TITLE: **Genetic Transformation of an Elite Indian Genotype of Cotton (*Gossypium hirsutum* L.) for Insect Resistance**

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ABSTRACT

Agrobacterium **mediated genetic transformation of an elite Indian Genotype (Bikaneri Nerma) of Cotton (*Gossypium hirsutum* L.) was achieved by using shoot apical meristems isolated from seedlings as explants and a synthetic gene encoding *Cry1Ac* δ-endotoxin of *Bacillus thuringiensis*. Regeneration of shoots was carried out in selection medium containing Kanamycin (100 mg l^{-1}) after co-cultivation of the explants with *Agrobacterium tumefaciens* (strain EHA 105). The rooting was accomplished on a medium containing naphthalene acetic acid and kanamycin. Progeny obtained by selfing T_0 plants were grown in the green house and screened for the presence of neomycin phosphotransferase (*npt* II), and *cry1Ac* genes by polymerase chain reaction (PCR) and Southern hybridization. Expression of *Cry1Ac* in the leaves of the transgenic plants was detected by Xpress strips and quantified by Quan-T ELISA kits (DesiGen). Insect bioassays were performed with the larvae of Cotton bollworm (*Helicoverpa armigera)*. Field tests of the most promising lines (T-2 and T-3 generations) were performed under contained conditions. The results of the field tests showed considerable potential of the transgenic cotton for resistance against cotton bollworm.**

KEY WORDS:

Gossypium hirsutum, Genetic transformation, shoot apical meristem, Agrobacterium, cry1AC, Insect resistance

Cotton is the most important source of natural fibre. India is the world's third largest cotton producer. One of the major limiting factors, which affect cotton production in India, is the incidence of pests, especially bollworms, causing more than 50 per cent yield loss (Atwal, 1976). The limited genetic variability for bollworm resistance in cotton land/wild races makes the task of developing pest-resistant lines very difficult. In the past decade insecticidal proteins of *Bacillus thuringiensis* (Bt), a Gram-positive soil bacterium, have been expressed in cotton and other crop species by genetic engineering with significant social, environmental and economic benefits to the farmers (Kumar, 2003). Bt-cotton expressing *Cry1Ac* protein of Bt was cultivated in an area of 20.0 million hectares in more than a dozen countries including India in 2005 (James, 2006).

Introduction of foreign genes in elite genotypes is limited by genotype-specific nature of gene transfer in cotton. Coker genotypes, which are amenable for regeneration *in vitro* by somatic embryogenesis, are widely used in genetic transformation experiments (Firoozabady et al., 1987; Umbeck et al., 1990; Finer et al., 1990). However, alternate procedures to transform non-Coker genotypes have been reported (McCabe and Martinell, 1993; Gould and Magallanes-Cedeno, 1998; Zapata et al., 1999; Styavati et al., 2002). In the present study, we report successful introduction of Bt-*cry1Ac* gene in an elite Indian genotype of cotton by following a modified shoot apical meristem procedure and significant protection from cotton bollworm in field conditions.

MATERIAL AND METHODS

Plant material. Cotton cv. Bikaneri Nerma (*G. hirsutum*) was selected because of its high commercial value. Bikaneri Nerma, the female parent of popular cotton hybrid, NHH-44, is also cultivated as a variety in many states viz., Punjab, Rajasthan and Haryana.

Explant preparation. Seeds were delinted with sulphuric acid and soaked in $HgCl_2$ (50 mg/L) for 30 minutes and kept for shaking (50 rpm) in a rotary shaker. Seeds were rinsed three times with sterile double-distilled water and germinated at 28°C in the dark for 3 days and later shifted to light and dark (16/8 hours) rotation for getting healthy seedlings. The seedlings (7-8 days old) grown aseptically on MS medium[11] were used for the isolation of shoot apex. The isolation of shoot apex was carried out as described by Gould et al (1991).

