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Development of transgenic cotton lines harboring a pesticidal gene (cry1Ab)

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Abstract

Each transgenic plant has its unique genetic architecture and level of expression. The present study was conducted to study each transgenic plant separately in order to develop pure transgenic lines to be further used in efficient cotton breeding programme. Earlier MNH-93 cultivar was transformed with the Bacillus thuringiensis (Bt) gene cry1Ab through Agrobacterium-mediated transformation method using mature cotton embryos as explants. Transformed plants obtained out of this transformation event were used to develop pure lines for five consecutive generations. The selection criteria contained confirmation of transgene through PCR and western dot blot, boll damage percentage, insect mortality percentage in lab bioassay with heliothis larvae, seed cotton yield (g), plant height (cm), number of monopodial branches per plant and number of sympodial branches per plant. All the characters were stably inherited in further generations. The expression of introduced genes was found to be variable; however it conferred protection against targeted insect pests. It was concluded that the transgenic pure lines are an excellent source of germplasm to be used in conventional breeding programme.

Key words: molecular evaluation; insect resistance; agronomic features; breeding programme

Introduction

Cotton is an important economic and fibre crop worldwide and likewise it is considered as backbone in Pakistan economy because of its major share in GDP (Economic Survey of Pakistan 20011-12). Genetically modified (GM) crops were cultivated on 148 million hectares globally in 2010. In Pakistan, insect Resistant cotton was grown on 2.4 million hectares out of 2.8 million hectares allocated land (James, 2011). Among them, transgenic cotton expressing insecticidal proteins from B. thuriengenesis (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 cry1Ac, cry1Ac + cry2Ab or cry1Ac + cry1F.

Many laboratories have obtained insect-resistant (Zhang and Feng 1998; Hussain, 2002; Rashid et al., 2008; Bakhsh, 2010) or herbicide-resistant transgenic cotton plants via Agrobacterium-mediated transformation (Umbeck et al., 1987; Rajasekaran et al., 1996), particle bombardment (Finer and McMullen 1990; McCabe and Martinell, 1993), or pollen tube pathway (Zhang et al., 2000).

Stable inheritance and expression of transgenes in transgenic plants is of paramount importance in the successful application of genetic engineering in crop improvement. Some research revealed that once foreign genes were integrated into host cells, they could be faithfully transmitted to progenies through sexual generations and retained high meiotic stability and expression stability (Duan et al., 1996; Fearing et al., 1997; Scott et al., 1998). However, there are also many instances where foreign genes were lost through meiosis or inactivated or silenced in the progenies of transgenic plants (Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Srivastava et al., 1996; Zhang et al., 1996).

Whether transgenes could be stably inherited and expressed in progenies of transgenic plants has remained a prime question during the course of the successful employment of transgenic plants into traditional breeding programs. The present study

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was conducted to develop pure transgenic cotton lines out of a transformation event (Khan et al., 2011) for consecutive five years. The various molecular (gene integration and expression assays) as well as agronomic approaches were utilized to screen and further develop these pure lines which can ultimately be used in an efficient transgenic cotton breeding programme.

Materials and Methods

MNH-93 (a good yielding local cotton variety but susceptible to lepidopterans insect pests) was transformed with *Bacillus thuringiensis* gene (cry1Ab) using Agrobacterium tumefaciens strain C58C1 (Khan et al., 2011) at National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. The sterilized seeds of variety 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 was done under vacuum using helium gas at a pressure of 4.13 bar. The bombarded embryos were co-cultivated with *A. tumefaciens* strain C58C1 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. The transgenic cotton plants obtained were further used to develop pure lines in field as well as green house conditions in five consecutive generations.

First Generation

The plants of cotton variety MNH-93, transformed with the gene cry1Ab were grown under field conditions in 2003. International biosafety guidelines were followed while designing field experiments. Experimental fields were surrounded with 5 rows of untransformed MNH-93 and CIM-482 (another locally approved cotton cultivar) to serve as refugia to prolong insect resistance. Sorghum bicolor was grown around the field to isolate field from surroundings (NBC, 1999). The total number of bolls was counted at the end of season. Since cotton is an indeterminate crop and new bolls keep on forming throughout the year, both mature and immature bolls were included in counting. In addition to it, the transgenes were meant to control lepidopteran insects; therefore no insecticidal spray against the lepidopteran insects was applied. The plants were monitored for degree of damage due to lepidopteran insects during the entire season. The boll damage was calculated in percentage by dividing the number of damaged bolls with the total number of bolls on the plant, multiplied by 100. Laboratory Bioassay assay were also performed against targeted insect pests.

