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Genetic transformation of an elite Indian genotype of cotton (*Gossypium hirsutum* L.) for insect resistance

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***Agrobacterium*-mediated genetic transformation of an elite Indian genotype (Bikaneri Nerma) of cotton (*Gossypium hirsutum* L.) was achieved 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) after co-cultivation of the explants with *Agrobacterium tumefaciens* (strain EHA 105). Rooting was accomplished on a medium containing naphthaleneacetic acid and kanamycin. Progeny obtained by selfing T_0 plants was grown in the greenhouse 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. Results of the field tests showed considerable potential of the transgenic cotton for resistance against cotton bollworm.**

Keywords: *Agrobacterium*, genetic transformation, *Gossypium hirsutum*, insect resistance, shoot apical meristem.

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 affects cotton production in India, is the incidence of pests, especially bollworms, causing more than 50% yield loss[1]. The limited genetic variability for bollworm resistance in cotton land/wild races makes the task of developing pest-resistant lines 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[2]. In 2005, *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[3].

Introduction of foreign genes in elite genotypes is limited by the 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[4–7]. However, alternate procedures to transform non-Coker genotypes have been reported[8–11]. In the present study, we report successful introduction of *Bt-cry1Ac* gene in an elite Indian genotype of cotton following a modified shoot apical meristem procedure[9] and significant protection from cotton bollworm in field conditions.

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

Seeds were delinted with sulphuric acid and soaked in $HgCl_2$ (50 mg/l) for 30 min and kept for shaking (50 rpm)

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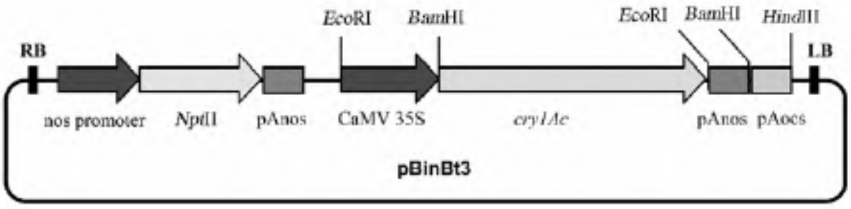

Figure 1. Restriction map of the binary vector pBinBt3 carrying truncated codon-modified *Bt-cry1Ac* gene.

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 h) rotation to obtain healthy seedlings. Seedlings (7–8-day-old) grown aseptically on MS medium[12], were used for isolation of shoot apex. Isolation of shoot apex was carried out as described by Gould *et al.*[13].

Agrobacterium tumefaciens (EHA 105) harbouring a binary vector (pBinBt3) was grown overnight at 28°C. The binary vector carries a codon-optimized *cry1Ac* gene driven by CaMV 35S promoter (Figure 1). 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.

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, 0.5 mg/l nicotinic acid, 0.5 mg/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 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 0.1 mg/l, NAA, 15 g/l sucrose and 400 mg/l cefotaxime.

Rooted plants were rinsed well with fungicide (bavistin, 0.2%) and transferred to 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 greenhouse.

Plants were grown in a transgenic greenhouse. Standard method of selfing was followed and seeds harvested from the T_0 plants were sown to raise T_1–T_3 generations in the field.

Genomic DNAs were isolated from the plants following the procedure of Doyle and Doyle[14]. 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 using radiolabelled *cry1Ac* gene (1.9 kb).

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

Laboratory bioassays were performed using neonate and first instar larvae of *H. armigera* reared on artificial diet[15]. Plants (T_2 and T_3 generations) were raised in open field conditions with permission of the Department of Biotechnology and following their guidelines. Natural infestation by various pests was allowed by not spraying any insecticides. Severe incidence of *Helicoverpa armigera* during 2003–04 (T_3 generation) facilitated analysis of the protection conferred by the expression of Cry1Ac.

Agrobacterium-mediated transformation of cotton was first reported by Firoozabady *et al.*[4] and Umbeck *et al.*[5]. Among the genotypes of *G. hirsutum*, Coker and Acala genotypes are amenable for genetic transformation[16] because of their high regeneration potential. Kumar *et al.*[17] have attempted to transfer the regenerative competence from Coker varieties to recalcitrant elite cultivars and developed a Coker 310FR line which could be used for genetic transformation. The transgene from the Coker 310FR can then be transferred to elite genotypes by conventional breeding. However, this could also lead to introgression of undesirable characters from Coker 310FR. Thus transformation of elite genotypes is desirable. Successful efforts to directly transform elite genotypes by alternate methods have been reported.[9,10] Satyavathi *et al.*[11] reported genetic transformation of two Indian genotypes of cotton using shoot apices from 3 to 5-day-old seedlings. 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.*[13] 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 the shoot apical meristem. The transformation efficiency was very low (0.2%) in contrast to 60–70% reported earlier[10]. Transformation efficiency was calculated based on physical presence of the transgene as analysed by PCR and its expression by ELISA and not on the number of kanamycin-resistant shoots.

The shoot apex explants infected with *Agrobacterium* and incubated on the selection medium gave rise to shoots in four weeks. The explants gave rise to dark cal-

Table 1. 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
Xpress strip 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 leaves of normal and T_1 generation plants of Bikaneri Nerma (BN) using first instar larvae of *Helicoverpa armigera*

Genotype	No. of replications*	No. of larvae died			Corrected mortality
		24 h	48 h	72 h	
Bt-BN (T1-4)	50	1	2	47	86
Bt-BN (T1-11)	50	2	2	46	84
N*Bt*-BN	50	1	2	2	0

*One replication means one larva feeding on the leaf disc of the selected plant.

