

TRANSGENIC COTTON: HARBORING BROAD TERM RESISTANCE AGAINST INSECT AND WEEDS THROUGH INCORPORATION OF CEMB DOUBLE Bt AND cp4EPSPS GENES

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Introduction of multiple traits in crop plants for desirable character is an essential tool of Biotechnological application. To overcome problem of insect pests and weeds cotton variety FBS-37 was genetically modified by transformation of CEMB Cry1Ac+Cry2A and GTGene cloned in different plant expression vectors under CaMV35S Promoter and nopaline synthase Nos Terminator. CEMB optimized protocol of cotton gene transformation i.e. Agrobacterium mediated shoot apex cut method was applied for transformation of these genes constructs in cotton. Overall transformation efficiency was found to be 1.05%. Putative transgenic cotton plants were shifted to soil pots and acclimatized in the green house conditions. PCR with gene specific primers amplified 190bp for EPSPS (Glyphosate GTG) and 1000bp for each of Cry1Ac and Cry2A. Quantification of Cry1Ac, Cry2A and GTG protein through ELISA determined maximum of 0.8µg/g, 1µg/g and 0.9µg/g of tissue respectively. 100% mortality was obtained in 2nd instar larvae of *Heliothus armigera* when compared with non-transgenic control cotton plants where maximum damaged was seen in leaf bioassay. Moreover, transgenic cotton plants successfully survived when sprayed with 1600ml/acre of glyphosate as compared to control where 100% mortality and necrosis occur after 7 days of spray assay in control conditions.

Keyword: Herbicide resistant, glyphosate, Cry1Ac, GTG, weedicide resistant

INTRODUCTION

Cotton is the backbone of economy and employment in Pakistan. The sector employs about 40% of total force and earns approximately US \$ 15 billion (Vogel, 2014). Nearly 45-60% of foreign exchange has been procured due to production and consumption of cotton (Batool *et al.*, 2010; Khan *et al.*, 2013; Khan and Khan, 2003). Pakistan being fourth largest producer of cotton suffered 14% of the losses in total agriculture due to insect pest of which 84% is in cotton (Shahid *et al.*, 2012; Awan *et al.*, 2015; Bale *et al.*, 2008), 25% yield losses occur due to weeds (Khan and Khan, 2003) while remaining 30% losses occur due to CLCuV (Farooq *et al.*, 2011) and 25% by abiotic factors. Initially, pre-sowing applications are applied to eliminate weeds already present and those that will appear before the crop (Farroq and Cheema, 2014).

Manual hoeing was the only reliable alternative for getting rid of weeds in the past, but it was time consuming and labour intensive (Cheema *et al.*, 2005). Total weed seed numbers in the soil was found to rise significantly after shifting from conventional chemical weed control to non-chemical means (Bond and Grundy, 2001). Hence, total

effort required using non-chemical methods are not viable or economically sustainable. Biotechnology provides a tool through which, this can happen easily compared with conventional method. Glyphosate (N-phosphonomethylglycine) belongs to herbicide group 'glycines' which is a broad-spectrum universal herbicide used to kill annual broad leaf weeds and grasses which are known to compete with commercial crops grown around the World. Glyphosate affects in the shikimate metabolic pathway by preventing the synthesis of 5-enolpyruvyle 3-phosphoshikimate (EPSPS). It inhibits the synthesis of three aromatic amino acids including tryptophan, phenylalanine and trypsin (Yamada *et al.*, 2009).

Resistance against insect pests and herbicide has been attained in high yield varieties by different ways now-a-days (Khan *et al.*, 2014). Conventional breeding though necessary but takes much time to attain main objective. Genetic engineering is also a modern way of breeding that guarantees to avoid the problems related with the transfer of large blocks of genetic materials between two parents (Bajaj, 1998).