Bacterial inoculation. *Agrobacterium tumefaciens* (EHA 105) harboring a binary vector (pBinBt3) was grown overnight at 28C. The binary vector carries a codon-optimized *cry1Ac* gene driven by CaMV 35S promoter. Healthy shoot apices were bisected from apex to base producing two asymmetrical halves. Both the halves were inoculated with *A. tumefaciens* diluted (1:20) in virulence induction medium (MS medium containing 2.0% glucose, Octopine 100 mg/ L and 100 mM Acetosyringone) followed by vacuum infiltration for 5 min. The explants were incubated on co-cultivation medium (MS medium containing 2 mg/L of Benzyladenine) for 3 days at 22°C.

Shoot regeneration and selection. After 3 days of co-cultivation shoots were transferred to shoot growth medium (MS medium containing 100 mg/L myo-inositol, 0.5 mg/l thiamine HCl, 0.5mg/L nicotinic acid, 0.5mg/L pyridoxine HCL and 2 % sucrose at pH 5.7) and incubated in diffuse light at 26 ± 2°C for a week followed by shifting them to selection medium (MS medium containing BA 0.2 mg/L, Cefotoxime 400 mg/L and Kanamycin, 100 mg/ L). Explants were sub-cultured on the same media at an interval of one week. Kanamycin resistant shoots were excised and rooted on MS medium containing NAA 0.1 mg/L, 15 g/L Sucrose and Cefotaxime 400 mg/L.

Rooted plants were rinsed well with fungicide (Bavistin, 0.2%) and transferred pots containing peat, soil and sand in 1:1:1 ratio. Seedlings were covered with plastic bags and kept in a plant growth chamber (65% RH) for two weeks before shifting to natural conditions in a transgenic green house.

Plant growth. Plants were grown in a transgenic green house. Standard method of selfing was followed and the seeds harvested from the T_0 plants were sown to raise T_1-T_3 generations in the field.

Molecular analysis. Genomic DNAs were isolated from the plants following the procedure of Doyle and Doyle, 1990. Polymerase chain reaction (PCR) analysis was carried out to detect the presence of *npt II* and *cry1Ac*. Southern hybridization analysis of *Hin*DIII restricted genomic DNAs was carried out by using radiolabelled *cry1Ac* gene.

Gene expression. Analysis of *cry1Ac* gene expression in the leaves was carried out by using Xpress strips (immunodiagnostic) and Quan-T (ELISA) kits (DesiGen, India) according to manufacturer's instructions.

Insect bioassays. Laboratory bioassays were performed by using neonate and first instar larvae of *H. armigera* reared on artificial diet (Chakrabarti, 1998). Plants (T_2 and T_3 generations) were raised in open field conditions with the permission of the Department of Biotechnology. Natural infestation by various pests was allowed by not spraying any insecticides. Severe incidence of *H. armigera* during 2003-2004 (T_3 generation) facilitated analysis of the protection conferred by the expression of *Cry1Ac*.

RESULTS AND DISCUSSION

Agrobacterium-mediated transformation of cotton was first reported by Firoozabady et al., 1987 and Umbeck et al., 1987. Among the genotypes of *G. hirsutum*, Coker and Acala genotypes are amenable for genetic transformation because of their high regeneration potential. Kumar et al. (1998) have attempted to transfer the regenerative competence from Coker varieties to recalcitrant elite cultivars. The introgression of the transgene from Coker varieties to elite genotypes is performed by conventional breeding which does not totally eliminate the presence of undesirable genes of Coker. Successful efforts to directly transform elite genotypes by alternate methods have been reported (McCabe and Martinell, 1993; Gould and Magallanes-Cedeno, 1998; Zapata et al., 1999; Satyavati et al., 2002). In the present study, we attempted to transform an elite Indian genotype of *G. hirsutum* by regenerating *Agrobacterium*-treated shoot apical meristems as described by Gould et al. (1998), with minor modifications. The modes of explant preparation and regeneration differ in these reports. In our study the explant was bisected vertically and shoot regeneration occurred from shoot apical meristem. The transformation efficiency was very low (0.2%) in contrast to 60-70 % reported earlier. In addition, we considered PCR and ELISA positive plants instead of kanamycin-resistant shoots to calculate transformation efficiency.

