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Introduction of *cry1Ab* gene into cotton (*Gossypium hirsutum*) enhances resistance against Lepidopteran pest (*Helicoverpa armigera*)

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Abstract

The significant breakthrough in plant biotechnology is the development of techniques to transform genes from unrelated sources into commercially important crop plants to develop resistance against insect pests. A local cotton cultivar MNH-93 was transformed through *Agrobacterium tumefaciens* strain $C58C_1$ assisted by bombardment with tungsten particles. The *Agrobacterium* strain contained the recombinant binary vector pKMAB harboring *cry1Ab* under 35S promoter. Neomycin phosphotransferase (*nptII*) gene was used as a selectable marker at a concentration of 50 mg L^{-1}. The transformation efficiency remained 0.26%. The primary transformants were analyzed for transgene integration and expression through PCR and Southern Blotting and Western dot blot. The gene copy number was determined by Southern analysis in order to find out the *cry1Ab* integration sites. The *Bt* protein being produced in the transgenic plants was quantified using ImageQuant software, which ranged from 0.00 to 1.35% of the total protein. The positive plant seeds obtained from T_0 progeny were further raised under greenhouse and field conditions to evaluate their field performance. Leaf biotoxicity assays were performed to determine the efficacy of introduced gene. The results showed that transgenic lines in T_1 progeny have appreciable level of resistance (40-60%) against lepidopteran pests in both green house and field conditions.

Additional key words: gene copy number; leaf toxicity bioassay; MS medium; transformation.

Resumen

La introducción de genes *cry1Ab* en algodón (*Gossypium hirsutum*) mejora la resistencia contra las plagas de lepidópteros (*Helicoverpa armigera*)

Un importante avance en biotecnología vegetal ha sido el desarrollo de técnicas para transformar genes provenientes de fuentes no relacionadas en cultivos de importancia comercial para desarrollar resistencia contra plagas de insectos. Se transformó el cultivar local de algodón MNH-93 por medio de la cepa $C58C_1$ de *Agrobacterium tumefaciens* mediante bombardeo con partículas de tungsteno. La cepa de *Agrobacterium* contenía el vector binario recombinante pKMAB que albergaba *cry1Ab* bajo el promotor 35S. Se utilizó el gen neomicina fosfotransferasa (*nptII*) a una concentración de 50 mg L^{-1} como marcador genético. La eficiencia de transformación se mantuvo en un 0,26%. Los transformantes primarios se analizaron para la integración y expresión del gen mediante PCR, Southern blotting y Western dot blot. El número de copias del gen se determinó por Southern blot a fin de conocer los sitios de integración del *cry1Ab*. Se cuantificó la proteína *Bt* producida en las plantas transgénicas utilizando el software ImageQuant, que varió de 0,00 hasta 1,35% de las proteínas totales. Las semillas de plantas positivas obtenidas a partir de la progenie T_0 se crecieron en condiciones de invernadero y de campo para evaluar su comportamiento en el campo. Se realizaron ensayos de biotoxicidad de la hoja para determinar la eficacia del gen introducido. Los resultados mostraron que las líneas transgénicas en la progenie T_1 tienen un nivel apreciable de resistencia (40-60%) contra las plagas de lepidópteros, tanto en condiciones de invernadero como de campo.

Palabras clave adicionales: bioensayo de toxicidad de la hoja; medio MS; número de copias de genes; transformación.

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Abbreviations used: CEMB (Centre of Excellence in Molecular Biology), GM (genetically modified), MS (Murashige and Skoog), PCR (polymerase chain reaction).

Introduction

The application of biotechnology tools to agriculture has allowed scientists to transform plants without the need for sexual compatibility between species, thus establishing the possibility of rapidly producing new crop varieties with traits beneficial to human health and the environment. Plants have been transformed successfully to improve their pest and disease resistance, herbicide tolerance, nutritional qualities, and stress tolerance (MacKey and Santerre, 2000).

