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Transformation of Cotton (*Gossypium hirsutyrn* L.) With Insect Resistant Gene by Particle Bombardment and Agrobacterium

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Abstract

A combination of two techniques i.e. particle bombardment and *Agrobacterium* method was applied to transfer marker *NPT-II* and insecticidal *cry1A(b)* gene into zygotic embryos of cotton variety MNH-93. Wounding in embryos produced by bombardment of tungsten particles increased the efficiency of *Agrobacterium* mediated transformation. Primary transformants selected on selection medium containing Kanamycin (100 µg/ml) for 6-8 week showed the presence and expression of *cry1A(b)* gene in the primary transformants. Insect Bioassays with 2nd instar larvae of of American bollworm *(Heliothis armigera), PCR* amplification,of the coding region of *cry1A(b)* gene in plant gennnle and ELISA with the antibodies against *cryt1A(c)* con·firmed the transformation.

Key words: Transformation, *Gossyplum hirsutum. cry1A(b),* Particle bombardment, *Agrobacterium*

Introduction

Cotton, the major cash crop of Pakistan, is the source of more than 60 percent of foreign exchange earning. Moreover 66 percent of the domestic edible oil production is obtained from cotton seed (Hashmi *et al*.,1995). Unfortunately, cotton is attacked by a number of insects causing a heavy yield loss. Cotton breeders have continually sought to improve the cultivar through conventional breeding methods (Christou,1993). However, limited germplasm is a bottleneck in the use of new genetic manipulation for insect resistance in crop plants. Meanwhile, genetic engineering allows researchers to introduce novel genes from any source (Bacterial, Viral and Animal etc.) into already existing cotton varieties. One of bacterial source for insect resistance is *Bacillus thuringiensis*. Various proteins found in *B. thuringiensis* as parasporal crystal inclusion, are insecticidal to certain insects. These bacterial genes can be transferred and expressed in plants yielding transgenic crops with resistance to insect attack (Fujimoto *et al*.,1993). A few investigators have reported successful transformation of cotton either by *Agrobacterium* (Firoozabady *et al*.,1987; Umbeck *et al*.,1987; Perlak *et al*.,1990; Cousins *et al*.,1991) or by biolistic approach (Finer and McMullen.,1990; McCabe and Martinell,1991; Chlan *et al.,* 1995; John and Keller,1996). In present study, a combination of both techniques i.e particle bombardment and *Agrobacterium* was used to transfer cotton embryos as wounding prior to cocultivation enhance the *Agrobacterium* mediated transformation (Bidney *et al.,1992;* Knittle *et al*.,1994). This strategy helped to obtain cotton with resistance to certain lepidopteron insects which may offer an additional weapon to be used in Integrated Pest Management to reduce insect damage to crop in an environmental friendly manner.

Materials and Methods

A) Transformation of Cotton: 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% $HgCl_2$ + 0.1% SDS by soaking for 16-20 hours in sterilized'conditions. Embryos were isolated carefully from these soaked seeds (40-50) and plated on simple MS full strength medium (Murashige and Skoog, 1962).

Bacterial Strain: *Agrobacterium tumefaciens* strain CAMB-2845 having co-integrative plasmid was used in this study. It harbors two genes, the neomycin phosphotransferase gene (NPT-II) which confer resistance to kanamycin regulated by Nos promoter and insecticidal *cry1A(b)* gene controlled by CaMV35S promoter (Van Aarssen *et al*.,1995).

Bombardment With Tungsten Particles: The Tungsten particles (60 µg/l were taken out from -20°C, thawed at 4°C, vortexed at high speed for 1 minute and then 60 µl was taken in a sterile eppendorff and spin down for 1/2-1 minute at 3000 rpm. The supernatant was removed and pellet was gently resuspended in 40 µl T.E buffer. From this suspension, 20 µl was coated on each filter assembly and allowed to dry for 1-2 minutes in laminar air flow. The samples (embryos) were placed at a distance of 22 cm from the shooting assembly and bombardment was done as described by Husnain *et al.* (1995).