To check the efficacy of endotoxins against targeted insect pests, laboratory biotoxicity assays of cotton leaves with *Heliothis* larvae (2nd instar) were conducted. Five leaves from upper, middle and lower portion of each lines were detached in perti plate ,placed on moist filter papers, taken to laboratory and 2 instar larvae of *Heliothis* was fed to them. After 2-3 days mortality rates of larvae were recorded.

Second Generation

The progenies of the 1st generation plants were grown in 2004. The field layout was planned according to the international guidelines for *Bt*-crop growing. Each plant progeny consisted of two rows (each row of 10m length) accommodating 66 plants. The plant to plant and row to row distances were kept at 30 and 75cm, respectively. The transgenic plants were surrounded by 4.5m wide belt of non-*Bt* cotton as refugia. The whole cotton field was further surrounded by 4.5m wide sorghum belt, as an isolation boundary.

Boll damage percentage due to natural infestation of bollworms, insect mortality percentage in lab bioassay with *Heliothis* larvae, seed cotton yield (g), plant height (cm), number of monopodial branches per plant, number of sympodial branches per plant, confirmation of transgene through PCR and western dot blot were recorded on individual plant basis. Single plant selection was made on the basis of the following criteria: - The plant must be positive in molecular screening tests viz. PCR and western dot blot. The

plant must be higher yielding in terms of seed cotton. The plant must bear less than 5% boll damage due to natural infestation of bollworms. The plant must have shown at least 40% insect mortality in the lab bioassays. The plant should give Ginning Outturn Percentage equal or more than the control plants and the plant should be equal or short in stature than control.

Third Generation

The progenies of the selected plants from 2nd generation were raised in the green house during winter 2004-05. The plants were grown in tumbler-shaped earthen pots of 60cm height and 45cm upper diameter. Each pot accommodated a single plant. Each progeny consisted of ten plants. The light, temperature and humidity conditions of the green house were maintained by using high-powered lights, heaters and humidifiers. During the entire plant growing period, the temperature and humidity ranged between 25-40ºC and 30-70%, respectively.

The plants were again subjected to individual studies and molecular analysis. Plant selections were made on the basis of the criteria stated above. The plants in this generation showed 50% to 100% homozygosity; therefore the seed of selected plants from each progeny row was bulked to have four pure lines.

Fourth and Fifth Generation

The four pure lines mentioned above were further analyzed at molecular level, for two successive generations to eliminate false positive plants and to check for errors in selection. A sample of 30 plants, at random, was taken from each pure line during every year and analyzed at molecular level through PCR and western dot blot.

Results and Discussion

The primary objective of developing transgenic pure lines was to develop an insect resistance source. The plant selections were made in each generation to choose plants with high resistance also accompanied by other characteristics as are desirable for an efficient plant breeding programme. The methods adopted during selections were according to the standard procedures laid down in the books by Poehlman (1978); Khan et al. (2001) and Singh (2005).

First Generation

The transformation event as described by Khan et al., 2011) resulted in 26 positive plants. The plants were further grown under field conditions at CEMB in 2004. The data were recorded on various plant characteristics; the most important at this stage were boll damage (%age) due to natural infestation of bollworms (Figure 1). A boll which was completely or partially damaged (at least 10%) by any bollworm was counted as the damaged one. The bolls which were totally undamaged were counted as healthy bolls. The boll damage ranged from 8% to 100% in the plants (Table 1). The plants were also subjected to insect bioassays with 2nd instar *Heliothis* larvae under laboratory conditions (Figure 2). The insect mortality in lab bioassay ranged from 20% to 100% (Table 1).

Figure 1. Comparative view of damaged and healthy cotton bolls.
Among various plant characteristics; the most important one was Boll Damage (%age) due to natural infestation of bollworms. A boll which was damaged at least 10% by any bollworm was counted as the damaged one (A&C). The totally undamaged bolls were counted as healthy bolls (B&D). The boll damage in the 1st generation plants ranged from 8% to 100% in the plants.

Table 1. Insect Resistance and No. of Bolls of 1st Generation Plants.