Genetic transformation of plants methodologies have been developed to upturn the efficiency of transformation and to

achieve stable expression of trans-genes in plants. In the present study, genes (Cry1Ac+Cry2A) and Glyphosate (GTG) that have resistance against the insect pests and weeds respectively were transformed in the cotton variety FBS-37 through *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Plant material: Cotton variety FBS-37 was selected for transformation of Cry1Ac+Cry2A genes along with cp4EPSPS genes because of its high germination. The seeds of cotton variety FBS-37 were collected from four brothers research station Multan. Delinting of cotton seeds was done by using concentrated H₂SO₄ while sterilization with 5% (W/V) HgCl₂ and 10% (w/v) SDS followed by 5-6 washings with autoclaved distilled water. Afterward, seeds were allowed to germinate by incubation at 30°C for 48 hours.

Transformation of cotton variety FBS-37 with Cry1Ac +Cry2A and CP4EPSPS gene: Transformation of Cry1Ac +Cry2A and cp4 EPSPS gene in cotton variety FBS-37 was done by following the method as done by (Rao *et al.*, 2011). Two constructs containing CaMV 35S constitutive promoter and Nos terminator were used for transformation of cotton. Screening of putative transgenic plants was done on kanamycine at the rate (50mg/ml) medium for two months, putative transgenic plants were shifted to selection free medium for shoot and root formation, as determined by Rao *et al.* (2009).

Isolation of DNA from putative transgenic cotton plants and confirmation through PCR: Isolation of DNA from screened cotton plants was done by taking the fresh leaves and following the protocol described by (Lenin *et al.*, 2001). Reaction mixture for PCR was prepared by using 10X PCR Buffer (2µl) with 2.5mM MgCl₂ (2ul), 300ng DNA template (1.5µl), 1mM dNTPs (2µl), 10 picomole primers (2µl) (forward and reverse) and 2.5U Taq DNA Polymerase for total volume of 20µl. The reaction was proceeded in ABI 9700 thermocycler having following conditions, initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 51°C for Cry2A and GTGene while 50°C for Cry1Ac for 1 min followed by extension at 72°C for 3 min. Final extension was done at 72°C for 5 minutes. PCR amplified products were resolved on 1 % (w/v) agarose gel and visualized by ethidium bromide staining.

Enzyme Linked Immuno Sorbent Assay (ELISA) of transgenic cotton plants: Cry1Ac, Cry2A and GTGene expression was evaluated through Enzyme Linked Immuno Sorbent Assay (ELISA) by using Enviroligix Kit (Cat # 051). One gram leaves samples of transgenic cotton plants were taken in liquid nitrogen and ground to fine powder in pestle mortar. 600 µl of protein extraction buffer (0.5M EDTA, Glycerol, 5M NaCl, 2M Tris-Cl, NH₄Cl, PMSF,

DTT) was added in the fine powder followed by incubation of 1hr on ice and centrifugation at 13000 rpm for 25 min.

Evaluation of Cry1Ac and Cry2A Toxicity through leaf bioassay: The transgenic cotton plants leaves were subjected to 2nd instar larvae of *Helicoverpa armigera*. Total five leaves from upper, middle and lower part of the transgenic cotton plants of 30, 60 and 90 day old (transformed and control, non-transgenic) were taken. The leaves were placed on moist filter paper present in petri plates. 2nd instar larvae of *Heliothis* were placed on the leaves for 2-3 days and their feeding was recorded. Insect mortality data was collected after 2-3 days feeding of insect on both transgenic and control plants.

Evaluation of herbicide tolerance of transgenic cotton plants through glyphosate spray assay: Total 1600ml/acre Glyphosate was applied to transgenic cotton plants in comparison to control. Herbicide Glyphosate is commercially available as Roundup™. Glyphosate was prepared up to final concentration of 1600ml/80L by dissolving it in water. Mixture was applied (1600ml/acre) to field grown transgenic cotton plants along with control non-transgenic cotton plants.

Table 1. Primer sequences used in the study:

Primer	Sequence (5'-3')	Size
Cry2A-F	AGATTACCCCAGTTCCAGAT	600bp
Cry2A-R	GTTCCCGAAGGACTTTCTAT	
GTG-F	CCCTGGTGACAAGTCCATCT	358bp
GTG-R	CTGCACACCCATCTCTCTGA	
Cry1Ac-F	ACAGAAGACCCTTCAATATC	565bp
Cry1Ac-R	GTTACCGAGTGAAGATGTAA	

RESULTS

Genetic transformation of cotton variety FBS-37: Cotton variety FBS-37 germination index was found to be 66.6%. Genetic transformation of cotton variety FBS-37 resulted in the development of 50 plantlets on selection medium after two month of screening through kanamycin. The overall transformation efficiency was found to be 1.05%.