Cotton (*Gossypium hirsutum* L.) is an important non-food cash crop in Pakistan and a significant source of foreign exchange earning. Cotton and cotton products contribute about 10% to gross domestic product (GDP) and 55% to the foreign exchange earnings of the country. The cotton crop was sown on the area of 2,820 thousand hectares; the production is estimated at 11.8 million bales (Economic Survey of Pakistan, 2008-09).

Cotton is susceptible of being attacked by more than 15 economically important insects, mainly belonging to the insect order Lepidoptera, Coleoptera and Homoptera. On overall basis, 13% of the cotton crop is lost due to insects (Gatehouse and Hilder, 1994) depending upon the weather conditions and other environmental factors every year. These insects are being controlled by chemical insecticides, which otherwise have serious environmental and human health threats. During the campaign 2008-09, 16,495 thousand tons of agricultural pesticides were imported in Pakistan with a cost of 68.7 millions US dollars (Economic Survey of Pakistan, 2008-09). Moreover, insects have also been evolving resistance against these chemicals. Over 500 species of insects have become resistant to one or multiple synthetic chemical insecticides (Schnepf *et al.,* 1998).

Using conventional plant breeding techniques, cotton breeders have continuously sought to improve the cotton which has introduced numerous improvements in crop yield during past centuries. However, resistance to insect pests and diseases does not exist in available germplasm; this has led to a limit in availability of new genetic information into plants and to create plant varieties with novel characters through plant breeding techniques (Hussain, 2002). Current approaches to cotton improvements include use of genetic engineering that is gaining momentum in developed as well as developing countries.

The advent of plant biotechnology has broken the barrier between species and has resulted in the introduction of genes from unrelated sources into commercially important crop plants to develop resistance against insect pests (Lycett and Grierson, 1990; Dhaliwal *et al.,* 1998). *Bacillus thuringiensis* (*Bt*) is perhaps, the most important source of insect resistant genes. Genetically modified (GM) crops were grown on 134 million hectares globally in 2009 (James, 2009). Among them, transgenic cotton expressing insecticidal proteins from *B. thuringiensis* (Bt) has been one of the most rapidly adopted GM crops in the world (James, 2002; Barwale *et al.,* 2004; Dong *et al.,* 2005) containing *cry* gene(s) such as c*ry1Ac*, *cry1Ac* + *cry2Ab* or *cry1Ac* + c*ry1F.*

The aim of this work was to transform the commercially approved cotton cultivar MNH-93 (a good yielding cultivar but susceptible to lepidopteran insect pest) with a locally isolated *B. thuringiensis* gene (*cry1Ab*) using *Agrobacterium tumefaciens* strain LB $C58C_1$ assisted by bombardment with tungsten particles.

Material and methods

Plasmid construction

A codon optimized 1.845 kb *cry1Ab* gene was cloned in pKMAB contruct under *BamHI* and *EcoRI* sites driven by 35S promoter and T-DNA gene terminator. Gene integration and orientation was verified by restriction digestion. The construct pKMAB also contained *Neomycin phosphotransferase* (kanamycin resistance) under NOS promoter and OCS terminator. CAB 22L was used as to enhance the expression of introduced gene. The construct was transferred into *Agrobacterium tumefaciens* strain $C58C_1$ by eletroporation to make co-integrate vector PGV2260: pKMAB (Fig. 1).

Cotton transformation

Cotton (*Gossypium hirsutum* L.) cv. MNH-93 was locally developed at Cotton Research Station, Multan and released for commercial cultivation in cotton zone of Punjab province (Pakistan). It was selected for transformation because it has a good regeneration potential through tissue culture, high yield potential and desired fibre characteristics. Moreover, it showed better genetic stability at field level as compared to other cultivars (Hussain, 2002). The seed of the cultivar MNH-93 was obtained from the Directorate of Cotton Research Institute, Faisalabad.

