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Particle Bombardment: Not a Good Approach for Gene Transfer into Embryonic Axes of Cotton (Gossypium hirsutum L.) Cultivars

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Tables

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Abstract

A particle bombardment mediated transformation protocol for direct gene transfer into cotton (Gossypium hirsutum L.) embryonic axes is described. Bt toxin gene, cry I A(c) under the control of CaMV 35S promoter and the npt-II gene as selectable marker is used for the experiment with cotton cv. NH 545. The embryonic axes explants were bombarded at various parameters of pressure and distance and incubated for 2 days at 28°C on MS medium supplemented with BAP (3 mg/l). Selection of transformed embryonic axes was conducted on MS medium containing 100 mg/l kanamycin supplemented with either BAP or kinetin and NAA after 15 days. A maximum regeneration frequency of 26% was achieved on selection medium when explants were bombarded with 900 psi at 6 and/or 9 cm distance and cultured on MS medium supplemented with BAP (1.6 mg/l) and Kinetin (0.4 mg/l) without NAA. The presence of transgene was detected by polymerase chain reaction (PCR) and southern blotting analysis. Out of total 432 bombarded explants, 112 survived the selection and only 3 transgenic cotton plantlets could be recovered. Overall, the gene transfer efficiency achieved through this method was only 3%.

Introduction

Cotton transformation has attracted attention of researchers due to its commercial significance (Anonymous 2000). Transfer of different genes into the cotton genome has been shown to enhance resistance to insects, herbicides, and fungal diseases (Perlak et al. 1990; Bayley et al. 1992; Lyon et al. 1993; Thomas et al. 1995; Nida et al. 1996; Rajasekaran et al. 1996 and Murray et al. 1999). However, cotton remains one of the challenging systems to transform, as there is a strong genotypic variability in cultivars with respect to their tissue culture amenability. Although some refinements in transformation protocols have been reported in recent years, cotton is still regarded as recalcitrant to transformation.

The methods originally developed for genetic transformation of cotton utilized Agrobacterium tumefaciens vectors (Firoozabady et al. 1987) and have been restricted to specific cultivars grown in respective countries. (Firoozabady et al. 1987; Umbeck et al. 1987; Finer and McMullen 1990; Cousins et al. 1991 and Bayley et al. 1992). Particle bombardment; however, promises cultivar independence, insertion of multiple genes and high transformation efficiency to cotton (McCabe and Martinell 1993). But the particle bombardment procedure especially for cotton involves cell suspension culture of somatic embryos. Both Coker and Acala varieties have been transformed using this procedure (Rajasekaran et al. 1996, and Rajasekaran et al. 2000). However, the maintenance of embryogenic suspension as well as their cryopreservation are skilled procedures and have not been widely used by many laboratories (John 1997). Also it has been observed in previous studies that, cotton is recalcitrant to regenerate through somatic embryogenesis and regeneration of whole plant from transformed cell is cultivar dependent (Trolinder and Goodin 1987). In contrary to this, Christou (1994) reported the advantages of particle bombardment such as transformation of organized tissue (eg. shoot meristem), relatively rapid recovery of transformed progeny and genotype independent transformation of crop plants.

Therefore in this study, we tried to test the potential of particle bombardment for gene transfer into cotton (*Gossypium hirsutum* L.) using its embryonic axes as explants. The δ-endotoxin producing *cry I A(c)* gene under the control of cauliflower mosaic virus (CaMV) 35S promoter and the neomycin phosphotransferase (*npt-II*) gene as selectable marker was attempted to transfer through particle bombardment using different parameters of pressure and distance. This gene had been previously shown to enhance resistance of cotton to lepidopteron pests such as bollworm (Perlak et al. 1990). The cotton cultivar NH 545 used in this study is a potential high yielding stable genotype and moderately resistant to sucking pests such as thrips, jassids, whitefly and bollworms (Bhatade and Ansingkar 2003).

