	36.55	21.28	15.28
Chuan 73-27	10	Control	142	92.17	0.00	14.81	74.07	11.11
		Bombed	468	77.98	86.17	32.68	14.82	13.14
Baoshandaling	11	Control	122	52.70	0.00	18.75	39.58	25.42
		Bombed	472	47.28	82.71	23.67	23.68	35.68
Xiangmian 13	12	Control	178	71.90	0.00	59.65	26.32	14.04
		Bombed	497	65.29	50.74	41.15	20.62	19.53
Xinmian 3	13	Control	170	48.00	0.00	35.56	44.44	15.56
		Bombed	478	30.23	80.45	23.99	17.74	15.70
Jizhi 82-1	14	Control	154	62.10	0.00	50.00	40.00	3.33
		Bombed	474	52.87	88.26	22.15	37.69	14.73
Zhongmiansuo 13	15	Control	180	59.09	0.00	39.58	33.33	14.58
		Bombed	560	48.19	85.86	34.82	26.68	15.12
Shanmian 7	16	Control	166	56.88	0.00	35.09	35.09	19.30
		Bombed	474	39.11	86.20	42.00	23.27	14.97
Jimian 1	17	Control	147	70.00	0.00	36.17	42.55	17.02
		Bombed	506	55.31	88.70	43.36	16.34	9.10
Sumian 2	18	Control	174	48.44	0.00	21.74	26.09	6.52
		Bombed	490	47.35	29.23	24.86	20.56	16.12
Liaomian 10	19	Control	181	91.58	0.00	12.79	83.72	2.33
		Bombed	537	68.09	75.15	41.24	40.16	6.61
Jinmian 2	20	Control	182	78.57	0.00	65.71	22.86	10.00
		Bombed	556	45.98	59.98	52.67	16.34	13.34
Liao 2152	21	Control	157	64.22	0.00	41.67	33.33	20.83
		Bombed	604	31.50	53.34	23.87	7.94	18.66
Xinku 80432	22	Control	196	85.42	0.00	30.77	46.15	13.46
		Bomded	510	23.03	64.93	28.63	7.12	23.20
Ji 938	23	Control	167	88.07	0.00	15.52	65.52	17.24
		Bombed	500	83.73	79.72	26.55	25.93	37.31
TM-1	24	Control	143	92.96	0.00	27.78	48.61	26.39
		Bombed	602	86.11	59.04	25.07	18.67	33.49

∗A pollen grain with an ejected tube equal to or longer than its diameter was considered as germinated, and shorter than that as germinating.

Table 3. Mean of bombarded pollen biological effect on Chinese cotton cultivar clusters.

Clusters	Codes of cultivar	Control/ bombarded	GUS positive pollen (%)	Pollen vigour (%)	*in vitro* germinated bombarded pollen (%)	Ecologic types included	Ancestors included
	14	Control	0.00	62.10	40.00	C	□
		Bombarded	88.26	52.87	37.69		
	9,10,11,13,15	Control	0.00	69.81	51.32	B,C,D,	□,□,□,□
	16,17,19,23	Bombarded	82.03	57.36	23.32		□, □
	1,2,12,20,	Control	0.00	77.51	37.92	B,C,D,E,	□,□,□ □
	21,22,	Bombarded	57.71	51.33	13.91		

3,4,5,6	Control	0.00	68.12	46.35	A,B,C,D,E,	□,□,□,□,□
7,8,18	Bombarded	33.78	62.45	17.26		

A, B, C, D and E represents South region, Yangtse River valley, Huanghe River valley, North earliest cotton region, and East land-Locked region of *G. hirsutium* cultivar in China, respectively. □, □, □, □, □, □, and □ represent Acacia, Deltapine, Foster, King, Lone Star, Triee and Uganda for *G. hirsutium* cultivar ancestors, respectively.

Table 4. Comparison of proportion on seed set following pollination with bombarded pollen in 11 cotton cultivars.