S.No.	NAME	Insect mortality % shown in lab bioassay with Heliothis	No. of Total Bolls (Mature + Immature)	Average Boll Damage %age During the Peak Infestation Period (August-October)
1	CEMB-1	90	72	24
2	CEMB-2	50	63	14
3	CEMB-3	60	71	24
4	CEMB-4	80	79	8
5	CEMB-5	80	51	37
6	CEMB-6	20	19	26
7	CEMB-7	50	11	64
8	CEMB-8	90	2	100
9	CEMB-9	50	10	40
10	CEMB-10	70	22	86
11	CEMB-11	60	60	18
12	CEMB-12	80	52	12
13	CEMB-13	90	65	34
14	CEMB-14	70	22	50
15	CEMB-15	60	13	92
16	CEMB-16	70	7	29
17	CEMB-17	100	46	17
18	CEMB-18	100	40	15
19	CEMB-19	80	39	46
20	CEMB-20	100	29	24
21	CEMB-21	80	24	21
22	CEMB-22	100	37	19
23	CEMB-23	70	41	27
24	CEMB-24	80	57	18
25	CEMB-25	70	30	37
26	CEMB-26	100	36	25
27	Control	20	8	63

The boll damage due to naturally occurring bollworms in the field ranged from 8% to 100% in the plants. The insect mortality in lab bioassay ranged from 20% to 100%

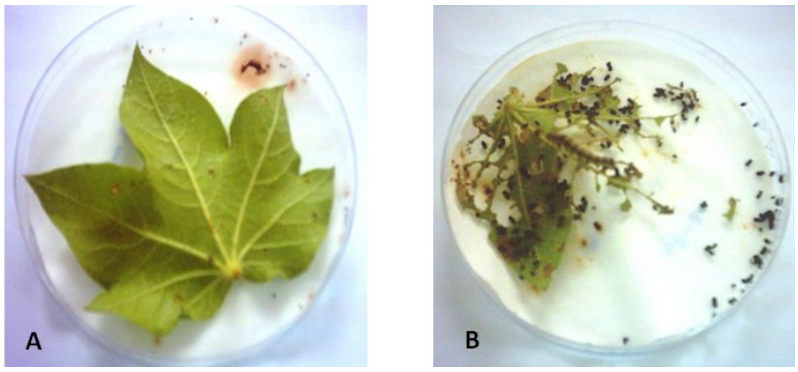

Figure 2. Laboratory bioassay with Heliothis larvae. The transgenic cotton plants were subjected to lab bioassays with American Bollworm (*Heliothis armigera*). Five fresh leaves from each plant were taken and placed on wet filter paper in petri plates accommodating one leaf per plate. One 1st/2nd instar larva, pre-fasted for 4-6 hours, was released in the each plate and allowed to feed on the leaf. After 48-72 hours feeding, the transgenic plants (A) showed insect mortality whereas the insect survived on control plant B.

The progeny rows of all 26 plants were desired to be grown but the seed setting in most of the plants did not take place under field conditions. The unfavorable weather conditions during the season besides late sowing hindered the bolls to mature. Moreover, most of the bolls which matured late season produced lighter seeds, which were discarded during seed selection for next sowing. At the end, sufficient seed of nine plants only could be obtained for further studies in progeny rows.

Second Generation

The progeny plants grown during in 2004 showed a considerable heterozygosity in plant morphology and insect resistance. The Boll Damage (%age) ranged from 0.0% to 100%. The *Heliothis* larval mortality %age in the lab bioassay ranged from 10% to 100%. The yield per plant ranged from 0.0 g to 165.1g. The plant height ranged from 47 to 230cm. The number of monopodial branches per plant ranged from 1 to 6 per plant. Similarly, the number of sympodial branches ranged from 4 to 40 per plant (Figure 3).