Evaluation of putative transgenic cotton plants through molecular analysis: Putative transgenic cotton plants were evaluated for (cp4EPSPS) GTGene through PCR. 190bp fragment was amplified for GTGene by using gene specific primers Figure 1. Similarly, 1000bp fragment was amplified for each of Cry1Ac and Cry2A as shown in Figure 2 and 3, respectively. Transgenic cotton plants namely V4, V5, V7, V8, V9, V10 and V11 were confirmed positive not only for GTGene but also for CEMB- double Bt genes while no amplification was seen in negative control Figure 2, 3 and 4. The results of ELISA by using Enviroligix kit clearly demonstrate successful expression of GTGene, Cry1Ac and Cry2A gene, respectively. Quantification of Cry1Ac, Cry2A

and GTG protein through ELISA determined maximum of 0.8µg/g, 1µg/g and 0.9µg/g of tissue, respectively (Fig. 4).

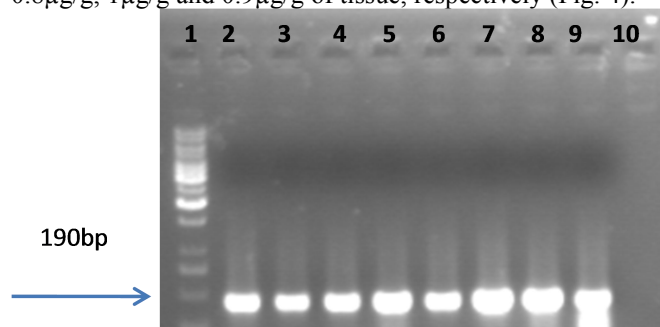


Figure 1. Amplification of cpEPSPS gene in transgenic cotton plants. Lane 1 1kb plus DNA Ladder, Lane 2-8 Transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11), Lane 9. Positive control plasmid, Lane 10. Negative control non transgenic cotton plant

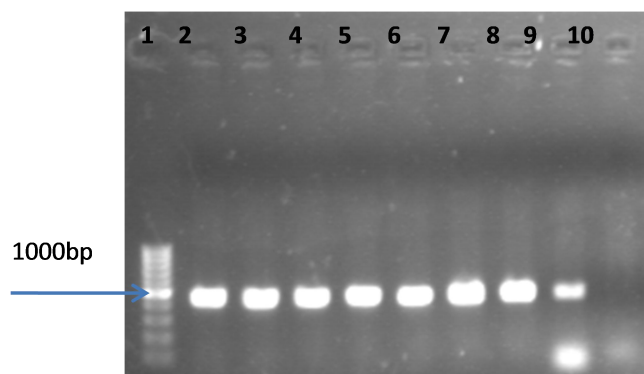


Figure 2. Amplification of Cry1Ac gene in transgenic cotton plants. Lane 1 1kb plus DNA ladder, Lane 2-8 transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11). Lane 9. Positive control plasmid, Lane 10. Negative control non transgenic cotton plant

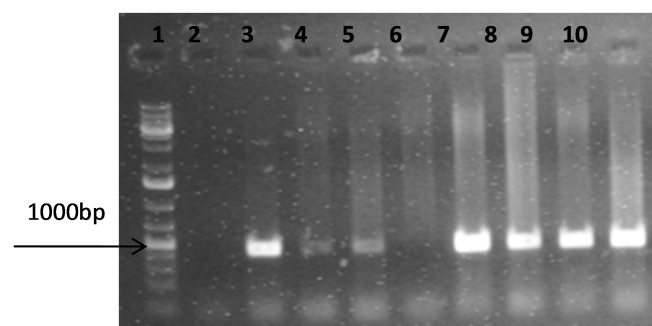


Figure 3. Amplification of Cry2A gene in transgenic cotton plants. Lane 1 1kb plus DNA Ladder, Lane 2. Negative control non transgenic cotton plant, Lane 3. Positive control plasmid, Lane 4-10