Cocultivation of Bombarded Explants With *Agrobacterium*: Bacterial culture (15 ml) was grown in YEP liquid medium containing Kanamycin 50 µg, spectinomycine 0.3 mg/ml and streptomycine 1 mg/ml at 28°C in a shaking incubator at 176 rpm for 48 hours. Overnight culture (10 ml) having

0.85-1.15 O.D. was centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml of MSO (without hormone) liquid medium. The bombarded embryos were dipped in the bacterial culture for 15-20 minutes on a shaker at 120 rpm speed, blotted dry and were shifted on simple MS medium. The embryos were allowed to grow with bacteria for 3-days at 28°C and then transferred to a selection medium i-e MS medium containing Kanamycine (100 μg/ml) and cefataxime (250 μg/μl). Subculturing was done after 2-3 weeks on the same selection medium.

B) Analysis of Transformed Plants: Insect Bioassay of Primary Transformants: Two to three leaves from each of the three month old plants were collected for insect bioassays. The leaves were washed with autoclaved distilled water, dried and placed in petriplates containing moistened filter papers. The second instar larvae of American Bollworm *(Heliothis armigera)* were used to feed on the leaves. After 72 hours, the insect mortality and other larval characters were recorded.

Polymerase Chain Reaotion(PCR) Analysis: In order to amplify *cry1A(b)* gene in primary transformants, the primers were designed for amplification of 359 bp by internal fragment of *cry1A(b)* gene. The two primers, forward 5' CCT CTC AAT GGG ACG CAT TTC and reverse 5' GAT AGT CGC GGC ATC AAA TCC were used in PCR amplification. Total genomic DNA was isolated from young leaves of the primary transformants and from a non-transformed (Control) plant by a method as described by Doyle and Doyle (1987) with some modifications.

Enzyme Linked Immunosorbant Assay(ELISA): The total protein was isolated from 4-5 leaves of primary transformants as described by Draper *et al.* (1988). The protein extract was coated to microtitre plate and antigen to antibody reaction (ELISA) was performed as described by Frederick *et al.* (1989).

Results and Discussion

Transformation of Marker and Insect Resistant Gene in Cotton var. MNH-93: In order to integrate *crylA(b)* gene into cotton gnome, independent experiments were conducted to ransform embryos. The bornbarded/cocultivated embryos were subjected to continuos selection on Kanamycin (100 mg/l) for 6-8 weeks. The embryos having only integrated NPT-II gene were capable of growing on selection medium while control explants growing on selection medium bleached and died (Fig. 1) so the healthy plants growing on selection medium were considered as putative transformants (Table 1). Transformation efficiency was calculated on the basis of Kanamycin selection which was 3.28 while survival efficiency of plants in soil was 20.65 percent only. This low survival rate was due to the delicate and weak rooting system of putative transformants as kanamycin inhibit root formation and more over cotton seedlings are very sensitive and do not like shifting.

Insect Bioassay: Out of 19 primary transformants, five plants showed 10-70 percent mortality against American bollworm while in three plants i.e. CAMB-407, 416 and 419, no larval mortality was observed rather larvae showed stunted growth due to their avoidance in feeding behavior and no significant increase in weight was observed as compared to control (Table 2). The larvae feeding on the leaves of control samples were seen healthy and motile while in experimental samples the larvae died after feeding portions of the supplied leaf (Fig. 2).

The results clearly demonstrated that larvae died due to the toxic effect of plants transformed with insecticidal gene. The mortality of lepidopteran insects with *cry1A(b)* gene in insect bioassays of transformed cotton has also been reported by Perlak *et al.* (1990), Altman *et al.* (1996) and Benedict *et al.* (1996).