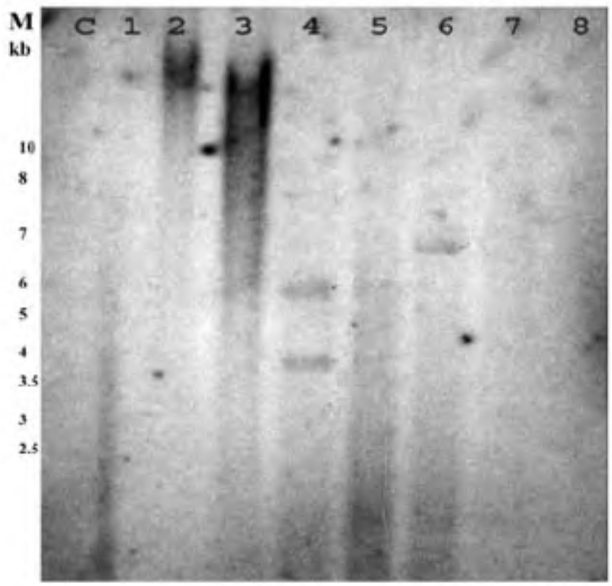

Figure 2. Southern hybridization analysis of genomic DNAs restricted by *Hin*dIII and probed by radiolabelled *cry1Ac* (C, Control; 1–8, transformants).

luses from which the shoots developed. Shoot growth was 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 medium 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 observations made 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 glasshouse and seeds were collected from the bolls set from the selfed flowers.

Various procedures followed to analyse the plants belonging to T_0 through 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; 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 (nos 4 and 6) and the copy number varied from one to two in the transformed plants (Figure 2). Although the other six plants were positive in PCR and immunological tests, no hybridization signals were detected in the 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

Table 3. Insect bioassay performed with leaves of normal and T_3 generation plants (T1-11) of Bikaneri Nerma using first instar larvae* of *H. armigera*

Genotype	Initial larval wt (mg)	Per cent larval survival to pupation	Per cent larval survival to adults	Per cent 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-N*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

*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

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

(2 μg/g 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. Plants in the T_2 generation (rapidly expanding leaves at 50 days after planting) showed high levels of Cry1Ac protein expression as measured by Quan-T ELISA (1.59 to 2.46 μg/g FW). In comparison, the levels of Cry1Ac protein in Mech 162 Bt ranged from 1.04 to 1.82 μg/g FW.

T_3 progeny of the T_2 plant (T1-11) was used for insect bioassay. Insect bioassays with first instar larvae of *H. armigera* on leaves collected from 45 to 55-day-old plants revealed high degree of larval mortality in T_3 generation, similar to that observed with the leaves of a commercial hybrid MECH-162 *Bt* (Table 3). Further, PCR and Quan-T ELISA analyses of T_3 generation plants showed the presence of *cry1Ac* gene, which indicated that the T1-11 line was homozygous (Table 1). Southern analysis of five plants of T_4 generation (T-1-11) was carried out and all the five plants tested positive, indicating the homozygosity (Figure 3). 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 g/plant as against 65 g/plant of N*Bt*-BN. The per cent boll shedding among *Bt*-BN plants was 31.3, while in N*Bt*-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 N*Bt*-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 in the two transgenic lines of Bikaneri Nerma was expressed at levels higher than that observed in the commercially available MECH-162 hybrid. It is important to achieve high

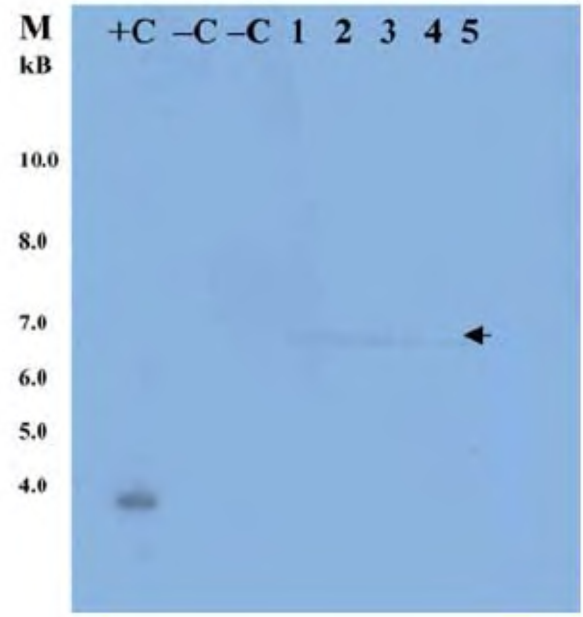

Figure 3. Southern hybridization analysis of genomic DNAs of Bikaneri Nerma (Normal and T-4) restricted by *Hin*dIII and probed by radiolabelled *cry1Ac* (+C, Positive control; –C, Normal cotton; 1–5, Transgenic plants).

levels of Cry1Ac expression in cotton tissues, especially terminal leaves, so as to maintain lethal levels of the toxin during boll development. Greenplate[18] 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[18].

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[10,19]. The degree of insect protection conferred by the expression of Cry1Ac protein in transformed Bikaneri Nerma was significant. Development of a *Bt*-cotton hybrid (NHH-44) by utilizing transgenic Bikaneri Nerma, which is the female parent, is in progress.

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