The seeds were delinted and surface sterilized with Tween-20 for 3 min and further subjected to 0.1%

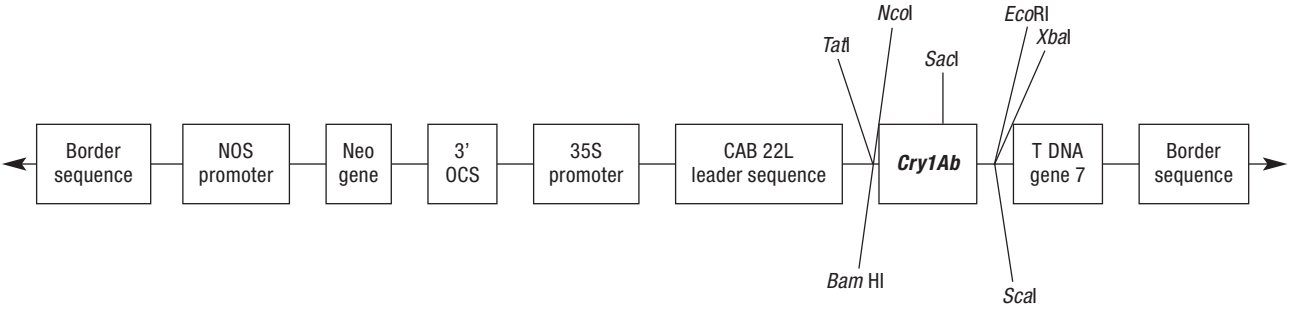

Figure 1. Schematic diagram of construct pKMAB showing: NOS (nopaline synthase) promoter, Neo (neomycin phosphotransferase (*nptII*) gene, OCS (octapine synthase) terminator gene, 35S (*Cauliflower mosaic virus* 35S) promoter, CAB 22L (enhancer).

$HgCl_2$ and 0.1% SDS solution mixture. The sterilized seeds were soaked placed in dark at 30°C overnight for the germination. The germinated embryos were used for the transformation.

Bombardment with tungsten particles was done to create small wounds on the surface of the embryos which would facilitate DNA transfer from *Agrobacterium*. The prepared tungsten particles were coated on a filter assembly and allowed to dry for 1-2 minutes in a laminar hood. The filter assembly was fixed in leur-lock of Particle Bombardment Gun. The mature embryos were placed at a pre-optimized distance of 22 cm and bombardment done under vacuum using helium gas at a pressure of 4.13 bar.

The bombarded embryos were co-cultivated with *A. tumefaciens* strain $C58C_1$ harboring pKMAB plasmid. The embryos were cultured on MS medium (Murashige and Skoog, 1962). Twenty five non-transformed embryos were cultured on MS medium as control. The plates were kept at 28 ± 2°C for 3 days. Thus, plantlets were sub-cultured on selection medium, *i.e.* MS containing 50 mg L^{-1} kanamycin. Cefataxime (250 mg L^{-1}) was also added to inhibit bacterial overgrowth. Subculturing was done after every 10 days. After 2 months selection, these seedlings were shifted to kanamycin-free MS medium until fully developed plantlets were obtained which were further shifted to pots containing soil of equal proportion of clay, sand and peat moss (1:1:1). Finally the plants were shifted to greenhouse and were subjected to different molecular analysis.

Confirmation of gene integration and expression

Polymerase chain reaction (PCR) was carried out using specific primer pairs to amplify c*ry1Ab* transgenes from transgenic cotton plants. For this purpose, genomic DNA was extracted and purified from leaves based on the protocol of Li *et al*. (2001). PCR was performed in a total reaction mixture volume of 20 µL containing 1X reaction buffer, 50 ng of DNA template, 1.5 mM $MgCl_2$, 1 mM of each of the dNTPs, 10 ng of each primer and one unit of *Taq* DNA polymerase.