Genotypes	Observed data				Statistics based on per dish		
	Dishes	Stigmata	Bolls	Seeds	Stigmata	Bolls	Seeds
Texas Marker 1	13.5	269	8	30	19.93	0.59	2.22
Zhongmiansuo 12	11.0	229	4	20	20.82	0.36	1.82
Ji 938	13.0	228	19	110	17.54	1.46	8.46
Huamian 7	5.5	80	1	2	14.55	0.18	0.36
Xinlu 201	5.0	93	2	2	18.60	0.40	0.40
Yuelu 1	4.0	69	3	4	17.25	0.75	1.00
Nantong 12	4.0	65	2	2	16.25	0.50	0.50
Zhongmian Suo 13	3.5	44	1	1	12.57	0.29	0.29
Liaomian 10	3.5	58	3	7	16.57	0.33	0.33
Jinmian 2	3.0	41	1	1	13.67	0.33	0.33
Xinku 80432	4.0	71	5	22	17.75	1.25	5.50
Total	70.0	1247	49	201	17.81	0.70	2.87

* Placed on the stigmata with screening solution with Kan before pollination. Due to limitation of available flowers on one day, the data for stigmata are less than 22.5 per dish.

Figure 1. Star site cluster based on biological effects due to bombardment of pollen of Chinese cotton (*G. hirsutum* L.) cultivars. An asterisk in the star site graph represents the coordinate of a cultivar. The 24 coordinates are clustered into four groups.

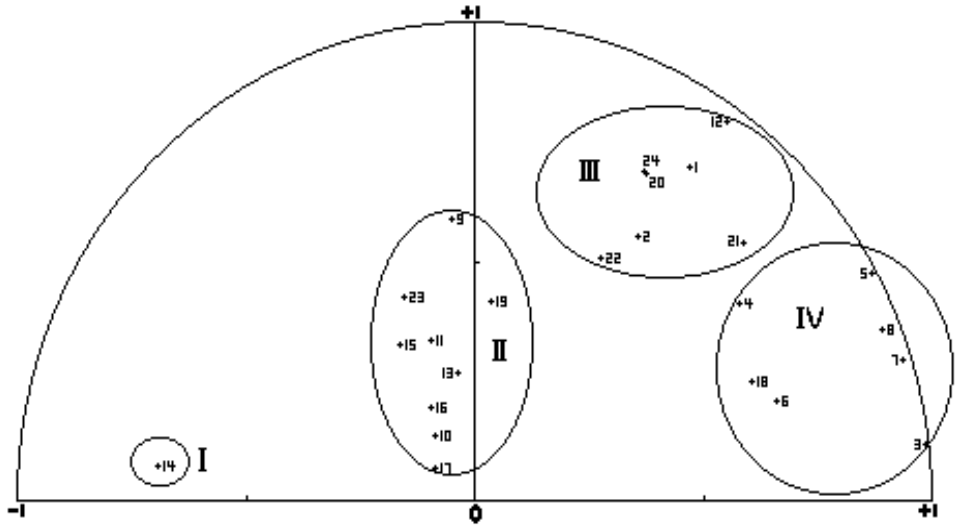

Plate1. Photomicrograph of *in vitro* germination of bombarded pollen by fluorescence microscopy, excited on A (BP340-380).

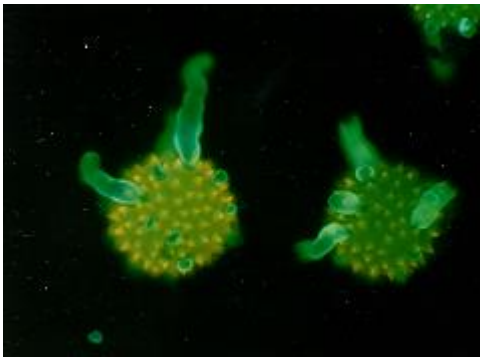

Plate 2. Boll setting status, indicated with a card, pollinated with transformed pollen.

Plate 3. Seedling screening status with kanamycin.

Plate 4. Transformants screened with kanamyci