FINAL RESEARCH PROGRESS REPORT

For

PARB's CGS PROJECT NO. 215

Development and commercialization of Cotton Leaf Curl Virus resistant/tolerant indigenous transgenic Bt and Glyphosate resistant cotton hybrids.

Name& Designation of Project Manager: Dr. Saghir Ahmad
Cotton Botanist

Name of Host Institution: Cotton Research Institute, Faisalabad.

(2016)

Table of contents

Basic Information of the Project:

Name of the project	Development and commercialization of Cotton Leaf Curl Virus resistant/tolerant indigenous transgenic Bt and Glyphosate resistant cotton hybrids.
Project period	01. April. 2011 to 31-March-2016
Total project duration	72 months
Total Project cost	Rs. **35.766 Million**
Total Expenditures	Rs. 16.632 Millions
Name of the Project Manager with designation	Dr. Saghir Ahmad Cotton Botanist Cotton Research Station, Multan.
Phone and Email	061-9200337; saghirahmad_1@yahoo.com
Host Institute	Cotton Research Institute, Faisalabad.
Name and Designation of the Team Leader with Name of the Collaborating institute	1. Ch. Muhammad Hanif, Chief Scientist, Four Brothers Seed Corporation Pakistan, Multan 2. Dr. Mehboob Ali, Director Research, Agri Farm Services Pvt. Ltd, Multan 3. Dr. Idrees Ahmad Nasir, Assistant Professor, National Centre of Excellence in Molecular Biology.

Executive Summary

Cotton is a main cash crop of Punjab/Pakistan. It has substantial share of 51% in foreign exchange earnings and thus plays the vital role in the economy of our country. Army worm, Pink Bollworm, American Bollworm and weeds are the main biological factors increasing cost of production and reducing yield of cotton in Punjab. In addition, Cotton Leaf Curl Virus Disease is also one of the impediments constituting limit to cotton production in Punjab. Hybrid cotton has proved its superiority in yield over non Hybrids cotton varieties in India. The present project titled: "DEVELOPMENT AND COMMERCIALIZATION OF COTTON LEAF CURL VIRUS RESISTANT/TOLERANT INDIGENOUS TRANSGENIC BT AND GLYPHOSATE RESISTANT COTTON HYBRIDS" was initiated in 2011 to resolve these issues of cotton production in Punjab. There are four components of this project i.e., Cotton Research Station, Multan, National Centre of Excellence in Molecular Biology (CEMB), Lahore, Four Brothers Seed Corporation Pakistan, Multan, Agri. Farm Services Pvt. Ltd, Multan. This project was funded by Punjab Agriculture Research Board initially for the duration of 60 months (April 2011 to 30 March 2016) with the total cost of Rs. 35.766 million. Later on it was further extended for 12 months without allocation of additional budget. Ten already identified cotton hybrids, 5 from Cotton Research Station Multan and 5 from Four Brothers Seed Corporation Pakistan, Multan were evaluated for yield potential, fiber quality and CLCV tolerance at 10 different locations (Agri Farm, CRI FSD, CRS Multan, CRS BWP, CRS RYK, CRS Vehari, CRS SWL, Kot Chutta, PSC Khanewal and 4B Multan) in 2012 and 2013. Two hybrids, one (H-6) from CRS Multan and one (H-4) from 4B Multan outyielded the other hybrids, and showed an ample amount of CLCV tolerance in evaluation trials. Average yield data of last three years showed that H-6 had an increase of 15% and 36% over standard varieties MNH-886 and Tarzan-1 respectively, while H-4 had an increase of 14% and 35% over standard varieties MNH-886 and Tarzan-1 respectively. Seed of the parental lines of these two hybrids (H-6 and H-3) was handed over to National Centre of Excellence in Molecular Biology (CEMB) in 2012 for the insertion of single cassette of two Bt genes i.e. Cry 1 Ac and Cry 2A for resistance against bollworms and glyphosate tolerant gene (GT) for herbicide resistance. The parental lines of H-6 Hybrid

are MNH-814 and MNH-886, whereas parental lines of H-4 Hybrid comprised of….?. The seed development of the transgenic version of the parental lines was delayed and these were handed over by CEMB in April 2015 to CRS Multan and 4B Multan instead of 2014. Owing to this seed multiplication of transgenic parental lines and development of hybrids was delayed. The seed of transgenic lines was planted in the field for multiplication and evaluation for the newly inserted Bt. and glyphosate resistant genes. The seed of transgenic lines handed over to 4B had poor germination and only a few plants survived due to which multiplication could not been possible. Contrary, the transgenic seed handed over to CRS Multan had good germination and gave rise to a good crop stand. However, a number of segregants were observed which showed susceptibility to glyphosate and bollworms. The production and commercialization of hybrid could not be possible because of variation and segregation observed in the transgenic parental lines. Single plant selections were made among the parental lines on the basis of their tolerance against bollworms and glyphosate, yield potential and fiber trait analysis. CRSP1/E9/5 (MNH-814) and CRSP3/E1/15 (MNH-886) possessed harmonious combination of yield and fiber traits. CRSP1/E9/5 (MNH-814) had yield/plant = 330g, GOT = 41%, Staple length = 30.6 mm, Fiber fineness = 4.2 µg/inch and Fiber strength = 30.9 g/tax; while CRSP3/E1/15 (MNH-886) had yield/plant = 326.3 g, GOT = 36.7%, Staple length = 29.2 mm, Fiber fineness = 4.1 µg/inch and Fiber strength = 29.2 g/tax. It is therefore requested to allow releasing these parental lines as varieties after purification and selection of true to types.

1. Introduction:

Cotton is the leading fibre crop not only in Pakistan but also in the world. It is confronted with various biotic stresses during its life cycle. Among them bollworms like heliothis, spotted and pink are the pests causing colossal damage resulting in 30 to 40 % yield decrease in cotton (Haque, 1991), and 20-60% decrease in market value of fiber (Verma, 1999). Moreover weeds are also among the major threats to sustainable cotton production causing about 40% yield losses. Grasses coupled with broad leaf weeds trim down 15 to 40% yield of cotton crop(Khan and Khan, 2003). Every year pesticides and herbicides amounting to Rs.12 billion are imported to control different pests and weeds in Pakistan and 80% of which is used for cotton (Mohyuddin *et al.* 1997). In Pakistan Bt cotton containing Cry 1AC gene is being cultivated on about 80% of total area of cotton. There are reports of insect resistance in cry 1AC against bollworms. As a result Cry 1 AC is not providing effective defense umbrella against bollworms. A potential problem associated with first generation of transgenic cotton expressing Cry1Ac is the possibility that the insect populations may evolve resistance to this toxin. In contrast to applications with chemical insecticides or with *B. thuringiensis* conventional products, the constitutive expression of the toxin in Bt-cotton allows very few escapes and thus exerts a strong selection pressure on the target population. For this reason, alternatives to Cry1Ac-cotton have been developed, such as Bt-cotton expressing other *B. thuringiensis* genes (a hybrid *cry1Ab/cry1Ac* gene, a *vip3* gene) or a combination of the *cry1Ac* gene with other genes either *cry2Ab* or *cry1F* (James, 2002). Starting in 2002, the first of such second-generation Bt-cotton, producing the Cry1Ac and Cry2Ab toxins, has been approved for commercial planting in Australia (James, 2002). The combined expression of these two toxins not only aims at preserving the effectiveness of Bt cotton in terms of delaying the evolution of resistance but also renders a more effective product against the major pests of this crop by combining the action of the two toxins. Bollworms and weeds problems need to be addressed in achieving satiated production of cotton in Pakistan.

Development of Bt hybrids has opened a new avenue to combat bollworms problem more effectively. According to Layton *et al.* (1997), overall performance of Bt. Cotton was better than conventional varieties. In India during last 6 years (2002 -2008), the area under Bt hybrids has expanded to more than 80% of the total cotton area and the yields

have increased from 302 kg/ha to 567 kg/ha (Karihaloo and Kumar, 2009). In China development and cultivation of Bt hybrids has been gaining much attention because of better yield and fibre quality compared to only Bt cotton and non-Bt hybrids. Vennila *et al.* (2004) found that Bt hybrids, MECH-184 and MECH-162 recorded significantly higher yield over their respective control (Bt variety). Bt cotton provides an alternative approach by replacing insecticides with a toxin within the plant (Annonymous, 2009). The results of field experiment that was conducted at College of Agriculture, Badnapur with three Bt hybrids of cotton viz., MECH 184, MECH 162 and MECH 12 and one non-Bt hybrid deciphered that percent bollworm infestation of squares, buds, flowers, bolls and fruiting bodies were significantly low in Bt hybrids as compared to non-Bt cotton hybrid. The same trend was observed in pink bollworm infestation. (Ilyas *et al*. 2010). Performance of Bt and their corresponding non-Bt cotton hybrids against sucking pests and yield was studied at Research Farm of CCS Haryana Agricultural University, Hisar. The yield of seed cotton was higher in Bt genotypes (MRC-6301, ANKUR-2226 and MRC-6304) than their corresponding non–Bt genotypes (ANKUR-2534, MRC-6304 and RCH-317). (Vijanderpal *et al*. 2010). F1 hybrid exhibited remarkable hetrosis for yield and yield components over Bt varieties **(**WenWu, *et al.,* 2006. Reddy, *et.al,* 2008) observed overall average yields of MECH 12 (1231 kg/acre) and MECH- 184 (1118 kg/acre) against control (non Bt) Bunny (1149 kg/acre) and Satya (1117 kg/acre). They also found that Bt hybrid required 1.5 sprays of bollworms compared to that of 5.3 sprays in case of non- Bt cotton. Dong *et al*., (2004) have estimated 20% increase in yield of Bt hybrids over simple Bt varieties in China. In India**, t**est Bt hybrids *viz*., Ankur Akka BG II,IT 923 Bt, Sudershan Bt, Rudra Bt, VICH 15 Bt, Dhruv Bt and Ankur Jai Bt were found to be superior to the check hybrids under both irrigated and rainfed situations in yield and fibre quality (Annonymous, 2006-07). In another study, Bt hybrids have also exhibited remarkable heterosis of 17.89% for lint yield over the Bt cotton (Annonymous, 2006-07). In India, Bt cotton hybrids viz., MRC 7301 BG II (2095 kg/ha) and Ajeet 11 BG II (1928 kg/ha) recorded highest seed cotton yield with significant superiority over checks, Ankur 651 BG I and NHH 44 (Non-Bt). These hybrids also depicted superior fibre quality. Bt cotton hybrids viz., MRC 7301 BG II, Ajeet 11 BG II, NCS 145 BG I (Bunny BG I), NCS 954 BG I, NCS 207 BG II, MRC 6301 BG I, RCH 144 BG I, RCH 386 BG I, SP 504 BG I and NCS 929 BG I are supportive to farmers to get the better

yield (Phad *et al.* 2010). Patil and Patel (2010) suggested that Bt hybrids viz., PRCH-31, Akka, RCH-2, MRC-6301, HM-322 and PCH-205 exhibited good stability with more responsiveness to seed cotton yield, lint yield, ginning outturn (%), boll weight and number of bolls per plant. Hence, these hybrids are highly adaptable and suitable for cultivation over a wide range of agro climatic conditions.

More over use of transgenic herbicide resistant (Glyphosate) in cotton has gained momentum for management of weeds in the advanced cotton growing countries of the world. Glyphosate resistant cotton is one of the most widely planted transgenic crops, whose resistance is obtained by overproducing CP4-EPSPS derived from the *Agrobacterium* spp. Strain CP4 (Nida *et al* 1996).

The development and commercialization of CLCV tolerant Bt hybrids with herbicide resistance on large scale will help Pakistani cotton growers not only protecting their crop against bollworms attack and weeds but also increase their net income. This technology offers promise of other benefits associated with reduction of broad spectrum pesticides/herbicides and conservation of natural enemies of bollworms, reduce soil and water contamination and health benefit to farm workers who would come in lesser contact with pesticides.

2. Project Objective:

Development & commercialization of boll worms and Glyphosate herbicide resistant transgenic cotton hybrids with Cotton Leaf Curl Virus resistance/ tolerance and desirable fiber traits.