The shoot apex explants infected with *Agrobacterium* and incubated on the selection medium gave rise to shoots in four weeks. The shoot growth was very slow and the Kanamycin-resistant shoots were excised and placed on rooting medium after three months. Seventy-nine independent transformed lines (T_0) of Bikaneri Nerma cotton were thus selected from 6520 shoot apex explants in the presence of Kanamycin. The leaf slices from the putative transformants remained green when incubated on media containing Kanamycin (100 mg/L). Analysis by PCR

to detect the presence of *npt* II and *cry1Ac* genes showed that twelve plants were positive (data not shown). However, these plants were chimeric in nature. The results were supported by the observations made by using Xpress immunodiagnostic strips and Quan-T ELISA. The transformation frequency is thus very low (0.2%). The positive plants were carefully nurtured in the glass house and seeds were collected from the bolls set from the selfed flowers.

Various procedures followed to analyze the plants belonging to T_0 – T_3 generations have been listed in Table 1. T_1 generation progeny (265) of the twelve PCR positive plants were raised in the transgenic greenhouse and subjected to various analyses. About 22 seeds from each T_0 plant were planted. PCR and Xpress Strip tests revealed gene segregation in T_1 generation and only 46 plants out of 256 showed positive results in PCR (*cry1Ac* primers) and Xpress Strip Test. Southern hybridization was performed with samples taken from eight out of 46 PCR positive plants, which belonged to different T_0 lines. The results showed that there were clear hybridization signals in only two samples (numbers 4 and 6) and the copy number varied from one to two in the transformed plants (Fig. 1). Although the other six plants were positive in PCR and immunological tests no hybridization signals were detected in Southern analysis. T_1 plants originating from two T_0 events (T1-4 and T1-11) corresponding to Southern positive plants and exhibiting high *Cry1Ac* content (2 μg g^{-1} FW) and insect protection (Table 2) were chosen and selfed. T_2 plants (13,136) were grown in the field and various analyses were conducted. The plants in T_2 generation (rapidly expanding leaves at 50 days after planting) showed very high levels of *Cry1Ac* protein expression as measured by Quan-T ELISA (1.59 to 2.46 μg g^{-1} FW).

Insect bioassays with first instar larvae of *H. armigera* on the leaves collected from 45-55 day old plants revealed very high degree of larval mortality in T_3 generation (Table 3). A comparison was also made with the mortality of the larvae fed with the leaves of a commercial

hybrid MECH-162 Bt. The levels of *Cry1Ac* protein in MECH-162 Bt ranged from 1.04 to 1.82 μg g^{-1} FW. PCR and Quan-T ELISA analyses of T_3 generation plants showed that all the plants were positive which indicated the homozygosity of the plants (Table 1). Observations on morphological characters, seed cotton yield per plant, boll damage and boll shedding due to natural *H. armigera* infestation of plants (T_3 generation) raised under unprotected condition were made. The results are presented in Table 4. The mean seed cotton yield of Bt-BN homozygous plants was 505 gm/plant as against 65 gm/plant of NBt-BN. The percent boll shedding among Bt-BN plants was 31.3 while in NBt-BN plants it was 88 per cent. The number of well-developed but damaged bolls due to Pink bollworm in Bt-BN was 1-2 per plant as against 8-11 in NBt-BN. Fibre parameters such as micronaire value, elongation, maturity ratio and tenacity were measured in both normal and transgenic plants. There were no differences among the normal and transgenic fibres (data not shown).

The results have shown that *Cry1Ac* protein was expressed at very high levels in the two transgenic lines of Bikaneri Nerma variety compared to the concentration of *Cry1Ac* in commercially available MECH-162 hybrid. It is important to achieve high levels of *Cry1Ac* expression in cotton tissues, especially terminal leaves, so as to maintain lethal levels of the toxin during boll development. Greenplate (1999) has carried out extensive analysis of *Cry1Ac* protein concentration in Bt cotton tissues and observed that the toxin levels decline steadily as the growing season progresses. The high levels of *Cry1Ac* observed in our study could be attributed to the truncated nature of the expressed toxin while the hybrids derived from the transformation event 531 (Monsanto Co., USA) express the *Cry1Ac* protoxin.