Transgenic cotton plants (V4, V5, V7, V8, V9, V10 and V11)

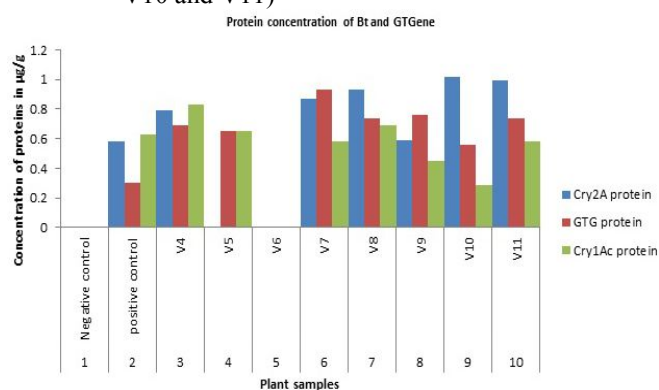


Figure 4. Graphical representation of quantification of GTG and Bt proteins.

Determination of efficacy of Cry1Ac and Cry2A through leaf bioassay of insects: Mortality percentage of *Heliothus armigera* 2nd larvae was found variable after 30, 60 and 90 days due to variation in level of expression at different time of growth of plants. Both dead and alive larvae were counted in each petri plate. Mortality 90-100% of larvae was found in larvae fed on transgenic plant leaves while no mortality was found in larvae fed on leaves of non-transgenic control plants and insects remain healthy and also advances their cycle to next instar (Fig. 5).

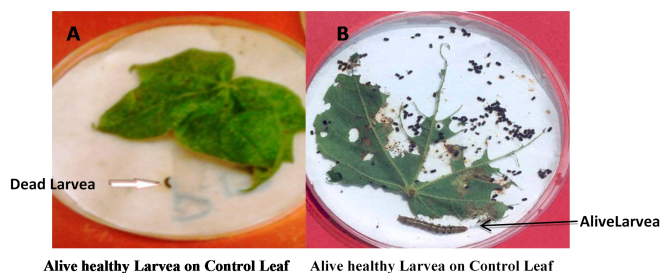


Figure 5. Bioassay of transgenic and control plant leaves.
A. A transgenic plant leaf and larva is killed after eating a small portion of leaf.
B. A control non-transgenic plant. Larva ate it and is alive.

Determination of herbicide resistance of transgenic cotton plants through glyphosate spray assay: Transgenic cotton plants were sown in field containment and no application of weed removal was applied until 3 months. After three months when cotton field was full of different kinds of weeds glyphosate spray at the rate of 1600ml/acre was applied. The necrotic effect of glyphosate became visible after 3-5 days post herbicide application (Fig. 6A, B). Symptoms which appear comprised of wilting, drooping and slight burning spot appearance and ultimate death of all

weeds along with non-transgenic control cotton plants (Fig. 6B).



Figure 6A. Field spray assay: Presence of weeds in field along with control and transgenic plants. Glyphosate was being sprayed.



Figure 6B. After five days weeds and control plants were dead while transgenic plants were healthy. It shows the presence of GTGene in cotton plants.

DISCUSSION

Cotton plays very significant role in economy of Pakistan by generating 30% of foreign exchange and contributing 80% of raw material to textile industry. Despite of its importance cotton is being harmed by attack of different insect pests and weeds. Total 20% yield losses in cotton occur due to insect pests (Gruere *et al.*, 2011) while 25% of yield losses in cotton occur due to weeds (Khan *et al.*, 2003; Monks *et al.*, 2007;). To overcome such losses an attempt was made in current study to develop insect and herbicide resistant cotton plants by introduction of Cry1Ac +Cry2A as done by Muzaffar *et al.* (2015) and cp4EPSPS gene as done by Awan *et al.* (2015).