Component-1 (Cotton Research Station, Multan)

Development and seed production of cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield/quality than the standard varieties

Component-2 (Four Brother Seed Corporation Multan)

Development, seed production and testing of cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield/quality than the standard varieties

Component-3 (Agri Farm Services)

Large scale multi-location evaluation of 10 hybrids from the combined list.

Component-4 (CEMB Lahore)

Transformation of parent lines of two potential F_1 cotton hybrids with GT Gene and CEMB-02 genes (Cry1Ac + Cry2Ab) and their Bio safety studies.

Outputs planned for the project: (As per project document)

Output/ Activity	**Description**	**Planned Completion date**	**Achievement Indicator as planned**	**Achievements** (Please attach data in brief as annexure if activity completed)	**Reasons for deviation if any**
Overall Project Objective	**Development & commercialization of boll worms and glyphosate herbicide resistant transgenic cotton hybrids with Cotton Leaf Curl Virus resistance/tolerance and desirable fibre traits.**	**31.03. 17**	**Two cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield than the standard varieties will be available.**	**Completed**	
Component -1 (Cotton Research Station, Multan)					
Component-1 objective	**Development and seed production of cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield/quality than the standard varieties.**	**31.03.17**	**Two hybrids resistant to boll worms and Glyphosate and with better/equal yield/quality than the standard varieties will be available.**	**Completed**	
Output-1	**Seed production and development of 5 hybrids better/equal than the existing varieties in yield and quality.**	**31.03.17**	**Availability of seed of 5 hybrids for large scale evaluation.**	**Completed**	
Activity-1	Seed production and development of 5 hybrids better/equal than the existing varieties in yield and quality.	31.12.11	Availability of 5 kg seed of each of the 5 hybrids for large scale evaluation.	Completed	

Activity-2	Seed production of 2 selected hybrids.	31.12.13 31.12.14 31.12.15 31-12-16	Availability of 5 kg seed of each of the 2 hybrids for large scale evaluation.	Completed	
Activity-3	Seed production of parental lines	31.12.11 31.12.12 31.12.13 31.12.14 31.12.15 31-12-16	Seed of parental lines enough to supply to CEMB and AGRI seed Farm Services and station use.	Completed	
Activity-4	Acquisition of Hybrid seed production Technology.	31.12.11	Training of one scientist on latest hybrid seed production technology from abroad	Completed	
Output-2	**Evaluation of 10 hybrids for quality traits.**	**31.12.13**	**Data on quality traits for 10 hybrids.**	**Completed**	
Activity-1	Evaluation of 10 hybrids for quality traits.	31.12.13	Data on fibre quality traits for 10 hybrids.	Completed	
Activity-2	Handing over seed of parental lines of top 2 hybrids to Agri Farm Services for hybrid seed production.	31.12.12	Record of handing over of 2 kg seed of each of the parental lines of 2 selected hybrids.	Completed	
Activity-3	Handing over seed of parental lines of top two hybrids to CEMB for insertion of Bt double genes and GT gene. Record of handing over of 5 kg seed of each of the parental lines of two selected hybrids.	31.12.12	Record of handing over of 5 kg seed of each of the parental lines of two selected hybrids.	Completed	
Activity-4	Receipt of transgenic version of	31.3.15	Record of receipt of parental lines of	Completed	

	parental lines of two selected hybrids from CEMB.		two selected hybrids.		
Activity-5	Seed multiplication and testing varietal integrity of transgenic version of parental lines of two selected hybrids in greenhouse/field.	31.3.15 31.12.14 31.12.15 31-12-16	At least one kg seed of each of the parental lines received from the CEMB	Partially Completed	
Activity-6	Final Report Writing	31-3-17		Completed	
Component-2 (Four Brother Seed Corporation Multan)					
Component-3 objective	**Development, seed production and testing of already identified five best hybrids and multi locations testing of ten pooled**	**31.3. 16**	**Two cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield than the standard varieties.**	**Completed**	
Output-1	**Seed production and development of 5 hybrids better/equal than the existing varieties in yield and quality.**	**31.3. 16**	**Two cotton hybrids resistant to boll worms and Glyphosate and with better/equal yield than the standard varieties.**	Completed	
Activity-1	Seed production and development of 5 hybrids better/equal than the existing varieties in yield and quality.	31.12.11 31.12.12	Availability of 5 kg seed of each of the 5 hybrids for large scale evaluation.	Completed	
Activity-2	Seed production of 2 selected	31.12.13 31.12.14 31.12.15	Availability of 5 kg seed of each of the 2 selected hybrids for large scale evaluation.	Completed	
Output-2	**Evaluation of 10 pooled hybrids for their yield**	**31.1.12**	**Data on yield and agronomic performance at 10 locations for 10 hybrids.**	Completed	
Activity-1	Evaluation of 10 pooled hybrids for its yield and agronomic	31.1.12	Data on yield and agronomic performance at 10 locations for 10	Completed	

	performance.		hybrids.		
Activity-2	Handing over seed of parental lines of top two hybrids to CEMB for insertion of Bt double genes and GT gene.	31.12.12	Record of handing over of 5 kg seed of each of the parental lines of two selected hybrids.	Completed	
Activity-3	Receipt of transgenic version of parental lines of two selected hybrids from CEMB.	31.3.14	Record of receipt of parental lines of two selected hybrids.	Completed	
Activity-4	Seed multiplication and varietal integrity testing of transgenic version of parental lines of two selected hybrids in greenhouse/field.	31.12.14 31.12.15	Data on varietal integrity of transgenic lines and at least one kg seed of each of the parental lines received from the CEMB	Partially Completed	
Activity-5	Seed production of 2 transgenic hybrids.	31.12.14 31.12.15	5 kg hybrid seed of each of the transgenic hybrid	Partially Completed	
Activity-6	Evaluation of 2 non transgenic hybrids for its yield and agronomic performance.	31.12.13	Data on yield and agronomic performance at 10 locations for 2 hybrids.	Completed	
Output-3	**Demonstration, Field days.**	**31.1.16**	**Record of demonstration and field days.**	Completed	
Activity-1	Demonstration of best 2 hybrids in core and non-core cotton area.	31.1.16	Record of demonstration and field days at 10 locations.	Partially Completed	
Component -3 (Agri Farm Services)					
Component-2 objective	**Large scale multi-location evaluation and commercialization of hybrids.**	**31.3.17**	**Data on yield and agronomic performance at 10 locations for 10 hybrids.**	Completed	
Output-1	**Large scale multi-location evaluation of 10 hybrids.**	**31.1.16**	**Data on yield and agronomic performance at 10 locations for 10 hybrids.**	Completed	
Activity-1	Evaluation of 10 pooled hybrids	31.1.14	Evaluation of 10 pooled hybrids for its	Completed	

	for its yield and agronomic performance..		yield and agronomic performance.		
Activity-2	Evaluation of 2 non transgenic hybrids for its yield and agronomic performance.	31.12.13 31.12.15	Data on yield and agronomic performance at 10 locations for 2 hybrids.	Completed	
Activity-3	Production of seed of 2 selected hybrids.	31.12.13 31.12.15	Availability of 05 kg seed of the each hybrid	Partially Completed	
Activity-4	Seed production of 2 transgenic hybrids.	31.12.14 31.12.15 31.12.16	5 kg hybrid seed of each of transgenic hybrid.	Not Completed	Segregation has been observed in parental lines whereas hybrid seed production requires pure lines/ inbred lines.
Output-2	**Demonstration, Field days.**	**31.1.17**	**Record of demonstration and field days.**	Partially Completed	
Activity-1	Demonstration of best 2 hybrids in core and non-core cotton area.	31.1.17	Record of demonstration and field days at 10 locations.	Partially Completed	
Component-4 (CEMB Lahore)					
Component-4 objective	**Transformation of parent lines of 2 potential F1 cotton hybrids with GTG and CEMB-02 genes (Cry1Ac + Cry2A) and their Bio safety studies**	**31.3.16**	**Transformed parental lines of 2 potential F1 cotton hybrids with GTG and CEMB-02 genes (Cry1Ac + Cry2A) and their Bio safety studies**	Completed	
Output-1	**Acquisition of advanced GMO detection Technology.**	**31.12.11**	**Training of one scientist on latest GMO detection technology from abroad (China / USA/Europe).**	Completed	
Activity-1	Acquisition of advanced GMO detection Technology.	31.12.11	Training of one scientist on latest GMO detection technology from abroad (China / USA/Europe).	Completed	
Output-2	**CEMB-Bt and GT Gene**	**31.3.14**	**Parental lines of 2 cotton hybrids**	Completed	

	transformation in selected parental lines of 2 best cotton hybrids.		**containing herbicide resistant gene and double Bt gene will be available.**		
Activity-1	Transformation of selected parental lines by Agrobacterium with CEMB-Bt and GTGene (at least 100 events) in appx.1000 embryos per parent.	30.11.13	100 transformed embryos per parent will be available. 30.11.13	Completed	
Activity-2	Regeneration of shoots and roots of Putative transgenic cotton plants on selection medium.	31.12.13	100 regenerated cotton plants from transformed embryos of each parent will be available.	Completed	
Activity-3	Raising of T0 generation in the green house	15.1.14	Seed of 10 selected transgenic plants	Completed	
Activity-4	Raising of T1 generation in the green house/tunnel	31.3.14	Seed of 10 homozygous transgenic plants	Completed	
Activity-5	Varietal integrity of transgenic parental lines.	31.3.14	Data on varietal integrity of transgenic parental lines.	Completed	
Activity-6	Cotton seed delivery to Cotton Research station/Four Brothers Seed containing CEMB-Bt and GTGene for hybrid seed development.	31.3.14	Seeds of 10 plants of each of the insertion events of each line will be supplied.	Completed	
Output-3	**Confirmation of transgenic cotton lines via molecular analyses and bioassays.**	**31.1.16**	**Transgenic cotton lines will be available.**	Completed	
Activity-1	Bioassay, ELISA and PCR analysis of transgenic plants during shoot regeneration, in-vitro multiplication, green house evaluation, field trials.	30.5.13 15.11.14 6.6.15	Confirmation of Integration of transgenes in plant genome, their multiplication and efficacy of Bt and GTGene in transgenic plants.	Completed	

Activity-2	FISH analysis of plants during in-vitro multiplication, green house evaluation, field trials.	31.3.14	Copy number of gene/ genome and multiplication of transgenic plants	Completed	
Output 4	**Collection of root and shoot samples from plants other than cotton from the transgenic fields and surroundings.**	**31.08.15**	**Sample will be available.**	Completed	
Activity 2	Analysis of samples collected to evaluate the presence of CEMB-Bt and GTGene protein.	31.3.16	Data for presence of CEMB-Bt and GTGene in samples collected will be available.	Completed	
Activity 3	Studies on protein expression in root exudates of transgenic plants	31.3.16	Data for presence/absence of proteins of transgenes in the water will be available	Completed	
Activity 4	Study of effect of CEMB-Bt and GTGene protein on Animals (Mice)	31.3.16	Data for effect of CEMB-Bt and GTGene on Animals (Mice) insects will be available.	Completed	
Activity 5	Report Writing	31.3.16		Completed	

3. Detailed component wise methodology adopted, data analyzed and results obtained (Attach raw data as annexure)

Component -1 (Cotton Research Station Multan)

a) Seed Production and Development of 5 hybrids better/equal than the existing varieties in yield and quality.

Plant material

Plant material comprised of six genotypes (MNH-814, MNH-909, MNH-886, CRSM-38, MNH-988 and MNH-809) of CRS Multan and one variety of CRS Vehari (VH-232).

Crossing program

The crosses were made using these seven in diall fashion. So produced 42 crosses were evaluated for yield trials at CRS Multan.