The forward primer used to amplify *cry1Ab* was 5'-GTTACCCTGATTGATAGGC-3' while the reverse primer was 5´ACAGAAGACCTTTCAATATC-3' (Khan, 2007). PCR was carried out in a thermal cycler using the following conditions: initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 40 seconds, extension at 72°C for 40 seconds, followed by a final extension at 72°C for 10 min. The plasmid pKMAB was used as positive control whereas the DNA isolated from untransformed plants was used as negative control. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light. The expected size of the band was 550 bp.

For southern blot analysis, genomic DNA from PCR positive plants was isolated using the protocol as given by Saha *et al*. (1997). To estimate copy number of the transgene in the transformed plants, 27 µg genomic DNA was digested with 320 U (about 12 U μg^{-1}) of *Sst*I which has a unique site in the plasmid pKMAB. Digested DNA fragments were separated on 0.8% agarose gel and DNA was transferred onto a nylon membrane (Hybond N+, Amersham, UK) by capillary blotting. Gene specific probe of *cry1Ab* was labeled using Fermentas Biotin DecaLabel™ DNA labeling kit. Hybridization was carried out overnight at 65°C. Detection procedure was followed as provided in Fermentas Biotin Chromogenic detection kit.

About 0.5 g fresh terminal leaves was ground in liquid nitrogen. One millilitre protein extraction buffer (0.04 M EDTA, 10% glycerol, 0.15 M NaCl, 0.01 M

Tris-ClH, 10 mM NH_4Cl, 20 mM PMSF (phenylmethylsulfonyl fluoride), 10 mM DTT (dithiotheritol) was added and centrifuged at 14,000 rpm at 4°C. The supernatant was harvested. The protein concentration was estimated by comparing OD value at 595 nm with an already plotted curve of known concentrations of BSA (bovine serum albumin).

Ten nanograms of protein were loaded on Hybond-C membranes. The membrane was completely air-dried and incubated in blocking buffer/reagent for 30 min at room temperature or overnight at 4°C. The membrane was thrice washed with 1X PBS. The membrane was probed with the diluted primary antibodies (1:2,500) for 1 h and washed three times with 1X PBS. Afterwards, the membrane was probed with the diluted secondary antibodies (1:2,000) for 1 h and washed again three times with 1X PBS. The color was developed in AP Buffer (NBT/BCIP). *Bt* contents were quantified after scanning the blots by using software Image Quant TL (Amersham Biosciences Co.).

Leaf biotoxicity assay

Transgenic cotton plants in T_0 progeny were subjected to lab bioassays with American bollworm (*Heliothis armigera*). Five fresh leaves from each plant were taken and placed on wet filter paper in petri plates accommodating one leaf per plate. One 2nd instar larva, prefasted for 4-6 h, was released in each plate and allowed to feed on the leaf. The data on insect mortality were recorded on daily basis up to 7th day.

Analysis of T_1 progeny

Transgenic cotton plants positive for c*ry1Ab* gene were carefully picked and their progenies were grown in the field, following international biosafety guidelines in which transgenic crop is surrounded by non transgenic crop. Pakistan has also achieved status of GMO country. *Sorghum bicolor* was used as refugia.

Plants in T_1 progeny were evaluated for the integration and expression of c*ry1Ab* using same molecular techniques. Leaf biotoxicity and field infestation assay were also performed once to determine the efficacy of inserted gene against targeted insect pests. Keeping in view the results of PCR, western blot and morphological and agronomic characteristics, further selections in the progenies were made.

Results

A total of 10,000 mature embryos of cotton were subjected to tungsten particle bombardment for microwound production and co-cultivation with the transformed *Agrobacterium*. After eight weeks of selection on 50 mg L^{-1} kanamycin, 26 plants were obtained. The transformation efficiency was 0.26%. These plants were then shifted to soil.

