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Optimization of Parameters for the Transfer of Foreign Gene to Cotton (*Gossypium hirsutum* L.) by Particle Bombardment

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Abstract

Zygotic embryos of cotton variety MNH-93 (*Gossypium hirsutum* L.) were bombarded with tungsten particles coated with plasmid pActin1-D carrying a reporter gene encoding *ß-glucuronidase* (GUS). A helium pressure of 60 kg/cm, vacuum of 28 inch of Hg, target distance 22 cm, DNA and tungsten ratio 0.50 µg: 240 µg and one-time shot showed maximum transformation efficiency by expressing a high detectable *ß-glucuronidase* activity of GUS gene. This study will help in developing a protocol for the transformation of cotton varieties with agronomically important genes.

Introduction

Gene-transfer is very difficult in cotton due to the non-availability of regeneration of transgenic cells. In contrast, mature embryos containing shoot apical meristem generate into whole plants *in vitro* in even very recalcitrant species, proving embryos to be an excellent tissue for transformation. Particle bombardment, wherein microscopic particles coated with genetically engineered DNA are explosively accelerated into cells, is the only procedure capable of delivering DNA into cells in virtually any tissue of any organism (Gray and Finer, 1993; Songstad *et al.*, 1995). To circumvent difficulties with cultivar-dependent regeneration, a few investigators have reported successful transformation of cotton meristem tissue using a biolistic approach (Finer and McMullen, 1990; McCabe and Martinelli, 1991; Chlan *et al.*, 1995). For successful particle bombardment mediated transformation, certain parameters have to be optimized through transient studies (Sanford *et al.*, 1987). In present study, different parameters of particle bombardment were optimized for the transfer of reporter gene (GUS) into mature embryos of cotton.

Materials and Methods

Plant material: Seeds of cotton variety MNH-93 were obtained from CCRI, Multan and were delinted with concentrated H_2SO_4. These seeds were sterilized with 0.1 percent $HgCl_2$ plus 0.1 percent SDS and soaked for 16-20 ours in sterilized conditions. The test of the seeds were carefully removed and the cotyledons were cut off to separate embryos. Mature embryos were used as explant for transformation. About 40-50 embryos were kept in 1-2 cm circle on MS full strength medium (Murashige and Skoog, 1962) in petri plate for bombardment.

Plasmid: The plasmid pAct1-D was obtained through the courtesy of Dr. Ray Wu, Cornell University, Ithica, USA containing (Fig.1) the 5' region of rice Act 1 gene, the Act1 5' intron, the Gus gene coding region and the 3' non-coding region of the nos gene (Zhang *et al.*, 1991).

Bombardment with beta-glucuronidase gene: For particle bombardment, Home made particle gun was used (Fig. 2) which has already been successfully used in transformation studies of rice and chickpea Husnain *et al.* (1995). Tungsten particles were sterilized and DNA was precipitated and coated on these particles as described Husnain *et al.* (1995). The filter assembly was fixed in the leur-lock. Samples to be bombarded i.e. mature embryos were placed at a distance of 18, 20, 22 and 24 cm in order to optimize the distance from the shooting filter assembly.

The chamber of the gun was closed carefully and evacuated till the needle showed 28 inch of Hg. Helium gas outlet was opened till it showed pressure of 60 kg/cm2. The fire switch was gently pressed three times after closing the prechamber. After bombardment, the vacuum was released slowly until the needle showed zero. The door was opened and the plates were taken out of the chamber of the Gun. These plates were wrapped with parafilm and incubated at 28 C for three days under the lux light for transient expression.

Histochemical assay of gus gene: Histochemical GUS assay (Jafferson *et al.*, 1987) was performed to visualize transformed tissues after 72 hours of bombardment. X-gluc substrate was prepared by dissolving 72 mg of X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) in 720µl DMF and then by adding 240 µl of 50 mM Potassium ferrocyanide and potassium fericyanide. Triton (X-100) 24µl of was also added and the final volume was made by adding 100 mM potassium phosphate buffer (pH-7.0). The tissue were incubated with X-gluc solution at 37 C for 16 hours and then were observed for the blue spots in mature embryos as a marker for selection.

Results and Discussion

Optimization of DNA and Tungsten ratio: Inconsistency in coating of DNA onto the particles and the quality and heterogeneity of the target tissues is the source of shot to shot variation (Christou, 1992; Vain *et al.*, 1993). The selection of right conditions of DNA and tungsten load is important to get higher transformation efficiency. It was noted that high DNA lead to aggregation and poorly coating

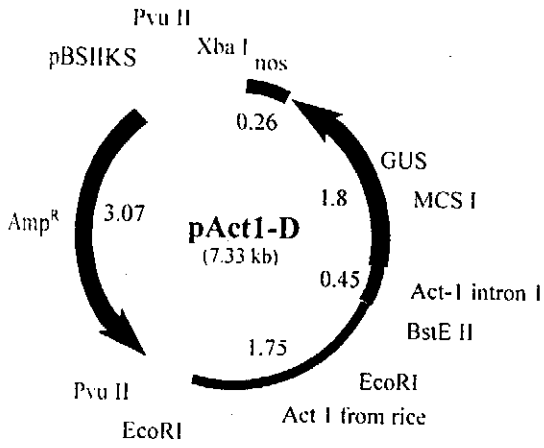

Fig. 1: The plasmid DNA (pAct 1-D)