Table . Crossing plan

	MNH-814	MNH-909	MNH-886	CRSM-38	MNH-988	MNH-809	VH-232
MNH-814	-	×	×	×	×	×	×
MNH-909	×	-	×	×	×	×	×
MNH-886	×	×	-	×	×	×	×
CRSM-38	×	×	×	-	×	×	×
MNH-988	×	×	×	×	-	×	×
MNH-809	×	×	×	×	×	-	×
VH-232	×	×	×	×	×	×	-

Selection of superior hybrids

Yield trials for these 42 hybrids were conducted at CRS Multan to evaluate their yield potential. Based on their yield performance fiber quality analysis and CLCV tolerance, five best hybrids (H6-H10) were selected. Parentage of these selected hybrids is given as under:

Hybrid Name	Parentage
H-6	MNH-814 × MNH-886
H-7	MNH-909 × MNH-886
H-8	VH-232 × MNH-909
H-9	MNH-456 × MNH-886
H-10	MNH-886 × MNH-456

Evaluation of selected hybrids

The already selected 5 hybrids were used as a base material for this project. Year wise evaluation of these hybrids is given as under:

- **2011-2012→**
- Five already selected hybrids, on the basis of their cross-ability, hybrid seed obtained, yield performance, CLCuV and heat tolerance of the parents, were planted in the field in 2011 along with their parental lines. 8 kg pure and self-seed of 5 parental lines was produced during 2011. For multiplication of hybrid seed, manual emasculation and pollination of flower buds of parental lines was carried out throughout the flowering season. A total of 84064 were attempted for the above mentioned purpose. High temperature caused massive shedding of emasculated flowerer buds and rottening of crossed bolls due to heavy rains in September ultimately it resulted in production of less quantity of hybrid seed than planned. Seed of these 5 hybrids was made available for further evaluation in 2012 and 2013. (Table.1)
- **2012-2013→**
- Seed multiplication of parental lines and 5 hybrids was also continued in the 2nd year of the project. Enough amount of seed of parental lines was produced and handed over to CEMB on 28-01-2013 vide letter no. 74 for development of transgenic lines, while to Agri Form Seed Services on 26-03-2013 vide letter no. 247 for production of hybrid seed. Other than this 10 hybrids (five from CRS Multan and 5 from 4B Multan) alongwith two checks (MNH-886 and Tarzan-1) were also planted at six locations instead of 10 due to of low quantity of seeds for evaluation trials. Two hybrids H-6 & H-7 of CRS Multan showed better performance over two standards (Tarzen-1, MNH-886). Hybrid-6 showed 4.46% and 25.2 % increase of seed cotton yield over MNH-886 and Tarzen-1 respectively, whereas H-7 exhibited 11.5 % and 33.72% increase over the standards respectively for the same trait (Table- 2). The staple length of both hybrids was higher than that of both the standards.
- **2013-2014→**

  The 10 selected hybrids along with two standards MNH-886 and Tarzan-1 were retested for the second year in 2013 at 10 locations in Punjab province. Data of 10 locations showed that the two hybrids of Cotton Research Station Multan, H-6 and H-7 performed significantly better than those of the standards both in terms of seed cotton yield and fiber traits. Hybrid-6 showed 44.71% and 10.66 % increase of seed cotton yield over Tarzen-1and MNH-886 respectively, whereas H-7 exhibited 54.83 % and 18.41% increase over the standards respectively for the same trait (Table 4-13). The

staple length, fiber strength and fiber fineness of H-6 was 28.3 mm, 36.4 g/tex and 4.7 µg/inch respectively whereas H-7 also possessed the harmonious combination of fiber traits (staple length = 28.4 mm, fiber strength = 35.9 g/tex and fiber fineness = 4.1 µg/inch). Both hybrids have better fineness than the standards but staple length of two hybrids is better than that of Tarzan-1 and at par with MNH-886. H-7 of CRS Multan was found the most tolerant hybrid against CLCuV. Moreover, 5 kg seed of two selected hybrids H-6 and H-7 was also produced.

CEMB Lahore had to provide the transgenic version of parental lines by 31 March 2014 but they could not provide it till the proposed date. As a result no seed multiplication and testing could be possible.

- **2014-2015→**

After two years evaluation of the 5 hybrids of CRS Multan at 10 locations two hybrids H-6 and H-7 were selected finally on the basis of higher seed yield, high CLCV tolerance and harmonious combination of fiber traits. A total of 4 hybrids along with two standards MNH-886 and Tarzan-1 were tested during 2014 at 8 locations in Punjab province. Two hybrids were developed by Cotton Research Station Multan and two by Four-Brother Seeds. Data of 8 locations showed that the two hybrids of Cotton Research Station Multan, H-6 and H-7 performed significantly better than those of the standards in terms of CLCuV tolerance, seed cotton yield and staple length. H-6 with 3815 kg/ha out yielded the check Tarzan-1 and MNH-886 by 24 % and 15% respectively while H-7 gave 3679 kg/ha as with an increase of 20% and 11% over Tarzan-1 and MNH-886 respectively. H-6 and H-7 showed CLCuV incidence of 40.0 % and 45.0 % respectively as compared to standard Tarzan-1 and MNH-886, 90 % and 70.0% respectively. Staple Length of H-6 was 28.7 (mm) which is better than both the checks, whereas H-7 had showed the lowest value of staple length (26 mm) (Table 4).

Moreover, 5 kg seed of two selected hybrids H-6 and H-7 was also produced. Seed multiplication of parental lines and hybrids could not be done because the transgenic parental lines were not supplied by CEMB, Lahore even at the end of 2015. Only fibre testing of Parental lines as well as two hybrids was done during the period under report.

- **2015-2016→**

The seed of the transgenic version of the parental lines (MNH-886 and MNH-814) got delayed and was handed over by CEMB in April 2015 to CRS Multan and 4B Multan instead of 2014. Only 200 gm seed of each two transgenic line was provided by CEMB. The seed of transgenic lines was planted in the field for multiplication and

evaluation for the newly inserted Bt. and glyphosate resistant genes. The seed of transgenic lines handed over to 4B had poor germination and only a few plants survived due to which multiplication could not been possible. Contrary, the transgenic seed handed over to CRS Multan had good germination and gave rise to a good crop stand. 10 kg seed of MNH-886 and 4.4 kg seed of MNH-814 was produced at Cotton Research Station Multan. The morphological data showed that no attack of Armyworm, Spotted bollworm and American bollworm infestation was observed except pink boll worm with average infestation of 30.5 % on MNH-886 and 40.6% in MNH-814 (Table-7). Variations were observed in all events of each transgenic parental line as mortality in plants was observed after Glyphosate spray. Therefore further selection and purification of parental lines is required. The production and commercialization of hybrid could not be possible because of variation and segregation observed in the transgenic parental lines. It is therefore requested to allow releasing these parental lines as varieties after purification and selection of true to types. In crop season 2016-17 four plants, two from each parent, with the highest seed cotton yield and harmonious combination of fiber traits have been selected. CRSP1/E7/9 (MNH-814) had yield/plant = 329.3g, GOT = 34%, Staple length = 28.4 mm, Fiber fineness = 3.6 µg/inch and Fiber strength = 29.4 g/tax; CRSP1/E9/5 (MNH-814) had yield/plant = 330g, GOT = 41%, Staple length = 30.6 mm, Fiber fineness = 4.2 µg/inch and Fiber strength = 30.9 g/tax; while CRSP3/E1/15 (MNH-886) had yield/plant = 326.3 g, GOT = 36.7%, Staple length = 29.2 mm, Fiber fineness = 4.1 µg/inch and Fiber strength = 29.2 g/tax and CRSP3/E4/4 (MNH-886) had yield/plant = 197.0 g, GOT = 37.6%, Staple length = 29.2 mm, Fiber fineness = 3.9 µg/inch and Fiber strength = 29.4 g/tax (Table 11).

Component -2 (Four Brothers Seed Corporation Pakistan)

Development of F1 Hybrids

It involved three steps as;

(i) Identification of best 5 hybrids
(ii) Seed Production of the F1 Hybrids
(iii) Testing and evaluation of hybrids

Identification of best 5 hybrids

As a first step fifty one (51) F1 hybrids were tested in replicated trial during winter (Ist January- 31st May 2011) at Four Brothers Seeds Research Centre, Multan. Out of these 51 combinations 9 hybrids were identified as significantly higher yielder than the standard check cotton varieties Tarzan-1 and MNH886. Out of these 51 F1 hybrids only 5 hybrids H-1, H-2, H-3, H-4 and H-5 were short listed keeping in view their field performance and fiber qualities.

Seed Production of the 5 F1 Hybrids

These five hybrids were reconstituted in 2012 for retesting and reconfirmation of their yield performance during the next crop season. As per plan 5 kg F_1 Seed of each best identified hybrid were to be produced but because of high temperatures & rains during crossing period boll setting was lower, therefore the less seed was produced than the desired quantity. Details of quantity of seed produced given in below table-1.

Testing and evaluation of 10 hybrids

The selected five hybrids (H-1 to H-5) along with five hybrids of CRS Multan (H-6 to H-10) and two standard varieties Tarzan-1 and MNH-886 were evaluated in the field in 2012 at six locations and 2013 at ten locations. Owing to low quantity of seed, these 10 hybrids were tested only at six locations instead of 10 locations in 2012. Seed multiplication of 5 hybrids of 4B along with the parental lines was also continued side by side. In both the years' two hybrids, H-3 and H-4, from 4B performed exceptionally well as compared to the standard varieties. In 2012, H-3 produced seed yield of 3062 kg/ha with an increase of 25% over check variety Tarzan-1 and 4% over MNH-886, while H-4 yielded 3145 kg/ha of seed cotton with an increase of 29% over Tarzan-1 and 7% over MNH-886. Yield data of 10 locations in 2013 showed that H-3 produced seed yield of 3328 kg/ha with an increase of

53% over Tarzan-1 and 17% over MNH-886, while H-4 produced the highest seed yield of 3459 kg/ha with an increase of 59% over check variety Tarzan-1 and 22% over MNH-886.

A set of four best hybrids two (H-3, H-4) developed by Four Brothers Seeds and two (H-6 & H-7) by Cotton Research Station Multan were further evaluated with two checks in 2014. H-3 gave 23% and 14% higher seed yield than that of Tarzan-1 and MNH-886 respectively, while H-4 was the highest yielding hybrid with seed yield of 4040 kg/ha showing an increase of 31% and 22% over Tarzan-1 and MNH-886 respectively.

Keep in view of three years (2012-2015) yield, fiber qualities and other characters of economic importance H-4 of 4B and H-6 of CRS Multan were found the best hybrids. Parent seed of these four hybrids were supplied to CEMB for incorporation of genes. CEMB returned back to us transgenic form of these parents after one year delay than the scheduled date. In addition the transgenic seed was in small quantity and also displayed poor germination that further worsened the situation. These transgenic lines were planted for hybrid seed production and to see their genetic stability with uniformity as their parental non transgenic version, unfortunately it was observed that all transgenic parental lines started segregation. True breeding parental lines are the basic requirement for hybrid seed production in cotton. These could not be used for hybrid seed production as per objectives of this project. However, PARB authorities were kind enough to grant one year extension in the project. Till the current crop season the second generation of parental lines is still showing segregation.

Component -3 (Agri Farm Services Pvt. Ltd, Multan)

Agrifarm services was the fourth component of the project. Its mandate was large scale evaluation of 5 hybrids from 4B and 5 hybrids from CRS Multan at 10 different locations in Punjab and commercialization of the best hybrids. Ten non transgenic hybrids were evaluated for the consective two years at different locations and four best hybrids (two from 4B and two from CRS Multan) were selected. These four hybrids were further evaluated and two best hybrids (H-4 from 4B and H-6 from CRS Multan) were selected on the basis of their high yield potential and tolerance against CLCV. The parental lines of these two hybrids were handed over to CEMB for insertion Bt + glyphosate resistant genes. The transgenic seed of parental lines of these hybrids was handed over to 4B and CRS Multan in 2015. These lines showed segregation in terms of bollworm and glyphosate resistance. For the exploitation of heterotic potential of any breeding material availability of homozygous inbred lines is a pre requisite. As the inbred lines in this case were not truly homozygous therefore seed production, multiplication and commercialization of so selected hybrids was not possible to carry out even after one more year,s extension. It will take extra time and budget to for purification of inbred lines and production of transgenic hybrids.