The results also demonstrated that it is possible to transform elite Indian genotypes of cotton (*G. hirsutum*) by adopting *Agrobacterium*-shoot apex technique which has been

successfully employed earlier in cotton, sunflower and maize. The degree of insect protection conferred by the expression of *Cry1Ac* protein in transformed Bikaneri Nerma was significant. Development of a national Bt-cotton hybrid, NHH-44 (Fig. 2) by utilizing transgenic Bikaneri Nerma, which is the female parent, is on anvil.

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Table 1. Number of plants subjected to various analytical tests.

Analysis	**T_0**		**T_1**		**T_2**		**T_3 (T1-11)**	
	Tested	+	**Tested**	+	**Tested**	+	**Tested**	+
Kanamycin test	**79**	**24**	**-**	**-**	**-**	**-**	**-**	**-**
PCR (*npt* II)	**79**	**12**	**-**	**-**	**-**	**-**	**-**	**-**
PCR (*cry1Ac*)	**79**	**12**	**265**	**46**	**45 (T1-4)** **41 (T1-11)**	**45** **41**	**136**	**136**
XpressStrip test	**79**	**12**	**265**	**46**	**170 (T1-4)** **182 (T1-11)**	**170** **182**	**917**	**917**
Quan-T ELISA	**79**	**12**	**46**	**46**	**45 (T1-4)** **41 (T1-11)**	**45** **41**	**97**	**97**
Southern analysis	**-**	**-**	**8**	**2**	**-**	**-**	**-**	**-**

Table 2. Insect bioassays performed with the leaves of normal and T_1 generation plants of Bikaneri Nerma (BN) using first instar larvae of *H. armigera*.

Genotype	No. of replications[z]	No. of larvae died			Corrected Mortality
		24 hrs	48 hrs	72 hrs	
Bt-BN (T1-4)	50	1	2	47	86
Bt-BN (T1-11)	50	2	2	46	84
NBt-BN	50	1	2	2	0

11

[z] One replication means one larva feeding on the leaf disc of the selected plant.

Table 3. Insect bioassays performed with the leaves of normal and T_3 generation plants (T1-11) of Bikaneri Nerma (BN) using first instar larvae[z] of *H. armigera.*

Genotype	Initial larval Wt (mg)	% larval survival to pupation	% larval survival to adults	% Pupal survival to adults	Larval Wt (mg) before pupation	Pupal Wt (mg)
BN-Bt (T1-11)	1.20	0.0	0.0	0.0	0.0	0.0
BN- Non Bt	1.40	84.50	79.10	82.0	159.0	89.2
MECH-162 Bt	1.35	0.0	0.0	0.0	0.0	0.0
Artificial diet	1.31	90.10	84.50	86.50	169.50	110.35
CD (1%)	NS	8.01	8.43	10.11	6.93	9.96
CV	6.91	4.47	4.93	8.06	9.29	6.15

[z] Fifty replications, each representing one larva feeding on the leaf disc of the selected plant.
Wt: Weight

Table 4. Economic characteristics of normal and some selected transgenic Bikaneri Nerma plants (T1-11; T_3 generation) grown in the field.

S.No.	Plant number (Code)	Seed cotton (g/plant)	Total number of fruiting points	No of squares/bolls shed	Shedding %
1	698	740	139	47	33.8
2	692	695	211	63	29.8
3	643	555	187	63	33.7
4	705	565	192	64	33.3
5	465	528	185	53	28.6
6	396	465	157	36	22.9
7	400	450	142	35	24.6
8	459	475	161	52	32.2
9	468	478	183	56	30.6
10	623	451	179	51	28.4
11	638	422	140	50	35.7
12	656	462	164	66	40.2
13	658	479	509	52	24.9
14	660	468	185	54	29.1
15	675	478	192	71	37.0
16	695	428	164	62	37.8
17	746	430	214	64	30.0
18	Mean	505	194	55	31.3
19	Non Bt (Mean of 30 plants)	65	166	146	88.0

FIGURE CAPTIONS

Figure 1. Southern hybridization analysis of genomic DNAs restricted by *Hin* DIII and probed by radiolabelled *cry1Ac* (C: Control; 1-8: Transformants).

Figure 2. Fig.2 NHH44 Bt cotton hybrid.

Figure 1.

Figure 2.