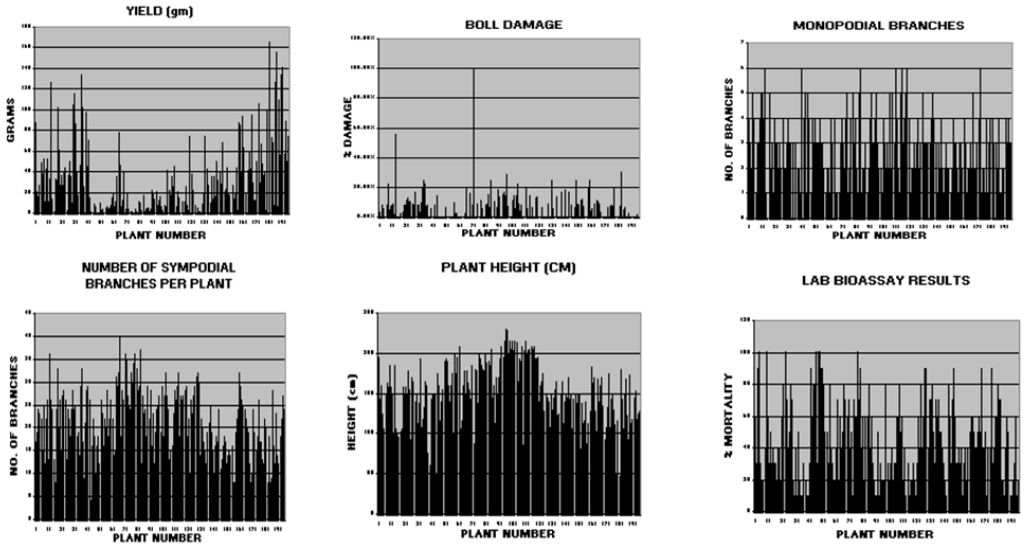

Figure 3. Data on different characters of all plants of 2nd generation, kharif, 2004The data on different characteristics of 2nd generation plants were recorded on individual plant basis.

Table 2. All plants of 2nd generation were subjected to screening on the basis of the data recorded regarding the above-mentioned traits. The data pertaining to finally selected five plants are shown here.

S. No.	Plant No.	PCR	Western Dot Blot	Insect Mortality - Lab Bioassay (%)	Boll Damage due to Natural Infestation (%age)	No. of Monopodial Branches	No. of Sympodial Branches	Plant Height (cm)	Yield (g)	Ginning Outturn (%age)
1	CEMB 3-2	+	+	60	3.33	4	17	195	87.8	43.96
2	CEMB 11-2	+	+	40	2.86	3	33	105	102.3	39.6
3	CEMB 16-10	+	+	50	2.63	1	12	172	109.3	41.0
4	CEMB 16-15	+	+	60	0.00	4	22	117	88.6	42.0
5	CEMB 17-25	+	+	50	3.45	5	19	170	95.3	38.5
6	Control	-	-	20	20	2	18	190	46.8	38.0

On the basis of the criteria stated above, selection of the best five plants was done. The data pertaining to the best five plants is given in the Table-2. The 1st plant CEMB 3-2 was positive in PCR and Western Dot Blot; it gave 87.8g seed cotton yield and 43.96% GOT; it had 4 monopodial branches, 17 sympodial branches, 195cm height; it showed 3.33% boll damage under natural conditions and killed 60% *Heliothis* larvae in the laboratory bioassay. The 2nd plant CEMB 11-2 was positive in PCR and Western Dot Blot; it gave 102.3g seed cotton yield and 39.6% GOT; it had 3 monopodial branches, 33 sympodial branches, 105cm height; it showed 2.86% boll damage under natural conditions and killed 40% *Heliothis* larvae in the laboratory bioassay. The 3rd plant CEMB 16-10 was positive in PCR and Western Dot Blot; it gave 109.3g seed cotton yield and 41.0% GOT; it had 1 monopodial branch, 12 sympodial branches, 172cm height; it showed 2.63% boll damage under natural conditions and killed 50% *Heliothis* larvae in the laboratory bioassay. The 4th plant CEMB 16-15 was positive in PCR and Western Dot Blot; it gave 88.6g seed cotton yield and 42.0% GOT; it had 4 monopodial branches, 22 sympodial branches, 117cm height; it showed 0.0% boll damage under natural conditions and killed 60% *Heliothis* larvae in the laboratory bioassay. Similarly, the 5th plant CEMB 17-25 was positive in PCR and Western Dot Blot; it gave 95.3g seed cotton yield and 38.5% GOT; it had 5 monopodial branches, 19 sympodial branches, 170cm height; it showed 3.45% boll damage under natural conditions and killed 50% *Heliothis* larvae in the laboratory bioassay.

Third Generation

The progenies of the selected five plants were raised in the green house during winter 2004-05. The plants were again subjected to individual studies and molecular analysis. The progeny plants were analyzed through PCR and Western Blotting. The plants of two progenies viz. CEMB 3-2 and CEMB 16-10 showed 100% positive results in PCR and Western Dot Blot. The plants of three progenies viz. CEMB 16-15, CEMB 11-2 and CEMB 17-25 showed 90% positive results in PCR and Western Dot Blot.

The *Bt* contents were quantified using Image Quant TL software of the Amersham BioSciences (Pvt). The *Bt* content ranged from 0.09% to 0.88% in the CEMB 3-2 plants, from 0.00% to 1.18% in the CEMB 11-2 plants, from 0.26% to 1.35%. in the CEMB 16-10 plants, from 0.00% to 0.83% in the CEMB 16-15 plants and from 0.00% to 0.76% in the CEMB 17-25 plants (Table 3).