Component -4 (National Centre of Excellence in Molecular Biology (CEMB), Lahore)

Transformation of parent lines of four potential F_1 cotton hybrids with GTG and CEMB-02 genes (Cry1Ac + Cry2A) and their Bio safety studies

Four cotton varieties FBS-37, M-1, CRSP-1 and CRSP-3 were transformed with Cry1Ac + Cry2A along withGTGene. The seeds of cotton varieties were collected from cotton research station Multan (Pakistan). Concentrated H2SO4 was used for delinting while sterilization of seeds was done with 5% HgCl2 and 10% SDS. Seeds were then allowed to germinate at 30 °C incubator overnight.

CEMB-Bt and GTGene transformation in selected parental lines of four best cotton hybrids

Cry1Ac + Cry2A and cp4EPSPS gene were transformed in CRSP-1 and CRSP-2 according to Rao et al. 2011. Two constructs having Bt and weedicide gene were used under CAMV35s promoter and NOS terminator for genetic transformation through Agrobacterium method of transformation. The genus of Agrobacterium has been divided into a number of species based on its disease symptomology and host range.*Agrobacterium tumefaciens* causes crown gall disease, *Agrobacterium rhizogenes*causes hairy root disease and a new species *Agrobacterium vitis* causes galls on grapes and a few other plant species. The host range of Agrobacterium is extensive. As a genus, Agrobacterium can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot angiosperm species and gymnosperms. The most widely used species in plant transformation is *A. tumefaciens*. *A. tumefaciens* is a naturally occurring soil borne pathogenic bacteria that causes grown gall. After transfer, T-DNA becomes integrated into the plant genome and its subsequent expression leads to the crown gall phenotype. There are two bacterial genetic elements required for TDNA transfer to plants. The first element is the T-DNA border sequence that consists of 25 bp direct repeats flanking defining the T-DNA. The borders are the only 12 sequences required in cis for T-DNA transfer. The second element consists of the virulence (vir) genes encoded by Ti Plasmid in a region outside of the T-DNA. The vir genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome. Transgenic plants generated through Agrobacterium were screened on kanamycin antibiotic selection at the application rate of 50 mg/L of medium for 1.5 month, putative transgenic plants were shifted on selection free medium for shoot and root generation as done by Muzaffar et al. 2015.

Acclimatization

Two-month old putative transgenic plants were shifted to pot from tubes and were exposed to light for 15 min at the first day and then 15 min increases onward up to one month daily. During acclimatization plants were exposed from 10 a.m. onwards and were daily watered

Transgenic analysis:

Various techniques such as ELISA, PCR, Southern Blot, Florescent in situ hybridization (FISH) and Bioassays were employed to confirm the integration and successful expression of transformed Bt and GTGene in putative transgenic plants.

Confirmation of transgenic plants through PCR

Genomic DNA from putative transgenic plants was isolated according to Lenin et al. 2016. Reaction mixture having 100 ng DNA (2 µl), 10X PCR buffer(2 µl), 2.5 mM MgCl2 (2 µl), 1 mM dNTPs (2 µl) one picomole each primer(2 µl) and 2.5 U Taq DNA polymerase for a total volume of 20 µl was prepared with gene specific primers shown in Table 1. The reaction was proceeded in ABI 9700 thermocycler having following conditions, initial denaturation at 94 °C for 5 min then 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for Cry2A and GTG while 50 °C for Cry1Ac for 1 min followed by extension at 72 °C for 3 min. After amplification products were resolved on 1% agarose gel and visualized by ethidium bromide staining.

Table 1.Primers with sequences used in this project

Primer name	Sequence (5′–3′)	Product size
Cry2A-F	AGATTACCCCAGTTCCAGAT	500bp
Cry2A-R	GTTCCCGAAGGACTTTCTAT	
GTG-F	CCCTGGTGACAAGTCCATCT	800bp
GTG-R	CTGCACACCCATCTCTCTGA	
Cry1Ac-F	ACAGAAGACCCTTCAATATC	1Kb
Cry1Ac-R	GTTACCGAGTGAAGATGTAA	

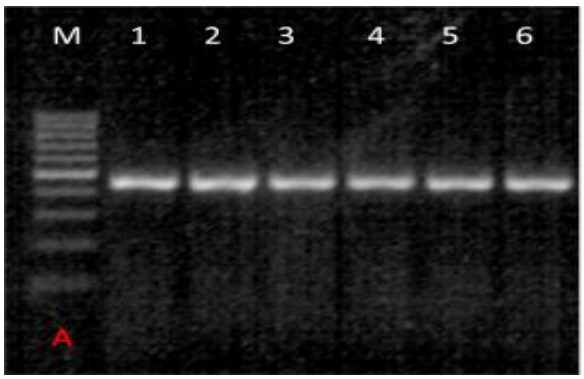

Figure. Confirmation of cry1Ac through PCR amplification. Gene Specific primers were used for PCR amplification: M-1 kb ladder, 1–6 positive plants for Cry1Ac.

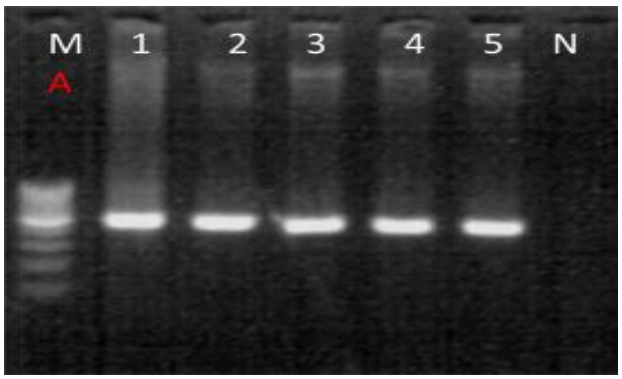

Figure. A: Cry2A 500 bp amplification with gene specific primers. M-100 bp ladder, 1–5 Cry2A positive, N—negative control

Enzyme linked immune sorbent assay (ELISA)

Envirologix Kit (cat# 051) was used for the enzyme linked immune sorbent assay of Cry1Ac, Cry2A and GT gene expression. One gram leaves samples from transgenic cotton plants were subjected to grinding and total crude protein was isolated by using protein isolation buffer (0.5 M EDTA, Glycerol, 5 M NaCl, 2 M Tris–Cl, NH_4Cl, PMSF, DTT (Dithiothreitol).

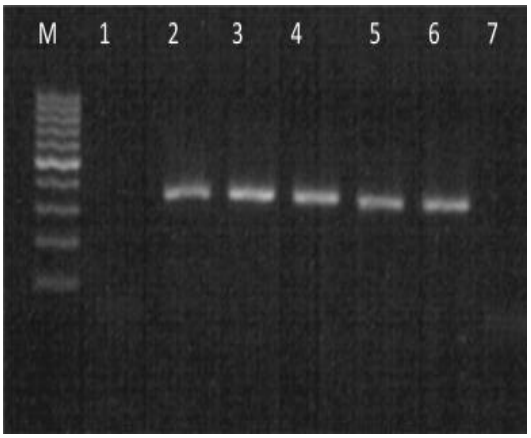

Figure. Glyphosate gene (350 bp) amplification with gene specific primers. M-1 kb plus DNA ladder, 1- negative control, 2–6 positive GTG transgenic plants, 7- GTG negative plant

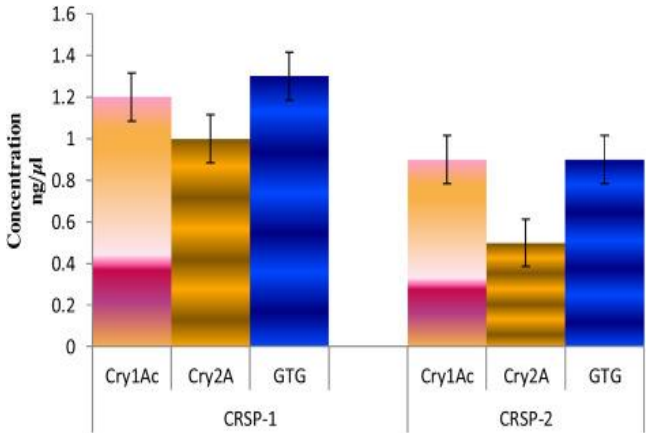

Figure: Graphical representation of quantification of Cry1Ac, Cry2A and GTG for both cultivars each representation is the average of three plants.

Fluorescence in situ hybridisation (FISH)

The PCR-based best positive transgenic plants of FBS-37, M-1, CRSP-1 and CRSP-3 were subjected to Fluorescence in situ hybridization (FISH) for determination of copy number according to the procedure described by Rao et al. 2011. Mirus Label IT® FISH Cy3 Kit (cat# MIR6510, MJS Bio Lynx Inc., P.O. Bag 1150, 300 Laurier Blvd. Brockville, ON K6V 5W1, Canada) was used for labeling of probes. Chromosomes from growing root tips were prepared and were hybridized with the specific probe. The fluorescent microscope (Carl Zeiss AXIO100) was used for the detection of fluorescent signals using appropriate filter set. The CCD camera was employed for capturing fluorescent signals and analyzed by using Genus 3.7 software provided by Cytovision Applied Imaging System. The same software package was utilized for karyotyping.

The best transgenic plant with better protein expression of Cry1Ac were subjected to FISHfor determining the copy number of the gene as well as a location of the gene on a chromosome. This analysis is very much important because copy number and the position of a gene on chromosomes are directly related with better expression and ultimately better control against insects.

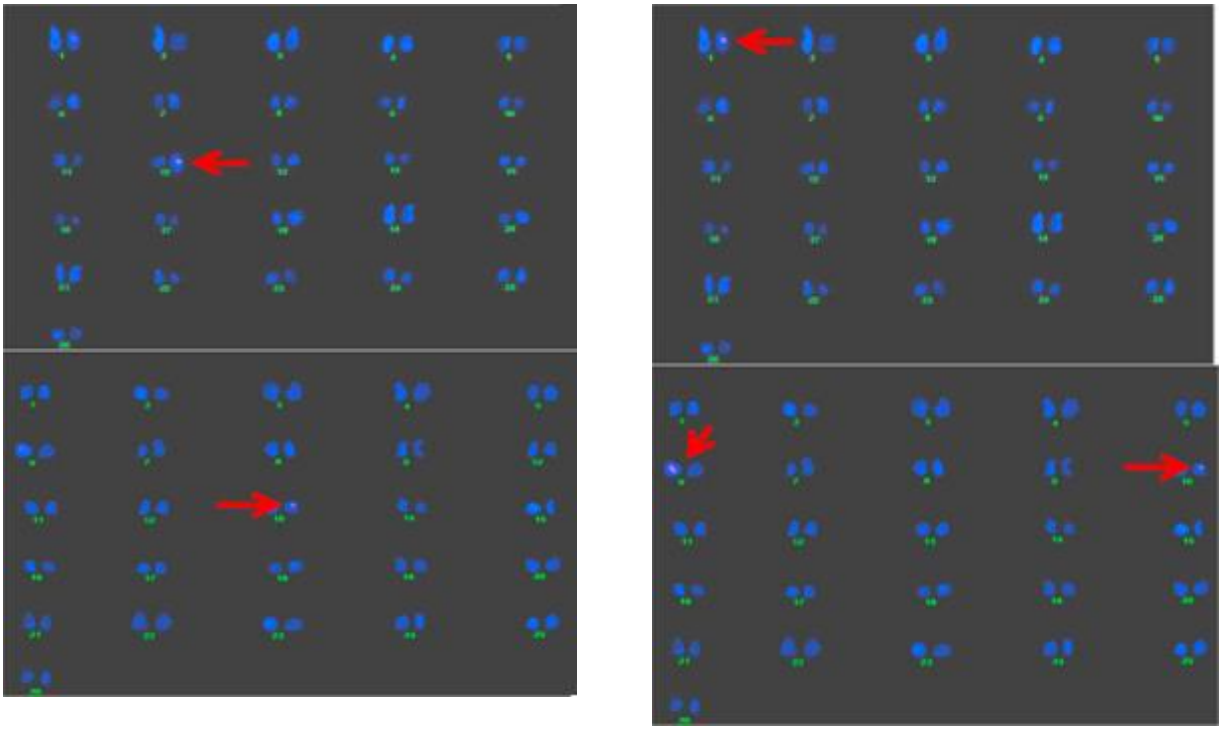

Figure: Copy no and location of Bttransgene of Cotton varieties FBS-37, M-1, CRSP1 and CRSP-3 respectively in figure A, B, C and D. Arrow determined the fluorescent signal.