Materials and Methods

Plant material - Seeds of the cotton (*Gossypium hirsutum* L.) cultivar NH 545 were obtained from the Cotton Research Scheme, Parbhani, India. Embryonic axes were excised as described previously (McCabe and Martinell 1993) from two-days old seedlings raised on MS medium (Murashige and Skoog 1962) without vitamins and growth hormones. The explants were placed on MS medium supplemented with 3 mg/l of 6-Benzylaminopurine (BAP) and incubated at 28°C for 24 hours prior to bombardment experiments.

Bombardment procedure: The plasmid pBin Bt-3 (12.60 kb), used in this study, contains the *Bt cry I A(c)* gene interrupted by an intron, and is flanked by the CaMV 35S promoter and 'nos' terminator. Also it has *npt-II* gene as the selectable marker (Fig. 1). The plasmid was provided by National Research Center on Plant Biotechnology, New Delhi, India under the collaborative research project on "Development of Bt-transgenic pigeon pea, rice and cotton for insect resistance."

Agrobacterium cells harboring above plasmid were cultured for 30 h in yeast extract mannitol (YEM) broth (Perlak et al. 1990) with 50-mg/l kanamycin and 10-mg/l rifampicin on a shaker (200-rpm) at 28°C. The log phase cultures were used for harvesting the cells for plasmid isolation by alkaline lysis method (Punia 1997). The plasmid was checked on 0.8% agarose gel for quality (Fig. 2A) and PCR amplified using *cry I A(c)* and *npt-II* genes specific primers to check its integrity (Fig. 2B). Purified plasmid DNA was coated on gold particles (1.0 µm in size) to prepare micro projectiles (Punia 1997). The micro projectiles were bombarded using PDS-1000/He Biolistic® Particle Delivery System (Bio-Rad Laboratorties, U.S.A.) on pre-incubated embryonic axes with different parameters of pressure and distance and the effective parameters for bombardment that resulted in transformation of embryonic axes were identified. The bombarded embryonic axes were then incubated at 28°C for 48 hours.

Selection and regeneration of putative transformants - Selection for transformed embryonic axes was conducted on MS medium containing 100 mg/l kanamycin supplemented with either BAP or Kinetin and 1-Naphthaleneacetic acid (NAA) after 15 days of the first selection of bombarded embryonic axes on MS medium containing 50 mg/l kanamycin. Subsequently, six different growth hormone combinations with MS medium were tested for the regeneration of putatively transformed embryonic axes.

Polymerase chain reaction - DNA was extracted by phenol chloroform method as described previously (Edwards et al. 1991) from 91 putatively transgenic plantlets growing on selection medium that were derived from different replicates of bombardment experiment (Table 2). PCR was performed in a total of 20 µl volume of Taq DNA polymerase (5 U/µl) and the corresponding buffer (1X), dNTPs (1mM), 50-ng/µl each of *npt-II* and *cry 1A(c)* specific primers in separate reactions. Banglore Genei Pvt. Ltd, India supplied all these materials. The primer sequences for PCR were: *npt-II* forward sequence (F) 5'-GAGGCTATTCGGCT-3', reverse sequence (R) 5'-ATCGGGAGGGGCGA-3' to yield a 1000-bp fragment (Firoozabady et al. 1987; Umbeck et al. 1987; Cousins et al. 1991 and Zapata et al. 1999), *cry I A(c)* (F) 5'-CCCAGAAGTTGAAGTACTTGGTGG-3', (R) 5'-CCGATATTGAAGGGTCTTCTGTAC-3' to yield an 1100-bp fragment (Perlak et al. 1990). The DNA was denatured at 94°C for 2 min followed by 45 cycles of amplification [1 min at 94°C; 30 sec at 61°C (*npt-II*) or 30 sec at 60°C (*cry I A(c)*); 30 sec at 72°C]. The final incubation at 72°C was extended to 5 min; and the reaction was cooled and kept at 4°C. The Biometra Thermal Cycler was used.