PCR analysis of newly transformed plants was performed for the amplification of c*ry1Ab* using specific primers. The amplification of the 550 bp band specific of c*ry1Ab* gene was achieved in all 26 plants showing that the plants had been successfully transformed. Inheritance of transgenic plants was investigated in further progeny (T1).

Four PCR positive plants were subjected to Southern blot to confirm the integration of c*ry1Ab* gene in the plants. Results revealed that the plant CEMB-3 had two copies of *cry1Ab* gene in its genome whereas the plants CEMB-11, CEMB-16 and CEMB-17 had three copies of the *cry1Ab* transgene (Fig. 2). The presence

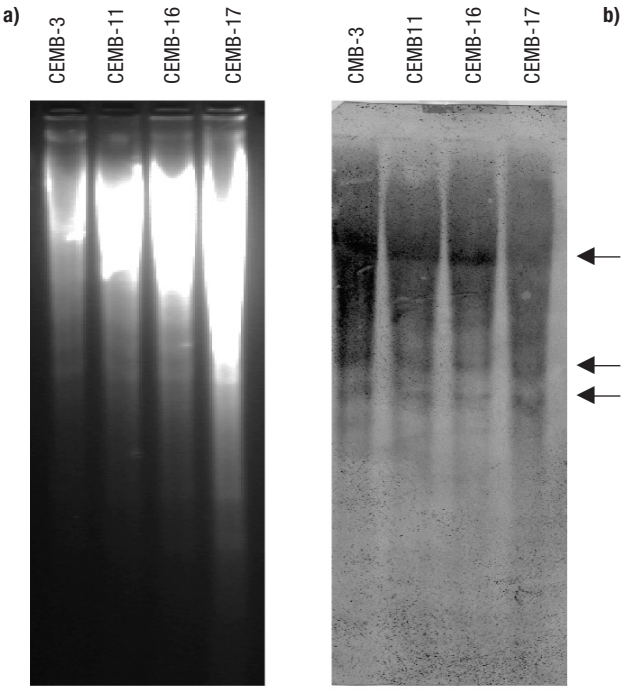

Figure 2. a) Genomic DNA of four selected plants digested with *Sst*I and b) probed with biotin-labeled *cry1Ab* DNA to assess copy number of the transgene in the transformed plants. The number of bands revealed that the plant CEMB-3 had two copies of the transgene in its genome whereas the plants CEMB-11, CEMB-16 and CEMB-17 had three copies of the transgene.

of *cry* protein was detected in all samples except negative control (Fig. 3).

Laboratory biotoxicity assays results with 2nd instar *H. armigera* larvae showed that c*ry1Ab* gene expression was sufficient to kill the targeted insects. A variation in resistance level (40-60%) against *H. armigera* larvae was observed in all transgenic plants. The mortality rate of larvae that ranged between 40-100% has been shown in Figure 4. The larvae which survived in few cases were too inactive or sluggish to be harmful for the plant. While in case of non transformed control, no mortality of larvae was noted.

T_1 progeny of the T_0 positive plants was sown in the field and its performance in field was evaluated. PCR and western dot blot was employed to confirm c*ry1Ab*