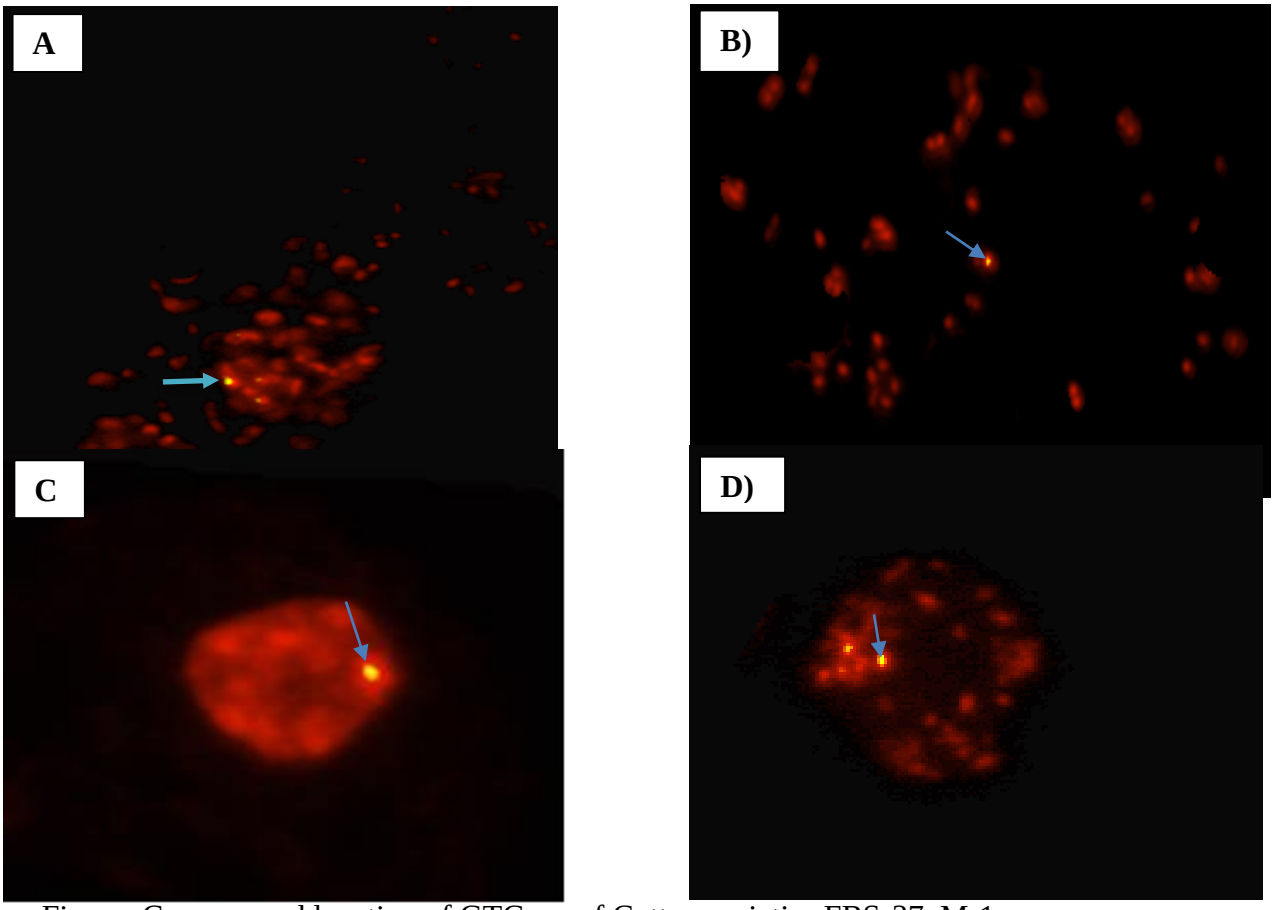

Figure: Copy no and location of GTGene of Cotton varieties FBS-37, M-1, CRSP1 and CRSP-3 respectively in figure A, B, C and D. Arrow determined the fluorescent signal.

Southern Blot Analysis for CEMB Construct out of PCR positive cotton plants

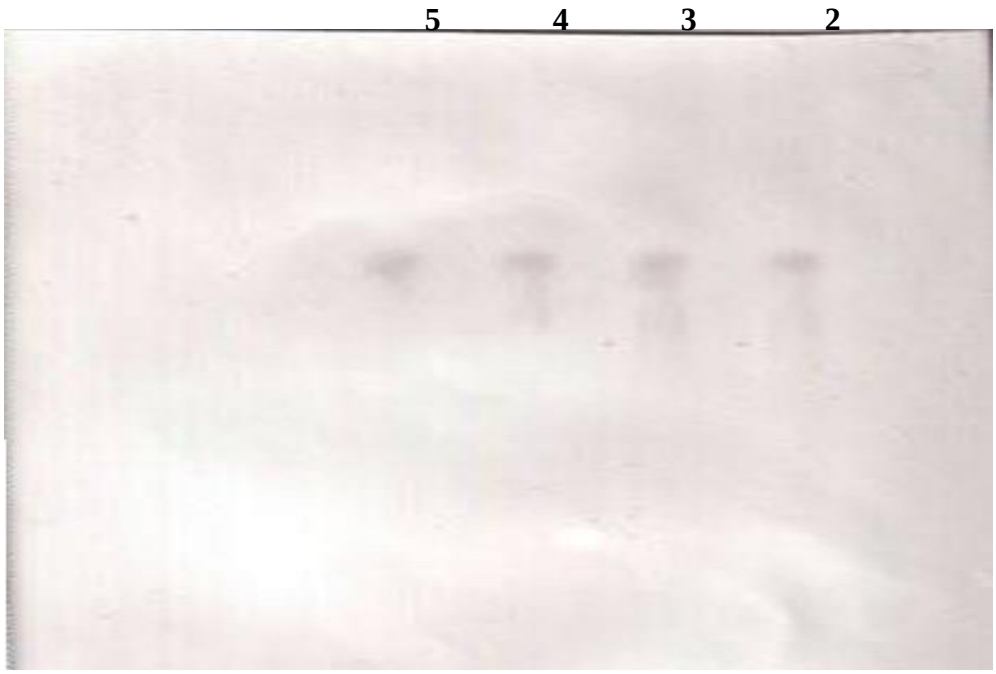

Figure: Southern Blot Analysis of Bt positive transgenic cotton plants.1: Negative plant, 2: FBS-37, 3: M-1, 4: CRSP-1, 5: CRSP-3

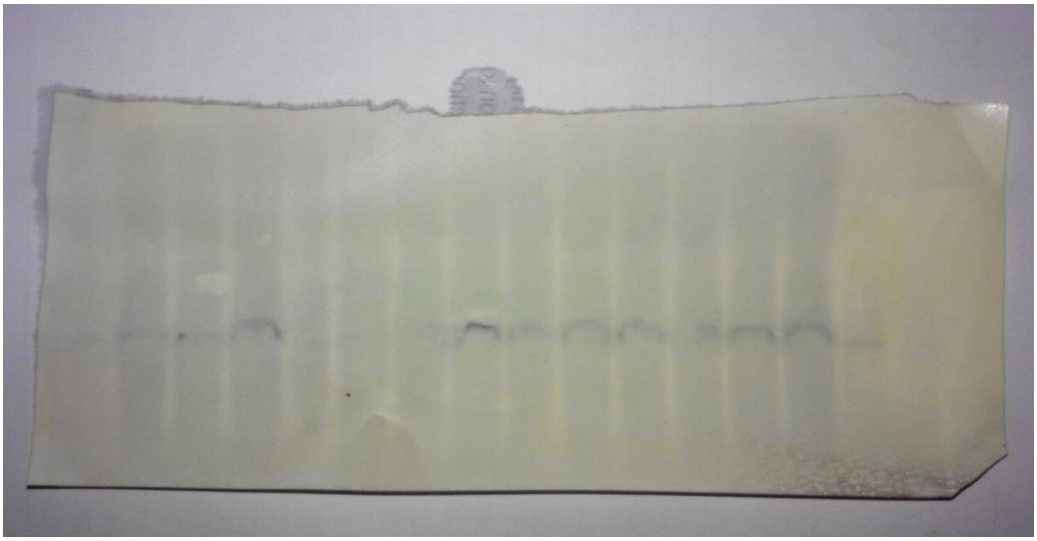

Figure: Southern Blot Analysis ofGTGene positive transgenic cotton plants.1: Negative plant, 2-4: FBS-37, 5-7: M-1, 8-10: CRSP-1, 11-13: CRSP-3, 14: Control Positive

Southern Blot for Cry2A gene

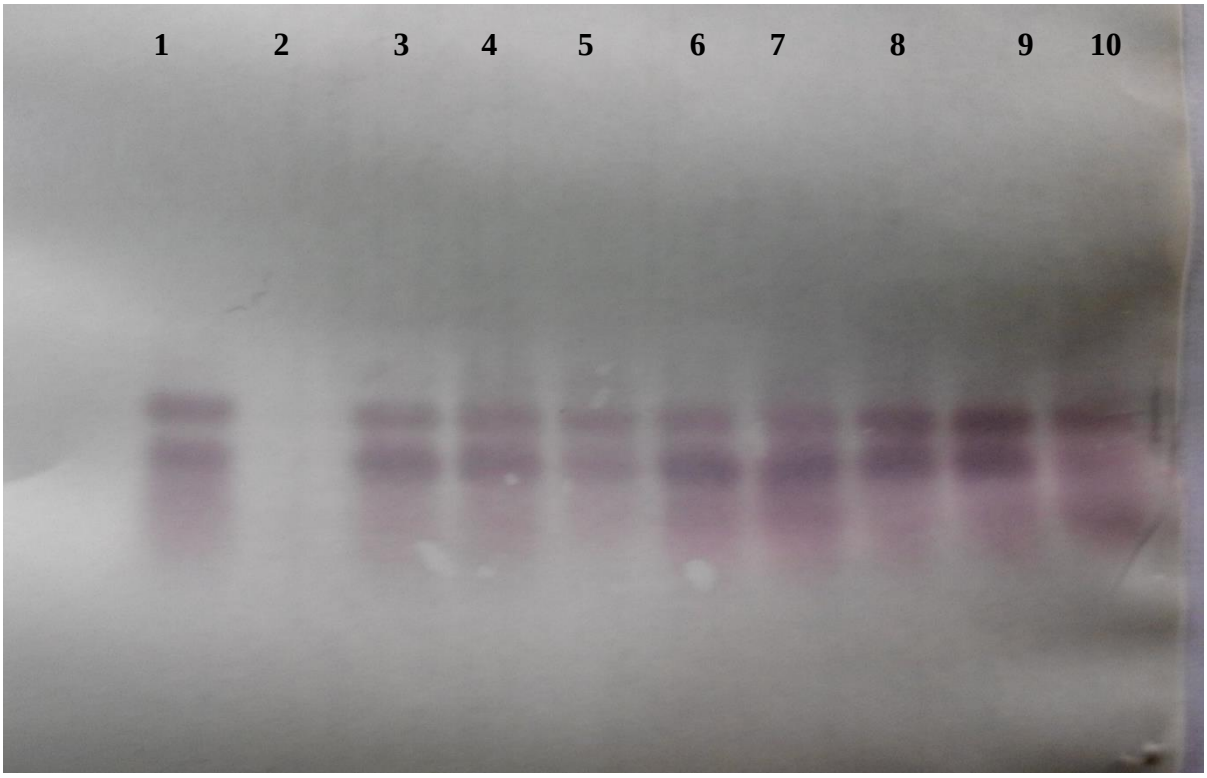

Figure: Southern Blot Analysis ofCry2A positive transgenic cotton plants.1: Control Positive, 2: Control Negative, 3-4: FBS-37, 5-6: M-1, 7-8: CRSP-1, 9-10: CRSP-3

Cry1Ac and Cry2A toxicity through leaf bioassay

Transgenic plants were subjected to 2nd instar larva of Helicoverpaarmigera to check their toxic level. A total three leaves from upper, middle and lower part of transgenic cotton plants of 25, 55 and 85 day old were allowed to attack by H. armigera. After 2–3 days

insect mortality picture was collected from transgenic and non-transgenic cotton plants.