Southern blotting - The gene integration was detected by southern blot analysis. The genomic DNA was digested with the restriction enzyme Xba I. Standard procedure was followed for southern blot (Sambrook et al. 1989).

Results

Development of bombarded embryonic axes on selection medium - Following excision of the embryonic axes from the cotton seedlings, bombardment experiments were performed and the bombarded embryonic axes were tested for regeneration on selection medium (kanamycin) composed of six different combinations of growth hormones with MS medium supplemented with inositol. Among the six combinations, embryonic axes were found to be regenerate on MS medium containing 1.6 mg/l of BAP and 0.4 mg/l of Kinetin (Table 1). The embryonic axes after 4 - 6 weeks growth elongated and developed 2 - 4 green leaves. Those embryonic axes were continuously sub-cultured on the same medium. The shoot elongation was direct from embryonic axes and there was no callus formation. Cultivar NH 545 responded well for direct organogenesis from embryonic axes (Fig. 3A). Healthy plantlets recovered from selection medium have been shown in Fig. 3B

The effect of bombardment and regeneration medium on the frequency of plantlets development on selection medium after 15 days is shown in table 2. The highest gene transfer and regeneration efficiency i.e., 3% and 26% respectively was obtained in cotton cv. NH 545, when explants were bombarded with 900-psi pressure at 6 and/or 9 cm distance (Table 2) and cultured on MS medium supplemented with 1.6 mg/l of BAP and 0.4 mg/l of Kinetin (Fig. 4A and B).

Molecular analyses of putative transformants - Ninety-one kanamycin resistant plantlets were analyzed by PCR. Three plantlets showing trans-gene signals with 1.1 kb *cry 1A(c)* fragment along with control have been presented in Fig. 5. Non-transgenic control plants did not show any bands.

Further the PCR-positive transformants were tested for stable gene integration by Southern blot analysis using the *cry 1 A(c)* gene as probe. The genomic DNA was digested with Xba I and hybridized with the probe. The hybridization of the probe was obtained with PCR positive progenies, which was absent in control DNA sample of untransformed cotton plants (Fig. 6).

Discussion

Protocols for gene transfer into cotton describe in the literature to date have been restricted to specific cultivars (Firoozabady et al. 1987; Umbeck et al. 1987; Finer and McMullen 1990; Cousins et al. 1991 and Bayley et al. 1992). The overriding problem each investigator faced was the necessity to regenerate a fertile, healthy plant from the callus or suspension culture phase, which their techniques required for both transformation and selection. The inability to use these protocols with the majority of elite cultivars has been a significant stumbling block for those who wish to expedite the use of genetic engineering to improve cotton.

Our choice of NH 545, which is a potential high yielding stable genotype and moderately resistant to sucking pests represents a broad range of cultivars in *G*. *hirsutum*, all important to the cotton industry. The present experiment with this cultivar revealed that cotton embryonic axes transformed with foreign genes could be directly regenerated into plants (direct organogenesis) on MS medium either with BAP or with BAP and kinetin in 4:1 proportion. Direct organogenesis such as multiple shoot induction from cotyledonary nodes of cotton on MS medium supplemented with BAP and kinetin in 2:1 proportion was also reported by Nandeshwar (2002). The pre-culture of explants on MS medium with BAP (3 mg/l) without NAA prior to bombardment has been reported to enhance transformation frequency in earlier study (McCabe and Martinell 1993). The pre-culture of cotton embryonic axes was thought to be useful in providing the turgidity so that enhances the penetration of the foreign gene. However, this did not work in the present experiment. Therefore, the process described here may not provide a method for transferring any gene into any cotton cultivar.

The present results showed that the *cry I A(c)* gene was stably integrated in the cotton genome. Also, the protocol described here appears extremely simple for transformation of cotton. However, due to its very less transformation efficiency and laborious and time consuming (Trolinder and Goodin 1987) nature, the particle bombardment mediated transformation of cotton is not reliable for developing improved lines of this important fibre crop.

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Illustrations

Illustration 1

Fig. 1: Structure of a plasmid construct containing the cry I A(c) gene and the selective marker npt II gene used in this study.