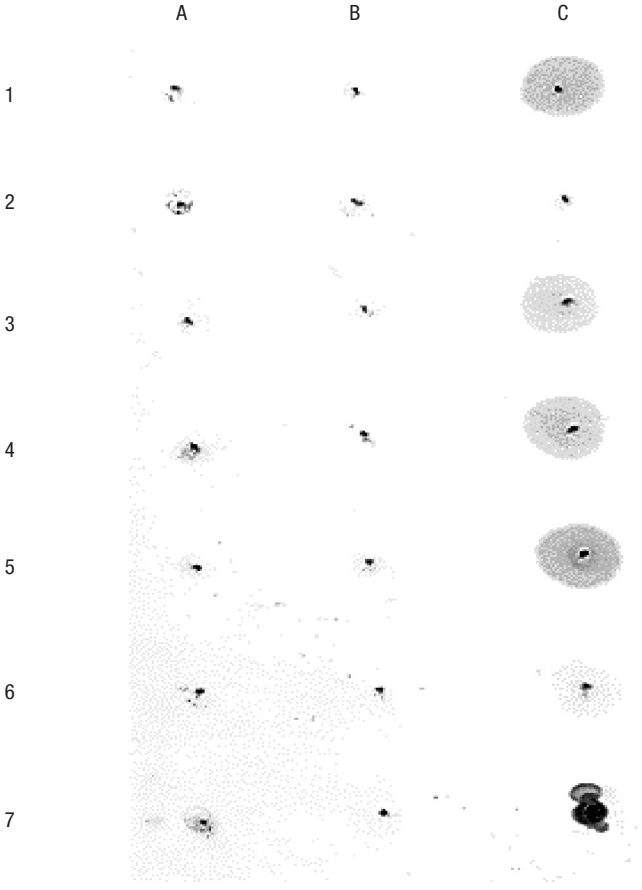

Figure 3. Western dot blot of transformed plants; A1 to A7 (CEMB-3), B1 to B7 (CEMB-11), C1 to C3 (CEMB-16), C4 to C5 (CEMB-17), C6 (negative control) and C7 (positive control). The transformed plants were screened on the basis of *Bt* gene expression assessed through Western Dot Blot. Among the plant samples (A1 to A7; B1 to B7 and C1 to C5) which developed colour using NBT/BCIP tablets, the presence of *Cry* protein was detected. The *Bt* contents were quantified using Image Quant TL software of the Amersham BioSciences (Pvt).

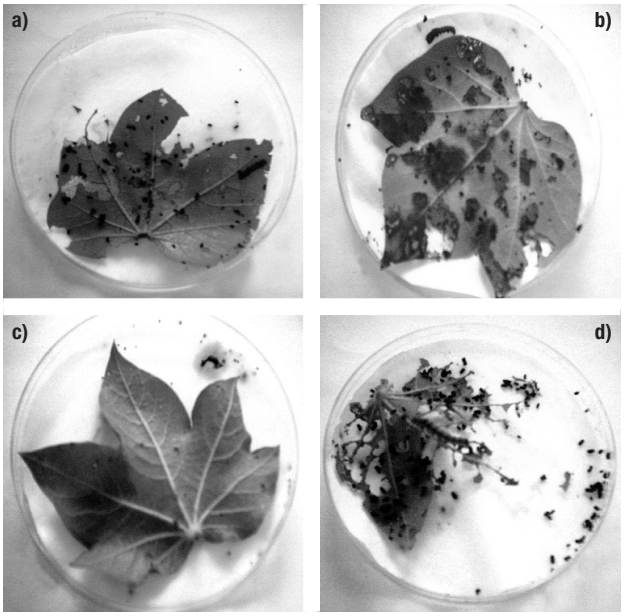

Figure 4. Transgenic cotton plants subjected to lab bioassays with American bollworm (*Heliothis armigera*). a), b), c) Represent the transgenic leaves of CEMB-3, CEMB, 11 and CEMB-16 plants, respectively, whereas d) shows the non transgenic leaf.

integration and expression is successive progeny. Morphological and agronomic characteristics of transgenic plants were recorded in T_1 progeny (Table 1).

Discussion

Genetic-engineering has enabled the isolation, amplification, and *in vitro* manipulation of genes. For a number of technical and practical reasons, resistance to herbicides was among the first traits introduced into crop plants (Mazur and Falco, 1989). Other characteristics of agronomic importance for cotton production are those related to insect resistance and fiber-quality modification (John and Keller, 1996).