PCR Analysis: The DNA isolated from the leaves of primary transformed plants was used in PCR reaction. The primers were designed to amplify 359 bp internal fragment of *cry1A(b)* gene. The DNA isolated from control plant was taken as negative control and DNA from plasmid containing *cry7A(b)* gene as positive control. The amplification of 359 by internal fragment of *cry1A(b)* gene (Fig. 3) concluded that *crylA(b)* gene has integrated in the genome of primary transformants.

Enzyme Linked Immunosorbant Assay: Gene expression could be studied by enzyme linked Immunosorbant assay, since their first description (Engvall and Perlman, 1971), ELISAs have become the system of choice when assaying soluble antigens and antibodies. In ELISA the desired protein expressed in a target plant was detected by binding with specific antibodies. The total protein was isolated from six transgenic plants and a control plant. These protein samples were transferred to microtitre plate (ELISA plate) and after treating the samples with specific antibodies the colour reaction was developed. The yellow colour indicated the expression of insecticidal, *CrylA(b),* protein in the primary transformants (Fig. 4) . Interestingly, the plants CAMB-416 and 419 also produced light yellow color in ELISA test showing the presence of protein. Although insect mortality in these plants was zero but there was a non significant increase in the weight of insects feeding on these plants as compared to insects feeding on control plant. This showed that ELISA is a sensitive technique and can detect lower expression of protein. Moreover it was also concluded that a high expression of Bt toxin is required for insect mortality. Our results are in same proportion as the results of Perlak *et al.* (1990) and Sims and Berberich (1996) who reported that ELISA analysis of transgenic plants of cotton produced same results as insect bioassays.

More results and confirmation of these results by southern analysis, western analysis and segregation analysis in the progenies will lead to the development of insect resistant cotton. This will ultimately result in the development of an

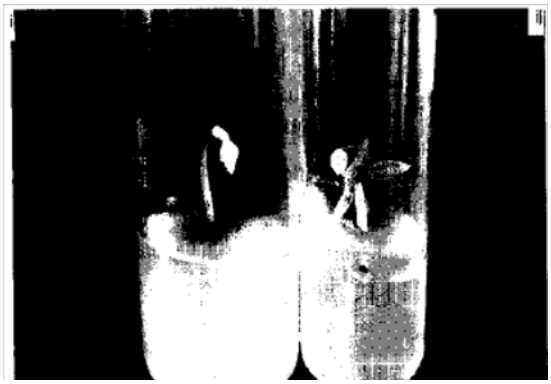

Fig. 1: Selection of transferred shoots on MS medium containing kanamycin (100 mg/l) i-Control ii-Co-cultivated shoots

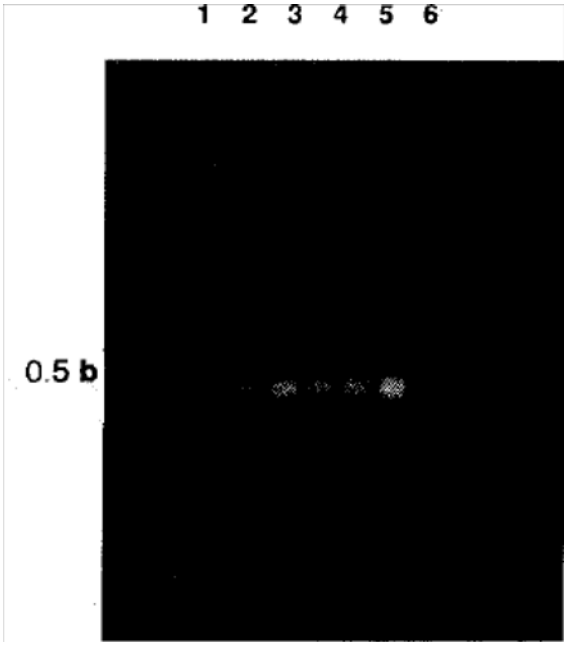

Fig. 3: PCR amplification of primary transformed plants (To)DNA
Lane 1: Marker lambda Hindi!
Lane 2: Amplified DNA from *crylA(b)*
Lane 3: Negative control
4&5: Amplified DNA from CAMB-402 and CAMS-403 cultivated shoots plants
Lane 6: Amplified DNA from control plant