Herbicide tolerance of transgenic cotton plants through Glyphosate spray assay

A total of 1900 ml/acre weedicide spray was done on both transgenic and non-transgenic cotton plants. Herbicide Glyphosate is commercially available as Roundup™. Glyphosate which was prepared a up-to final concentration of 1900 ml/acre by dissolving it in water. Transgenic cotton plants were subjected to a full pressure of weeds in containment without manual hoeing until 3 month. After the three months when the cotton field was full of different kinds of weeds glyphosate spray at the rate of 1900 ml/acre (300 ml of 99% Glyphosate of Galaxy brand FMC mixed with 1900 L and 700 ml of water in the tank) was applied. The necrotic effect was seen on weeds along with nontransgenic cotton plants which ultimately lead to death. However, no effect of spray was observed on FBS-37, M-1, CRSP-1 and CRSP-3 plants which remained healthy and showed the full potential of growth.

Figure: 19 different types of weeds found in field growing alongside transgenic cotton plants


Glyphosate Spray test on (Triple Gene cotton)
Before Spray
After 2 weeks


On-site inspection visit of PARB team

Figure: After six days weeds were died in contained field condition at CEMB.

Evaluation of transgenic cotton plants for resistance against insectsthrough insect leaf bioassay

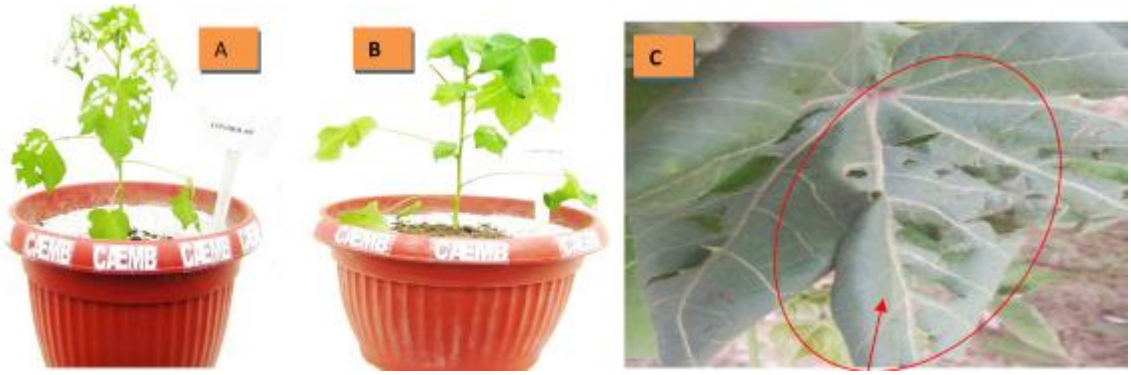

Figure: Bioassay of Transgenic and non-Transgenic plants. A: Non-transgenic plant almost fully damaged, B: Transgenic CRSP-1 variety stayed healthy, no insect attack, C: CRSP-2 transgenic variety, a portion damaged by insects.

Variation in mortality % age of *H. armigera* 2nd instar larvae was observed after 30, 60 and 90 days depending upon expression of Cry proteins.

Multiplication of CEMB Bt and herbicide tolerant plants:

Initial seed increase of transgenic cotton varietiesFBS-37, M-1, CRSP-1 and CRSP-3 containing Bt and herbicide (Glyphosate) tolerant gene were done at

CEMB. After one-month kanamycin selection putative transgenic cotton plants were made selection free by shifting on simple MS medium for new root formation followed by shifting in soil pots. The putative transgenic cotton plants were kept covered with plastic bags for three days.The acclimatization therapy was initially started by opening of plants for 15 min followed by a further increase of fifteen minutes up-to one month. During fist, five days plants showed a slight wilting due to dehydration which was recovered with the passage of time in both cultivars. The healthy survived putative transgenic cotton plants were shifted to the contained field of CEMB.

Green house/ tunnel evaluation:

Green house/tunnel evaluation of all transformed cotton lines as compared with non-transformed versions will be done at CEMB, Lahore. During field trial the plants will be analyzed at molecular level and through bioassay for confirmation of gene expression. Data for fibre yield and other morphological characters will also be collected and analyzed with comparison to control.

Biosafety Studies:

The following steps were performed during biosafety studies of transgenic cotton cultivars.

Gene Flow

Gene flow frequency: Root and shoot samples from plants other than cotton (weeds, etc.) were collected at CEMB from the transgenic cotton fields and surroundings and analyzed for presence and expression of transgenes.

Frequency of gene flow were calculated by following formula and expressed in % Gene Flow.

$$\% \text{ Gene Flow} = \frac{\text{Number of samples showing transgenes}}{\text{Total number of samples collected}} \times 100$$

Figure: Lane 1 (from left to right): Ladder (1kb DNA ladder), lane 2: positive CEMB-GTGene control, lane 3: positive GTG cotton plant, Lane 4-8: Samples from tunnel (other than cotton plants), Lane 9: Negative Control

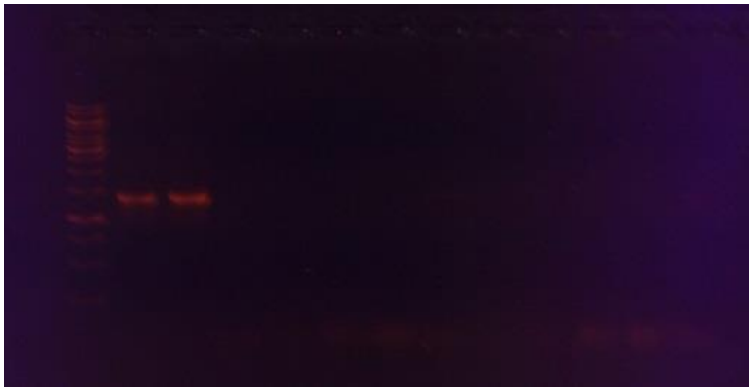

Figure: Lane 1 (from left to right): Ladder (1kb DNA ladder), lane 2: positive CEMB-Bt control, lane 3: positive Bt cotton plant, Lane 4-12: Samples from tunnel (other than cotton plants), Lane 13: Negative Control. **No gene flow was observed in non-target species**

Protein Expression in Root exudates of transgenic plants: 100 gram pre-washed roots of transgenic plants were chopped into 1" pieces and dipped into 200 ml distilled water for 24 hours. The water will be tested for the presence of proteins of transgenes through ELISA.

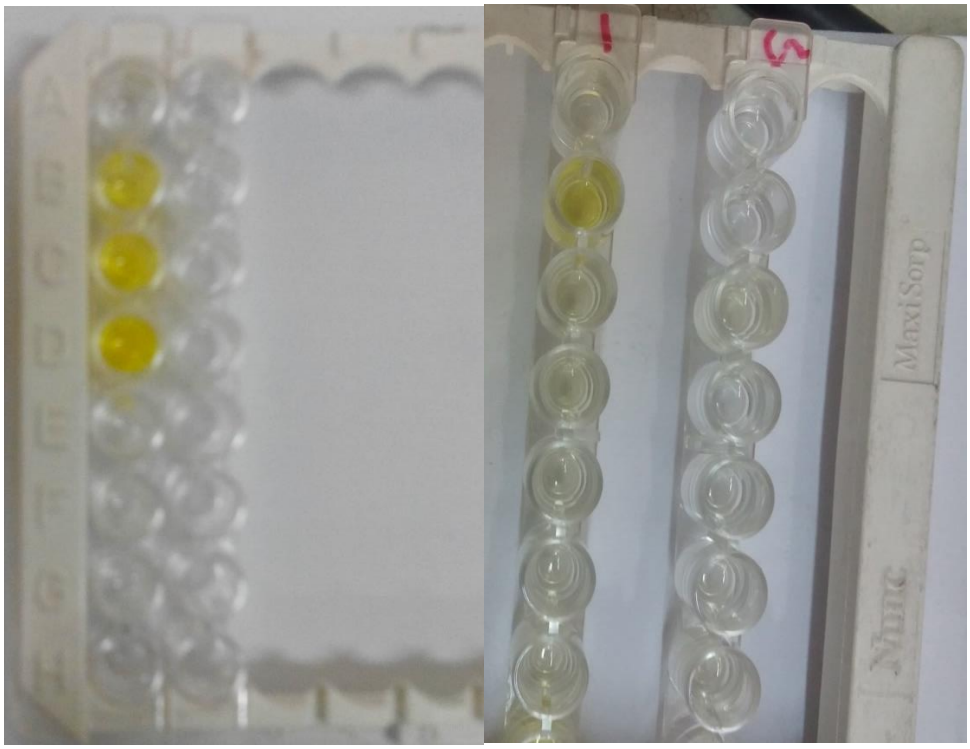

Figure: FOR CEMB-BtProtien: A1: Negative Control, B1: Positive Control (5 ng), C1: Positive Control (10 ng), D1: Positive Control (25 ng), From E1-H2: Plants samples other than transgenic Cotton plants from tunnel (Kit Used: Enivirologix USA for Quantitative ELISA)

Figure: FOR CEMB-GTGeneProtien: 1: Negative Control, 2: Positive Control, 3-12: Plants samples other than transgenic Cotton plants from tunnel (Kit Used: Enivirologix USA for Qualitative ELISA)

Biosafety studies on Animals: To evaluate the potential risk of transgenic Bt and GTGene on experimental mice, data on histological studies for vital organs (heart) after 30 & 60 Days Feeding on diet containing transgenic seed wererecorded.

Histological analysis of vital organs:

At the termination of feeding trial, animals were sacrificed and their heart were removed for further morphological and histological studies. Morphological variations in terms of organ structure, weight and maturity were observed. Sectioning was done with microtome followed by staining with hematoxyline and eosine. These sections were observed under microscope for comparison.

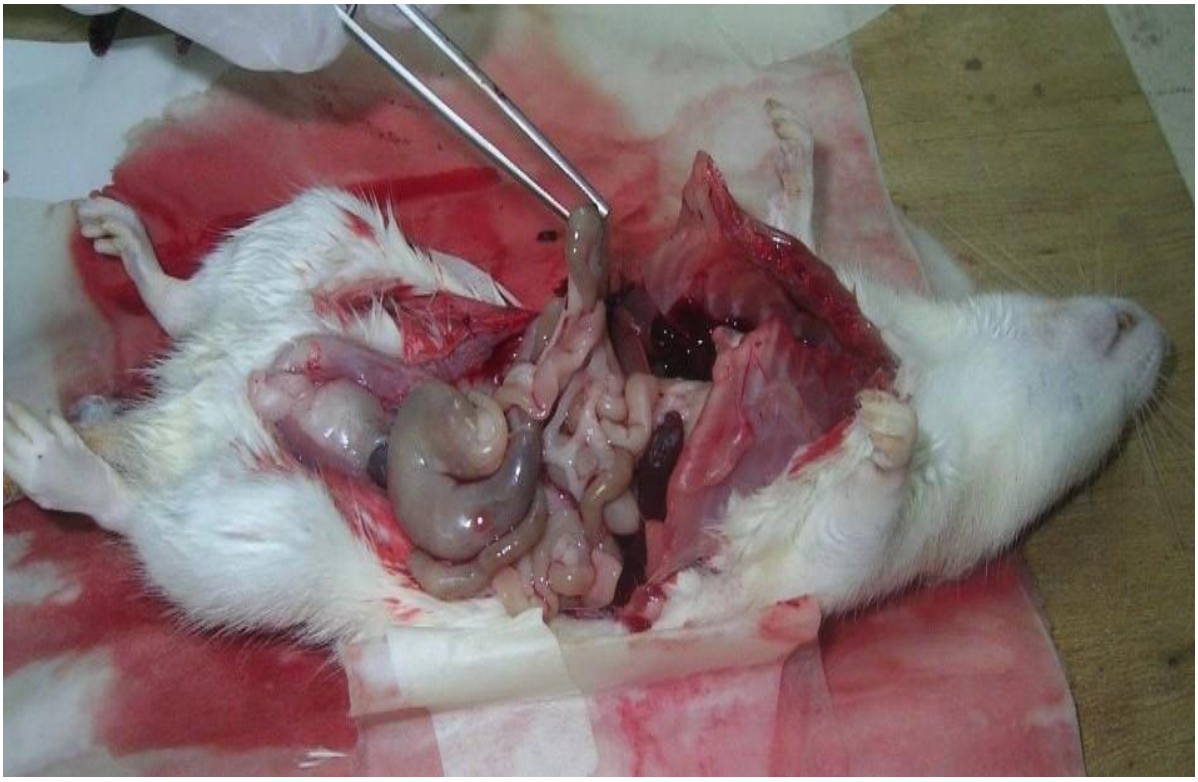

Figure: Dissection of mice fed on transgenic feed containing CEMB-Bt and CEMB-GTGene cotton seed. Morphological observation dictated that all the apparent vital organs remained un-effected.