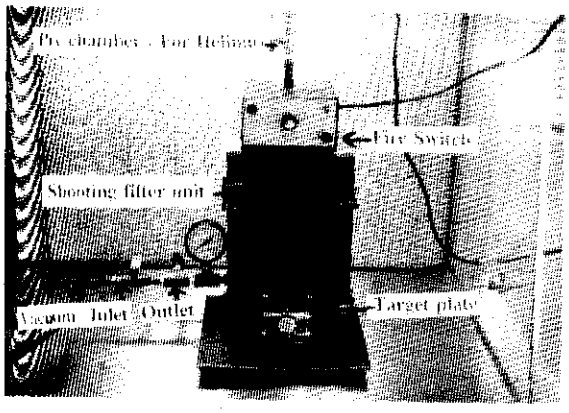

Fig. 2: Home made biolistic gun

Table 1: Effect of DNA tungsten ratio on transformation efficiency in cotton variety (MNH-93)

DNA: Tungsten Ratio (μg)	Distance (cm)	Transient Expression (No. of embryos with blue spots)
0.50:240	20	36.33 ± 2.081
1.00:240	20	33.66 ± 2.465
1.50:240	20	28.33 ± 3.055

Table 2: Analysis of variance for DNA: tungsten ratio and for number of shots

Source of variation	Degree of freedom	Sum of squares	Mean of squares
DNA: Tungsten ratio (1)	2	99.56	79.78*
No. of shots (2)	1	8.16	8.16N.S
Error 1	6	28.00	4.66
Error 2	4	29.33	7.33

* Significant ($P<0.05$)

N.S. Non-significant at both 5 % and 1 % level

Fig. 3: Transient expression of β-glucuronidase (GUS) i. control embryos ii. Bombarded embryos

Table 3: Effect of number of shots and distance on transformation efficiency in cotton (Variety MNH-93)

Serial No.	Bomb. Cond.	Distance (cm)	Total No. of embryos	Transient Expression (No. of Embryos with blue color)
1	ONE	18	50	28.33 ± 2.081
2	TWO	18	50	29.66 ± 3.214
3	ONE	20	50	31.33 ± 2.516
4	TWO	20	50	34.33 ± 1.000
5	ONE	22	50	37.33 ± 2.081*
6	TWO	22	50	39.66 ± 3.214
7	ONE	24	50	25.33 ± 3.057
8	TWO	24	50	29.33 ± 5.291
9	TUNGSTEN ONLY	22	50	NIL
10	NO BOMB.	--	50	NIL

DNA pActin1-D was used with DNA tungsten ratio as 0.5 ug: 240 ug

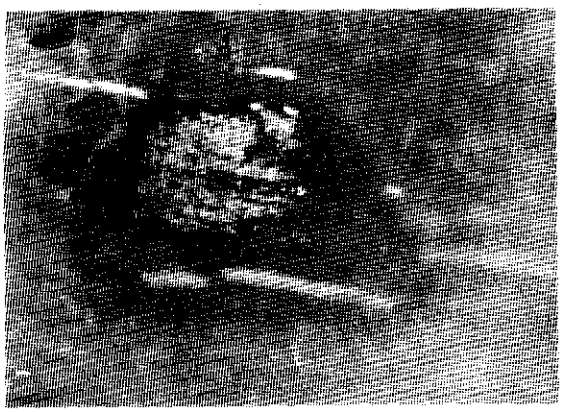

Fig. 4: Transverse section of cotton embryo showing transient expression of GUS gene

on particles which ultimately resulted in lower expression as less number of embryos showed blue spots. The DNA and tungsten ratio 0.5 μg: 240 μg was proved to be the best (Table 1) with respect to the transient expression in embryos (Fig. 3). Statistical analysis (ANOVA) has shown a significant difference among treatments of DNA to tungsten ratio (Table 2).

Optimization of distance from shooting unit to target explants: The efficiency is greatly influenced by the distance of the target tissue from shooting filter assembly. The target embryos were placed at different distances i.e., 16, 18, 20 and 22 cm below the syringe filter unit in order to determine the optimum distance for higher transient GUS expression. The Gus transient expression (Fig. 4) showed more efficiency at the distance of 22 cm (Table 3). At this distance, embryos sustained more injuries because a high number of particles hit embryos per unit area and when the distance of the target to the filter assembly was less (18 cm) the helium pressure damaged tissues more and less number of embryos were hit. At the higher distance (26 cm), the particles dispersed to greater extent across the target and the momentum of particles hitting the embryos was also reduced. Targeting the tissue at longer air resistance for longer time will result in lower velocity (Klein *et al.*, 1988).

Optimization of number of shots: The different treatments i.e. one shot and two shots were applied. There was no significant difference between one and two shots on the average number of embryos with blue spots (Table 3). Statistical analysis (ANOVA) has also shown that the difference between one and two shots was non-significant (Table 2). Statistical analysis (ANOVA) has also shown that the difference between one and two shots was Non-significant (Table 2).

Level of vacuum and pressure: The helium pressure of 28 inch of Hg and pressure of 60 Kg/cm2 was optimized already in previous study (Husnain *et al.*, 1995) and the same were used in this study. These results will be helpful in developing a transformation procedure for cotton genetic engineering to transform agronomically important characters in commercial cultivars.

Acknowledgments

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