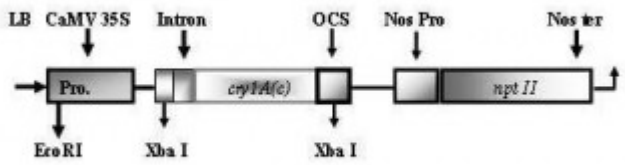

Illustration 2

Fig. 2: A- Band of a plasmid pBinBt3 carrying Bt, cry I A(c) gene obtained on 0.8% agarose gel, B- PCR amplification of a plasmid pBinBt3 with npt II (Lanes 1, 2, 3) and cry1A(c) (Lanes 4, 5, 6) specific primers (M = Supermix DNA ladder).

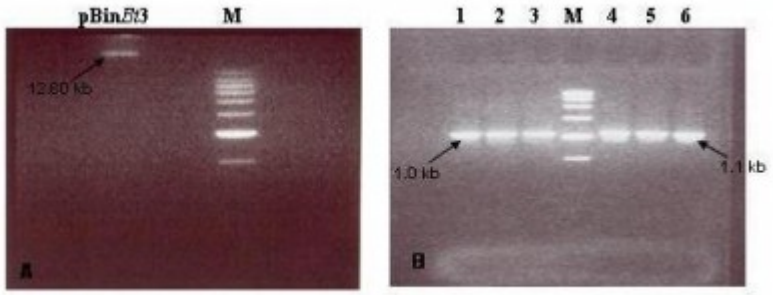

Illustration 3

Fig. 3: A- Fifteen days old putatively transformed embryonic axes sub-cultured on selection medium (100 mg/l kanamycin)

Illustration 4

Fig. 3: B- Healthy plantlets recovered from selection medium.

Illustration 5

Fig. 4: A and B- Putatively transgenic plants on MS medium supplemented with BAP (1.6 mg/l) and Kinetin (0.4 mg/l).

Illustration 6

Fig. 5: PCR analysis for confirmation of cry I A(c) gene integration in transformed embryonic axes [Lane 1 and 8- Markers, Lane 2- Plasmid amplified with cry I A(c) gene specific primer, Lane 3 and 6- Positive controls, Lane 4, 5 and 7- Transformed embryonic axes with cry I A(c) gene].

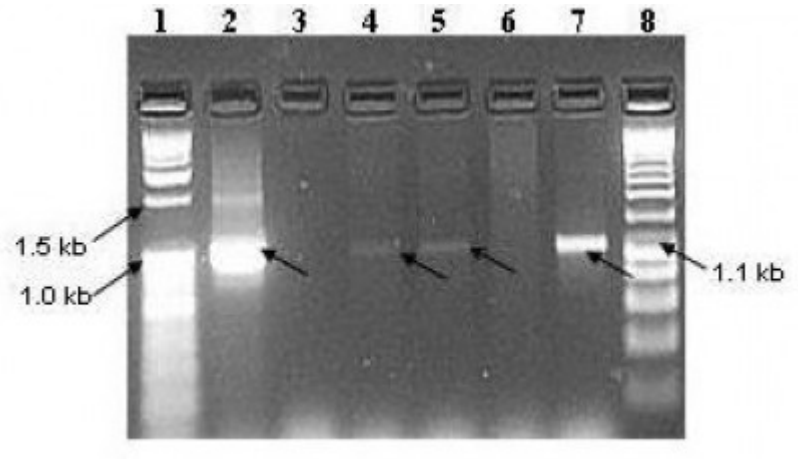

Illustration 7

Fig. 6: Southern blot analysis of T1 cotton plants using the cry I A(c) probe. Lane 1 - 6- DNA samples of transformed cotton plants digested with Xba I and hybridized with the probes, Lane 7- Control DNA sample of untransformed cotton plants digested with Xba I and hybridized with the probe, M- Marker (1.3 kb).