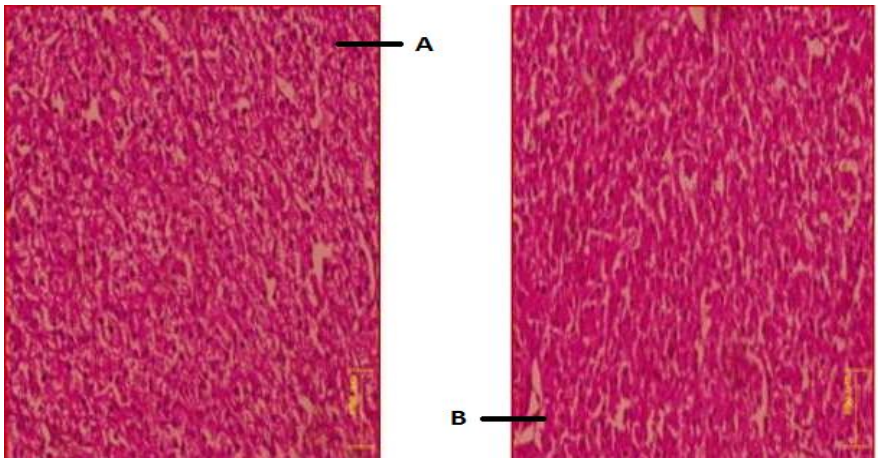

Figure: Comparison of cellular architecture of heart between randomly assorted groups (a): The cellular structure of cardiac tissues of the mice in control group fed with CEMB-Bt and CEMB-GTGene transgenic cotton (b) indicates the cellular structure of cardiac tissues of the chickens fed with non- transgenic cotton, group-II

Transfer of the Transgenic Cotton Seed to Project Director

CEMB transgenic seed of four cotton varieties i.e. M1, FBS-37, CRSP-1, CRSP-3 have been handed over to the concerned partner in the PARB Project 215. The seed was transferred by signing a MTA for “Transfer of Biological Material” at CEMB in the presence of Cotton Commissioner, Director CEMB and other dignitaries.

Detail of transferred material is as under:

Cotton Variety	Transgenic Events	Weight	Handed over to
M1	10	100 gms (approx 10 gram each event)	Project Collaborator
FBS-37	10	100 gms (approx 10 gram each event)	Project Collaborator
CRSP-1	10	100 gms (approx 10 gram each event)	Project Collaborator
CRSP-3	10	100 gms (approx 10 gram each event)	Project Collaborator

Photograph of the event is as under:

4. Component wise salient achievements

(Statements only)

1. Bt + Glyphosate resistant genes have been successfully transformed in two lines (MNH-886 and MNH-814) of CRS Multan.
2. Bt + Glyphosate resistant genes have been successfully transformed in two lines () of 4B Multan.
3. CEMB had successfully transformed Bt + Glyphosate resistant genes in four lines of CRS Multan and 4B Multan.

5. Overall progress of the problem searched

Bt + Glyphosate resistant genes have been successfully transformed in four parental lines (two from 4B and two from CRS Multan). However, due to segregation after transformation the transgenic hybrids could not be developed.

6. Varieties, breeds, vaccines or products developed and patented

Four lines two from CRS Multan (MNH-886 and MNH-814) and two from 4B Multan have been transformed successfully for Bt. and Glyphosate resistant genes and can be utilized in further breeding program.

7. No. of national and international papers published one paper submitted

8. No. of Ph.D/M.Phil. produced

Nill

9. Any other achievement

Nill

10. Current status of commercialization of the project. How many stakeholders adopted this technology along with monitory benefits

Variations were observed in all events of each transgenic parental line as mortality in plants was observed after Glyphosate spray. Therefore further selection and purification of parental lines is required. The production and commercialization of hybrid could not be possible because of variation and segregation observed in the transgenic parental lines. It is therefore requested to allow releasing these parental lines as varieties after purification and selection of true to types.

11. Impact of the project on strengthening of the institutional infrastructure, machinery, equipment and human resources

a. Availability of Bt + glyphosate resistant genes
b. Strengthening of green houses

12. Constraints in the:

(a) Implementation of the project

The transgenic seed of parental lines was delayed due to which multiplication of parental lines and hybrids is delayed. Secondly the parental lines are still showing segregation and further

purification is required for development of hybrids which is not possible in the proposed timeline of the project.

(b) Commercialization of the project

As the hybrid development is not possible because of the segregation observed in parental material therefore commercialization of these hybrids is not possible.

13. Suggestions for future research and development

It is suggested to allow releasing these parental lines as varieties after purification and selection of true to types.

Dated: 13-10-2016

(Signature of Project Manager)

Dated: 14-10-2016

(Signature of Head of Organization)

Annexures

Table 1 No. of Pollinations attempted for Hybrid Seed Production April 2011 to March 2012

Months	1st week	2nd week	3rd week	4th week	Total
June	3240	3255	3401	3401	13297
July	2810	3450	35911	3498	45669
August	2090	2180	2214	2301	8785
September	1500	1880	1915	1990	7285
October	3578	3470	1980	0	9028
Grand Total	13218	14235	45421	11190	84064

Table 2. Seed Cotton Yield (Kg/ha) data of 10 cotton hybrids along with standard tested at 6 locations in Punjab during 2012-13.

Hybrids	CRS Multan	4B Multan	Agri Farm	PSC KWL	CRS Vehari	CRS BWL	Avg.	% inc/dec over standard	
								Tarzan-1	MNH-886
H-1	2080	4584	1883	4591	1847	1865	2808	15	-4
H-2	2403	4501	2152	5523	1793	2116	3081	26	5
H-3	1435	4368	2331	6169	1650	2421	3062	25	4
H-4	2224	6151	2152	3981	1309	3052	3145	29	7
H-5	681	3529	1686	2403	574	176	1508	-38	-49
H-6	2188	4921	2511	6205	1686	2116	3271	34	12
H-7	2403	4824	2080	4071	1686	3314	3063	25	4
H-8	2403	4659	2690	4913	1130	1779	2929	20	0
H-9	825	742	538	2905	108	237	892	-64	-70
H-10	1148	1506	1219	4429	466	1083	1642	-33	-44
Tarzan-1	1650	2726	2152	4627	1004	2518	2446		
MNH-886	2116	4315	2331	5272	1255	2303	2932		

Annexure-1

Average No of Bolls/plant,Boll weight,Got% and expected yield per Acre

Name of hybrid	Ave.No of Bolls/plant	Ave.Boll Weight	Got%	Expected yield per Acre(kg)
H-1	35.8	3.18 gm	40.04%	2443
H-2	33.6	3.43	42.96%	2175
H-3	68.6	3.28	41.80%	2660
H-4	70.8	3.44	40.28%	2707
H-5	59.8	3.10	39.64%	2403
H-6	69.0	3.45	41.86%	2699
H-7	72.0	3.79	41.20%	2995
H-8	38.8	3.44	40.01%	2482
H-9	38.6	2.62	36.585	1957
H-10	58.2	3.31	42.04%	2584
H-11(control)	45.4	2.66	40.99%	1866
H-12(control)	32.6	3.62	38.87%	2093

Annexure-2

Pakistan Cotton Standards Inst. Multan

Lot ID: Dr.Idrees Cemb Lahore. Bale Group: A HVI SW Version: 3.1.1.0
Operator: Ajmal Serial Number: 0603016
Date: 12/7/2012
Time 12:01:33PM 1

Bale ID	Amt	SCI	Grade	Moist	Mic	Mat	UHML	UI	SFI	Str	Elg	Rd	+b	C Grade	Tr Cnt	Tr Area	Tr ID
				[dry%]			[in]	[%]	[0.5in]	[g/tex]	[%]			[Upland]		[%]	Tr-Grd
H.6/143	577	139			4.36	0.87	1.111	83.2	8.53	32.6	5.4	73.6	7.0	41-2	54.0	0.55	4
H.7/145	568	136			4.50	0.87	1.098	83.1	8.74	32.3	5.5	75.3	6.5	41-2	30.0	0.35	3
H.8/147	414	121			5.09	0.88	1.052	82.4	8.58	30.9	5.9	74.5	7.0	41-2	24.0	0.19	2
H.10/151	407	133			4.20	0.87	1.121	82.2	8.78	31.4	5.3	75.3	6.8	41-2	34.0	0.47	4
H.12/155	506	138			4.64	0.87	1.099	84.2	7.56	32.4	5.9	72.2	6.5	51-1	74.0	1.08	6
H.2/R3/123	523	123			5.10	0.89	1.106	82.1	8.70	32.2	5.8	70.0	6.7	51-1	90.0	1.69	8
H.1/R.1/113	476	110			5.09	0.88	1.104	80.4	10.88	30.4	5.8	70.5	7.0	51-1	54.0	0.71	5
H.1/R.2/115	538	122			4.75	0.88	1.093	81.3	9.59	32.4	5.5	70.1	6.5	51-1	79.0	1.06	6
H.1/R.3/117	526	123			4.61	0.87	1.100	81.6	10.72	31.3	5.9	71.5	7.3	51-1	31.0	0.85	5
H.2/R.1/119	414	103			5.25	0.88	1.057	81.6	11.19	27.4	6.1	70.2	6.7	51-1	65.0	0.75	5
H.2/R.2/121	542	128			4.71	0.88	1.090	81.9	8.93	33.0	5.3	72.1	6.7	51-1	56.0	0.93	6
H.3/R.1/125	473	121			4.74	0.87	1.123	81.9	10.37	29.7	6.0	72.9	6.8	51-1	80.0	0.87	6
H.3/R.2/127	495	135			4.50	0.87	1.123	82.3	8.66	33.7	6.0	70.5	6.6	51-1	95.0	1.23	7
H.3/R.3/129	506	131			4.69	0.88	1.140	82.3	9.32	32.7	5.8	70.7	6.5	51-1	70.0	0.69	5
H.4/R.1/131	467	130			5.08	0.88	1.147	82.4	8.66	32.6	5.9	73.6	6.9	41-2	35.0	0.38	3
H.4/R.2/133	451	135			4.54	0.87	1.166	82.0	8.29	33.4	5.6	73.1	6.5	51-1	105.0	1.29	7
H.4/R.3/135	461	147			4.67	0.87	1.195	82.9	7.21	35.4	6.0	74.6	6.8	41-2	46.0	0.69	5
H.5/R.2/139	501	119			3.67	0.85	1.042	80.8	11.80	30.5	6.3	68.5	7.1	51-1	79.0	0.97	6
H.5/R.3/141	533	135			3.68	0.85	1.088	81.1	12.03	34.5	6.2	66.3	6.6	61-1	118.0	1.75	8
n 19																	
Max	577	147			5.25	0.89	1.195	84.2	12.03	35.4	6.3	75.3	7.3	61-1	118.0	1.75	8
Min	407	103			3.68	0.85	1.042	80.4	7.21	27.4	5.3	66.3	6.5	41-2	24.0	0.19	2
Average	**494**	**128**			**4.63**	**0.87**	**1.108**	**82.1**	**9.40**	**32.0**	**5.8**	**71.9**	**6.8**	**51-1**	**64.2**	**0.87**	**6**
Std.Dev.	49	10			0.40	0.01	0.037	0.9	1.34	1.8	0.3	2.3	0.2		0.0	0.41	
CV%	**10**	**8**			**8.63**	**1.12**	**3.306**	**1.1**	**14.29**	**5.5**	**4.8**	**3.2**	**3.5**		**0.1**	**47.09**	
Q99% +/-	28	6		0.0	0.23	0.01	0.021	0.5	0.78	1.0	0.2	1.4	0.1		0.0	0.24	

NOT VALID FOR COURT

Note: The above test results are issued on the basis of samples provided by the clients.