The seeds of the positive plants were picked in each descent and bulked at this stage. Since the progenies of the plant nos. 16-10 and 16-15 were identical and also share a common ancestor, the seed of these two lines was bulked jointly. The plants in the 3rd generation were phenotypically similar and were screened at molecular level; there was no need for further selfing. The bulked seed was thus declared to be pure lines and named as CEMB-3, CEMB-11, CEMB-16 and CEMB-17.

Table 3. Bt Protein %age in 3rd Generation Plants 2004-2005. The Bt contents were quantified using Image Quant TL software of the Amersham BioSciences (Pvt). The seeds of the positive plants were picked in each descent and bulked at this stage. Since the progenies of the plant nos. 16-10 and 16-15 were identical and also share a common ancestor, the seed of these two lines was bulked jointly.

	CEMB 3-2	CEMB 11-2	CEMB 16-10	CEMB 16-15	CEMB 17-25
Plant No.1	0.56	1.07	0.53	0.40	0.76
Plant No.2	0.88	1.02	0.32	0.44	0.54
Plant No.3	0.55	0.16	1.35	0.40	0.56
Plant No.4	0.40	1.18	0.26	0.39	0.58
Plant No.5	0.70	0.94	0.27	0.62	0.00
Plant No.6	0.09	0.85	0.32	0.62	0.39
Plant No.7	0.11	0.61	0.49	0.26	0.63
Plant No.8	0.20	0.20	0.39	0.00	0.40
Plant No.9	0.60	1.06	0.33	0.83	0.52
Plant No.10	0.46	0.00	0.30	0.68	0.55
Standard Deviation	0.26	0.44	0.33	0.24	0.20

Fourth and Fifth Generation

Besides included in the field trials, the pure lines mentioned above were further analyzed at molecular level, for two successive generations to eliminate false positive plants and to check for errors in selection. A sample of 30 plants, at random, was taken from each line during every year and analyzed at molecular level through PCR and western dot blot. All plants in both generations were found to be positive in molecular screening.

Most of the transgenic lines were superior as compared to untransformed control variety MNH-93 with respect to average number of bolls, boll damage %age, early in maturing and yield of plant. It is already reported that transgenic lines may be early or late in maturity (Jiang et al., 2000). Possible reason for this morphological variation may be somaclonal variation (Larkin et al., 1981), breakdown of plant genes caused by transgene insertion or insertion mutagenesis (Lijsebettens et al., 1991), pleiotropy or transgene induced endogenous silencing (Matzke et al., 2000).

In the present studies, Bt contents were quantified and expressed as percent of total protein. The *Bt* contents showed variation in successive generations. In the 3rd generation, the *Bt* contents ranged from 0.09% to 1.35% of the total protein whereas in the successive two generations, the *Bt* contents ranged from 0.21% to 0.29% of the total protein. Our findings were in confirmation of the findings of Sachs et al., (1998) who found that *Cry1A* gene expression was variable and strongly influenced by environmental factors. The expression of transgenes in our lines varied in different generations. These results are in agreement with previous studies conducted by Wu et al. (2002), Mahon et al. (2002), Xia et al. (2005), Adamczyk et al. (2009), Bakhsh et al. (2010) and Bakhsh et al. (2011, 2012) who have reported inconsistency in insecticidal gene expression over the crop growth period and in various generations. However, investigation at molecular, genetic, as well as physiological levels should help in understanding the differential expression of transgenes and the quantitative changes in insecticidal proteins in insect resistant cotton plants. Similarly, a varying behaviour of transgenic progeny plants in respect of resistance was recorded laboratory bioassays against *Heliothis* larvae. This has been in confirmation of many research workers. Chunlin et al. (1999) and Zeng et al. (2002) reported that some of the homozygous *Cry1Ac* transgenic rice plants of T_2 progeny showed high level resistance against striped stem borer (*Chilo suppressalis*) at field trial.

The transgenic pure lines expressing insecticidal gene *cry1Ab* were selected and further propagated to attain purity. These lines provided protection against lepidopterans insects throughout the growth period till the harvesting and were desirable for agronomic and morphological characteristics. Based on the molecular and agronomic data of five consecutive years, these transgenic lines proved to be an excellent source of germplasm for an efficient cotton breeding programme.

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