Fig. 2: Insect bioassay on the leaves of cotton var. MNH93. i-Control (Leaf of untransforrried plant); ii-Leaf of transformed plant

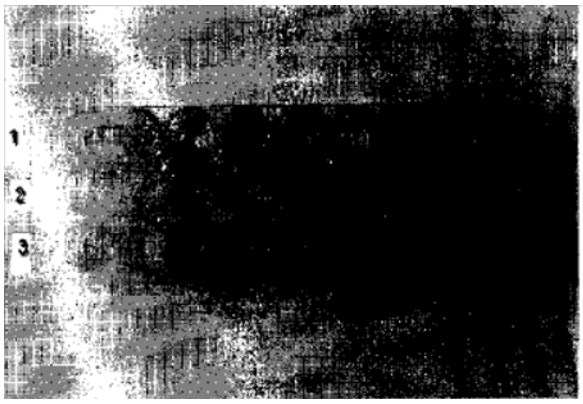

Fig. 4: Enzyme linked immunosorbant assay of transgenic plants
Lane 1 (Well 4 & 7): Protein expression of *crylA(b)* in CAMB-402, CAMB-405
Lane 2: (Well 3): Protein isolated from control plant Lane 2: (Well 5): Protein control *crylA(c)* protein
Lane 3 (Well 3, 4 & 8): Protein expression of *crylAfb)* in CAMB-414, CAMB-416 and CAMB-419 plants

Table 1: Transformation of insecticidal *crylA(b)* gene into cotton variety MNH-93 by particle bombaidment and cocultivation with *agrobacterium* strain

S. No.	No. of embryos	Plant after 6-8 weeks on selection medium* Cont-0(25)	Cont-I1(25)	Expt.(400)	Transformation efficiency (%) plants	No. of plants In soil	Survival efficiency (%)
1	450	18	0	12	3.00	3	25.00
2	450	21	0	15	3.75	2	13.33
3	450	22	0	8	2.00	2	25.00
4	450	22	0	19	4.75	4	21.06
5	450	13	0	19	3.25	2	15.38
6	450	15	0	9	2.25	3	33.33
7	450	14	0	16	4.00	3	18.75
	3150	-	-	92	3.28	19	20.65

Control - I: Plants growing on simple MS medium; Control - II: Plants growing on selection medium; *Selection medium containing Kanamycin (100 mg/l)

Table 2: Mortality of larvae of *Haliothis armigera* after 72 hours. Feeding on putative transformants of MNH-93 with Insect Resistant *crylA(b)* Gene

No. of Plants	Weight Inc. (Folds)	Mortality (%)	Other observations of larvae
CAMB 401	8.25	10	Eating the leaf
CAMB-402	1.25	70	Lazy, Black and dead
CAMB-403	1.75	40	Lazy Black and dead
CAMB-405	8.10	33	Lazy, eating
CAMB 407	1.55	0	Lazy, Not eating
CAM B-408	9.00	0	Eating well
CAM B-411	0.00	0	Eating well
CAMB-414	1.21	33	Lazy, Not eating
CAMB-416	1.28	0	Lazy, Retarded growth
CAMB-419	1.16	0	Lazy, Retarded growth
Control-I	9.75	0	Active, Eating
Control-II	9.00	0	Active, Eating

Control-I = Plant obtained without bombardment; Control-II = Plant obtained after bombardment with tungsten particles without DNA

alternative measure in the country against insect pests. Our results showed the probability of transformation of cotton with any gene of interest as this study will provide a foundation work for the future advancements in transgenic technology of cotton in Pakistan.

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