M. Ajmal Chughtai
Officer Incharge
PCSI Cotton Fiber Testing Lab
Ministry of Textile Industry
Govt of Pakistan

8/12/12

System Testing

Page 1 of 1

Table 3. Seed Cotton Yield (Kg/ha) data of 10 cotton hybrids along with standard tested at 10 locations in Punjab during 2013-14

Hybrid	Aziz Farm	CRI FSD.	CRS BWL.	CRS MLT.	R Y Khan	CRS SWL.	CRS VHR	Kot Chuta	P.S.C. KWL.	4 Brothers	Average	% inc/dec over standard	
												Tarzan-1	MNH-886
H-1	5059	1401	3588	2153	1078	754	4485	1130	5810	3838	2929	35	3
H-2	4198	2051	4593	2583	1026	431	3696	1183	5129	5129	3002	38	6
H-3	4808	2496	3660	2404	1813	1543	4485	1238	5882	4949	3328	53	17
H-4	5705	2541	3660	2451	1027	1345	5131	1220	5918	5595	3459	59	22
H-5	1220	233	754	215	1086	154	1220	479	2260	144	776	-64	-73
H-6	5956	1808	3050	1722	2050	1538	5490	1606	5487	4985	3369	55	18
H-7	5633	1266	2619	1758	1980	933	5274	1426	4842	5667	3148	45	11
H-8	5095	1582	2835	2651	1881	1754	5095	1233	5810	3945	3187	46	12
H-9	4916	1517	2189	1686	1915	466	4629	795	5165	4555	2783	28	-2
H-10	4198	843	1256	431	1401	646	4198	1263	3658	4017	2191	1	-23
Tarzan-1	2763	889	2117	1148	1685	502	3947	675	4124	3909	2176		
MNH-886	5346	2209	2835	1758	1971	933	4019	920	4160	4304	2845		

Table-4:- Seed cotton yield (kg/ha) data of four cotton hybrids along with standards tested at eight locations in Punjab cotton belt during 2014-15.

Hybrids	Aziz Farm Multan	Khitrarn Farm Pir Mehal	4b Farm	Galewal Farm	Bangash Farm	Rojhan Farm	Rahim Yar Khan	CRS Multan	Average yield kg/hac	Tarzan-1	MNH-886
H-3	2614	3074	3083	4514	4115	4992	5738	2235	3796	23	14
H-4	2832	3316	3365	4380	4258	5150	6482	2535	4040	31	22
H-6	2462	3033	3167	4335	4087	5429	5640	2369	3815	24	15
H-7	2500	3117	3053	3747	3927	5136	5916	2035	3679	20	11
TARZAN-1	1966	2449	2634	3078	3089	4292	5065	2035	3076		
MNH-886	2199	2665	2814	3399	3596	4615	5231	2068	3323		

Table-5:- Averaged data of seed cotton yield (kg/ha) of two selected cotton hybrids along with standards from 2012-2014 tested at different locations in Punjab..

Hybrid	Yield (Kg/ha)			Average	% Increase/Decrease	
	2012	2013	2014		Tarzan-1	MNH-886
H-4	3145	3459	3796	3467	35	14
H-6	3271	3369	3815	3485	36	15
Tarzan-1	2446	2176	3076	2566		
MNH-886	2932	2845	3323	3033		

Table 6. Averaged morphological data of 8 locations of 2 cotton hybrids of CRS Multan along with standards during 2014-15.

Hybrid	Plant Height (cm)	CLCuV %	Bolls/plant	Boll Weight (g)	Seed Cotton Yield (kg/ha)	G.O.T %	Staple Length (mm)	Staple Strength (g/tex)	Mike (µg/inch)
H-6	140.2	40	27.2	3.69	3815	43.50	28.70	31.10	5.30
H-7	140	45	23.2	3.82	3679	43.50	26.00	34.70	5.00
Tarzan-1	136	90	23.2	2.95	3076	43.90	26.90	26.90	6.00
MNH-886	137.2	70	24.3	3.97	3323	43.40	27.80	26.30	5.00

Table 7. Pink bollworm data of two transgenic parental lines at CRS, Multan during 2015-16

Event #	Pink Bollworm Infestation (%)	
	CRSP-1	**CRSP-3**
1	30.0	53.8
2	15.0	47.4
3	10.0	53.8
4	13.3	33.3
5	73.3	35.3
6	16.7	35.3
7	41.2	38.1
8	30.8	40.1
9	38.1	29.4
10	36.1	39.1
Average	**30.5**	**40.6**

Table 8. Plant mapping data of two transgenic parental lines at CRS, Multan during 2015-16

CRS P1										
Event No.	**Plant Height (cm)**	**No. of nodes/pl**	**Total plants**	**Days to 1st flower**	**Days to 1st boll open**	**No. of bolls/pl**	**Boll wt. (g)**	**Yield/plot (g)**	**Yield/pl (g)**	**CLCuV (%)**
E_1	85	33	33	57	95	15.4	3.2	1645.1	49.9	34.5
E_2	94	30	8	56	101	24.9	4.6	915.1	114.4	34.4
E_3	77.8	28.4	9	55	103	23.3	3.7	771	85.7	37.5
E_4	81.6	29.2	21	56	97	26.8	4.2	2343.5	111.6	18.4
E_5	86.4	28.8	40	52	95	30	3.08	2471.1	61.8	36.7
E_6	74.2	30.4	16	57	98	18.4	4.4	1293.6	80.9	21.6
E_7	99.4	33.1	27	53	94	21.1	3.1	1786.5	66.2	26.2
E_8	116.2	34.5	21	52	95	25.7	4.1	2194.5	104.5	30.3
E_9	111.4	35	14	57	102	18	5	1253.7	89.6	18.2
E_{10}	78.8	29.4	16	60	102	21.8	4.4	1531.1	95.7	35.4
Average	**82.6**	**31.2**	**20.5**	**55.5**	**98.2**	**23**	**4**	**1620**	**86**	**29.3**
CRS P_3										
Event No.	**Plant Height (cm)**	**No. of nodes/pl**	**Total plants**	**Days to 1st flower**	**Days to 1st boll open**	**No. of bolls/pl**	**Boll wt. (g)**	**Yield/plot (g)**	**Yield/pl (g)**	**CLCuV (%)**
E_1	87.6	29	12	59	100	15.2	3.4	622	51.8	44.4
E_2	102	29.2	13	60	102	18.2	5	1173.4	90.3	48
E_3	81.8	28	6	59	102	15.8	2.8	260.4	43.4	33.3
E_4	63	25	16	57	101	17.6	3.8	1063.1	66.4	42.8
E_5	85.2	28.8	15	60	104	26.7	4.5	1806.4	120.4	40.6
E_6	74.4	27.9	15	51	95	20.6	2.7	845.7	56.4	43.7
E_7	84.4	28.5	21	54	93	20.3	3	1239.9	59	21.4
E_8	78.6	28.2	31	54	99	17.4	3.1	1652.2	53.3	16.9
E_9	71.8	26	8	54	98	15	2.8	333	41.6	20
E_{10}	70.8	26.5	5	57	101	14	4	291	58	30
Average	**80**	**27.7**	**14.2**	**56.5**	**99.5**	**18.1**	**3.5**	**928.7**	**64.1**	**34.1**

Table 9. Data Recording CEMB triple gene cotton after application of 1900ml/acre Glyphosate (Galaxy)

Event No.	Total No. of plants before spray	Plants partially affected after spray	Plants completely resistant after sprayed	Days to 1st flower opening	Plant height (cm)	Weeds species survived after spray
(CRSP-1)						
Event 1	45	10	34	-	8	Deela
Event 2	14	3	10	-	8.8	Deela
Event 3	16	5	11	-	8.6	Deela
Event 4	30	1	29	-	9	Deela
Event 5	62	11	51	52	10	Deela
Event 6	20	4	16	-	9.8	Deela
Event 7	36	12	24	53	11.8	Deela
Event 8	21	2	19	52	10.6	Deela
Event 9	18	5	13	-	8.6	Deela
Event 10	21	4	16	-	10.2	Deela
(CRSP-3)						
Event 1	35	17	15	-	10.4	Deela
Event 2	35	20	13	-	8.8	Deela
Event 3	25	13	10	-	9.2	Deela
Event 4	42	26	13	-	9.4	Deela
Event 5	45	23	14	-	10.4	Deela
Event 6	44	16	19	51	9.2	Deela
Event 7	40	19	19	-	10.6	Deela
Event 8	38	17	20	-	12.8	Deela
Event 9	32	17	12	-	10	Deela
Event 10	23	14	7	-	7.6	Deela

Sowing date: 22-04-2015
Date of Glyphosate 1st spray: 04-06-2015
Date of Glyphosate 2nd spray: 18-06-2015

Table 10. Fiber traits of Glyphosate + Boll worm resistant material of cotton at CRS Multan 2015

(CRSP-1)

Event #	GOT (%)	S.L(mm)	Fiber Fineness (µg/inch)	F.Str. (g/tax)	Pink Bollworm (%)
E_1	38.2	25.1	4.9	34.0	30.0
E_2	31.1	24.0	5.2	40.9	12.3
E_3	39.1	24.1	5.1	35.4	5.0
E_4	38.4	27.3	5.2	40.2	13.33
E_5	38.8	24.8	4.9	38.5	73.33
E_6	37.7	25.6	5.4	29.9	16.66
E_7	36.4	27.6	4.7	35.6	41.17
E_8	40.7	24.0	5.2	32.9	30.76

E_9	42.0	27.2	5.4	37.4	38.09
E_{10}	39.7	23.1	4.8	27.8	36.82

(CRSP-3)

Event #	GOT (%)	S.L(mm)	Fiber Fineness (µg/inch)	F.Str. (g/tax)	Pink Bollworm(%)
E_1	41.7	24.1	5.2	33.9	53.8
E_2	39.1	24.7	5.1	30.3	47.4
E_3	39.8	22.8	4.2	31.3	53.8
E_4	37.4	25.2	5.0	33.8	33.4
E_5	39.5	25.9	5.0	32.0	35.3
E_6	39.4	23.8	4.8	33.1	35.5
E_7	36.8	24.7	4.7	32.8	38.1
E_8	37.2	24.0	4.6	32.3	40.1
E_9	38.0	23.8	4.3	33.0	29.4
E_{10}	38.1	24.2	4.7	33.5	39.1

Table 11. Yield and fiber traits of selected Glyphosate + Boll worm resistant material of cotton at CRS Multan 2016

Event #	Yield/pl (g)	GOT (%)	S.L(mm)	Fiber Fineness (µg/inch)	F.Str. (g/tax)
CRSP-1					
E_7/9	329.3	34.0	28.4	3.6	29.4
E_9/5	330.3	41.0	30.6	4.2	30.9
CRSP-3					
E_1/15	326.3	36.7	29.2	4.1	29.2
E_4/4	197.0	37.6	29.2	3.9	29.4