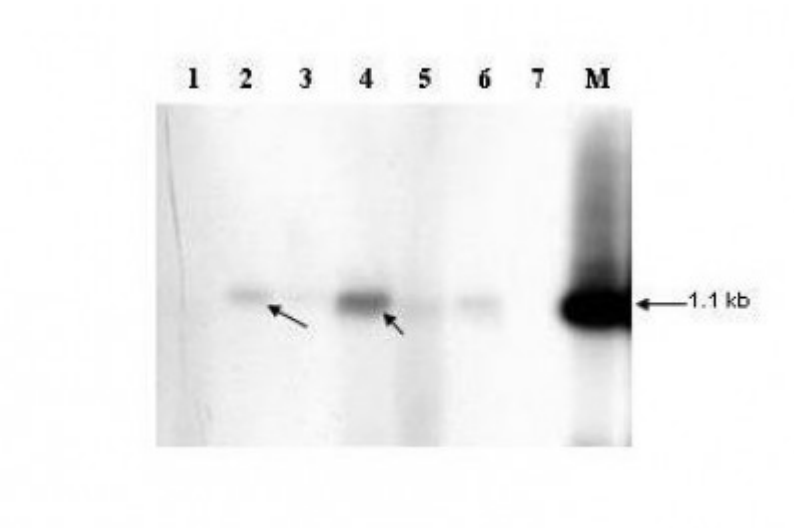

Illustration 8

Hormonal combination tested for regeneration of putatively transformed embryonic axes of cotton on MS medium

Sr. No.	Basal medium	BAP (mg/l)	Kinetin (mg/l)	NAA (mg/l)	Organic vitamins
1.	MS (1 lit.)	0.2	0	0.2	Myo-inositol (100 mg/l)
2.		0.8	0.2	0	
3.		1.0	0	0	
4.		1.2	0.3	0	
5.		1.6*	0.4*	0*	
6.		2.0	0.5	0	

*Quantity of growth hormones at which highest regeneration frequency achieved.

Illustration 9

Summary of the methodology used and results obtained in the present experiment of cotton transformation through particle bombardment

Lot	No. of seeds germinated	No. of embryonic axes excised	No. of embryonic axes used for bombardment/ plate		Pressure (psi)	Distance (cm.)	Quantity of microcarrier (µl/plate)	Selection on 1st level of kanamycin (50 mg/l)	Selection on 2nd level of kanamycin (100 mg/l)	No of samples used for confirmation test	No. of samples found positive with PCR
			Plate	No. of explants							
N1	75	40	A	20	450	9	15	17	6*	9	-
			B	20	900	9	15	14	3*		1
N2	165	151	C	12	900	6	10	10	4*	32	1
			D	14	900	6	10	7	5*		-
			E	18	900	6	10	14	5*		-
			F	15	1350	9	10	12	0		-
			G	17	1350	9	10	11	6*		-
			H	12	1350	9	10	10	3*		-
			I	17	1350	9	10	12	4*		-
			J	15	1350	12	10	10	0		-
			K	18	1350	12	10	14	5*		-
			L	13	1350	12	10	12	0		-

N3	115	115	M	13	450	6	7	13	7*	24	-
			N	12	900	6	7	12	8*		
			O	15	1350	9	7	15	9*		
			P	15	450	9	7	11	0		
			Q	12	900	12	7	12	3		
			R	15	1350	12	7	13	5		
			S	17	450	6	7	12	4		
			T	16	900	9	7	14	0		
N4	79	66	U	13	1350	9	8	11	5	12	-
			V	15	1350	9	8	11	4		1
			W	15	900	9	8	12	0		
			X	9	900	6	8	9	4*		
			Y	14	450	6	8	14	8*		
N5	83	60	Z	10	450	6	10	10	5*	14	-
			A_1	15	900	9	10	11	3*		
			B_1	18	1350	12	10	13	2*		
			C_1	17	450	6	10	12	4*		
TOTAL	517	432		432				348	112	91	3

*Putative transformants used for confirmation test (test of gene integration)

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