A number of local cultivars has been screened at CEMB, on the basis of their regeneration capability through tissue culture for subsequent transformation with foreign genes (Hussain, 2002). However, the lengthy procedures of transformation and development of pure lines could not reap full benefits afterwards. The main reasons to this loss have been the replacement of old cultivars with the new high yielding cultivars, or the lower genetic stability at field level. It was therefore imperative to select for transformation a cotton cultivar that had shown better adaptability and more genetic stability at field level besides having a good yield and

Table 1. Characteristics of five selected plants in T_1 progeny

Plant no.	PCR	Gene copy number	Western dot blot results	Insect mortality shown in the lab bioassay (%)	No. of monopodia	No. of sympodia	Plant height (cm)	Yield (g plant^{-1})	Ginning outturn (%)
CEMB 3-2	+	2	+	60	4	17	195	87.8	43.96
CEMB 11-2	+	3	+	40	3	33	105	102.3	39.6
CEMB 16-10	+	3	+	50	1	12	172	109.3	41.0
CEMB 16-15	+	3	+	60	4	22	117	88.6	42.0
CEMB 17-25	+	3	+	50	5	19	170	95.3	38.5
Control	–	—	–	20	2	18	190	46.8	38.0

regeneration potential. The cultivar MNH-93 was thus the most suitable material for transformation purposes as it was evident from the field performance and screening data of Hussain (2002).

In the present study, transformation method used was primarily *Agrobacterium*-mediated. However, it was supplemented with bombardment of tungsten particles through biolistic gun. The tungsten particles were bombarded just to create micro-wounds on the embryos to facilitate DNA transfer by *Agrobacterium*. Our strategy was in line with earlier research workers such as Fraley *et al.* (1983), Finer and McMullen (1990) and Bidney *et al.* (1992). According to Finer and McMullen (1990), *Agrobacterium*-mediated transformation is the most common method to transform dicotyledonous plants. The other method being used to transform cotton is microprojectile bombardment. Earlier, Fraley *et al*. (1983) had reported the *Agrobacterium*-mediated transformation of petunia and tobacco. Bidney *et al*. (1992) have shown that efficiency of *Agrobacterium*-mediated gene transfer could be increased by wounding the explants by bombardment with naked particles. Similar results were found by Khan (2007).

The transformation efficiency in the present study has been 0.26% which was however very low as compared to Smith and Towsend (1997), Zapata *et al.* (1999), Majeed *et al.* (2000) and Rao (2009). This may be attributed to the regeneration capability through tissue culture of the cv. MNH-93, which was good but comparatively lower than the cultivars transformed by these researchers.

The transformed plants were analyzed for DNA integration through PCR and Southern Blotting. The copy number was also found through Southern Blot. Our results are in line with several research workers including Schrammeijer *et al*. (1990) and Cousins *et al*. (1991), who confirmed the integration of foreign DNA through PCR. Gould and Magallanes-Cedeno (1998) and Zapata *et al.* (1999) have also reported transformed plants analyzed through DNA blots for evidence of integration of transgenes and their copy number.

The transgene expression was confirmed through Western Dot Blot. Our findings were in line with Bashir *et al.* (2004) and Tohidfar *et al.* (2008), who confirmed the expression of *Cry* proteins through ELISA and Western Blotting while evaluating transgenic lines of indica basmati rice and cotton respectively.

There has been a varying behaviour of transgenic progeny plants in respect of resistance shown in different laboratory bioassays against *Heliothis* larvae. Chunlin *et al.* (1999), Zeng *et al*. (2002) and Bakhsh *et al.* (2010) reported that some of the homozygous c*ry1Ac* rice and cotton transgenic plants showed high level resistance against targeted insect pest at field trials.

Progeny analysis of transgenic lines suggested that genes were successfully introduced and stably inherited; showing high level of resistance against targeted insect pests (Table 1). These results are in agreement with the findings of Zapata *et al.* (1999), Bashir *et al.* (2004) and Bakhsh *et al.* (2009). Based on the molecular data obtained from the laboratory and morphological and agronomic data recorded from the field it is believed that these transgenic lines are an excellent source of germplasm to be used in conventional breeding programme.

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