Transformation of double Bt and GTGene was done by shoot apex cut method as done by Rao *et al.* (2011). Transformation efficiency of experiments was calculated to be 1.05% which is almost similar to the results obtained by Bakhsh *et al.* (2012); Rao *et al.* (2011). Amplification of 190bp fragment for GTGene and 1000bp for each of Cry1Ac + Cry2A confirmed successful transformation of three gene in cotton variety FBS-37 conferring resistance to the broad-spectrum glyphosate and insects, respectively (Ali *et al.*, 2016); Rao *et al.*, 2013).

The results of ELISA of Bt and GTGene in transgenic cotton plants confirmed their successful expression. Quantification of Cry1Ac, Cry2A and GTG protein through ELISA determined maximum of 0.8µg/g, 1µg/g and 0.9µg/g of tissue, respectively (Fig. 4). The efficacy of transgenes in transgenic plants were further confirmed by insect bioassay and glyphosate spray assay as shown in Figure 5 and 6, respectively. Mortality of insects (100%) on transgenic cotton plant leaves while complete survival on non-transgenic control plant leaves determined successful expression of Cry1Ac +Cry2A genes as was obtained by Kiani *et al.* (2013). Similarly, complete survival of transgenic cotton plants against 1600ml/acre glyphosate spray in comparison to control and weeds where 100% death was obtained and determines efficacy of transgene cpEPSPS against glyphosate herbicide. The results are in accordance as obtained by Zhao *et al.* (2011) and Awan *et al.* (2015).

Conclusion: FBS-37 harboring CEMB Cry1Ac + Cry2A genes along with cp4EPSPS gene holds good potential to combat with serious problems of insects and weeds. It may be proved to be a good asset for national breeders to utilize this material for development of high yielding insect and weedicide resistant cotton varieties which can pave their role in boosting up of national economy.

REFERENCES

- Ali, A., Ahmad, S., Ahmad Nasir, I., Qayyum Rao, A. and Husnain, T., 2016. Evaluation of two cotton varieties CRSP1 and CRSP2 for genetic transformation efficiency, expression of transgenes Cry1Ac+ Cry2A, GT gene and insect mortality. doi:10.1016/j.btre.2016.01.001Biotech. Rep.
- Awan, M.F., M.A. Abass, A. Muzaffar, A. Ali, B. Tabassum, A.Q. Rao and T. Husnain. 2015. Transformation of insect and herbicide resistance genes in cotton (*Gossypium hirsutum* L.). J. Agri. Sci. Technol. 17:287-298.
- Bajaj, Y.P.S. 1998. Biotechnology in Agriculture and Forestry (cotton). Springer, Berlin.
- Bakhsh, A., S. Siddique and T. Husnain. 2012. A molecular approach to combat spatio-temporal variation in

- insecticidal gene (Cry1Ac) expression in cotton. *Euphytica* 183:65-74.
- Bale, J.S., J.C. Van Lenteren and F. Bigler. 2008. Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B: Biol. Sci.* 363:761-776.
- Batool, S., N.U. Khan and K. Makhdoom. 2010. Heritability and genetic potential of upland cotton genotypes for morpho-yield traits. *Pak. J. Bot.* 42:1057-1064.
- Bond, W. and A. Grundy. 2001. Non-chemical weed management in organic farming systems. *Weed Res.* 41:383-405.
- Cheema, M.S., M. Akhtar and M.S. Iqbal. 2005. Evaluation of chemical, mechanical and manual weed control methods in cotton. *Pak. J. Weed Sci. Res.* 11:47-50.
- Farooq, A., J. Farooq, A. Mahmood, A. Shakeel, A. Rehman, A. Batool, M. Riaz, M.T.H. Shahid and S. Mehboob. 2011. An overview of cotton leaf curl virus disease (CLCuD) a serious threat to cotton productivity. *Aust. J. Crop Sci.* 5:1823-1831.
- Farooq, O. and Z.A. Cheema. 2014. Influence of sowing dates and planting methods on weed dynamics in wheat crop. *Pak. J. Agri. Sci.* 51:817-825.
- Gruere, G. and D. Sengupta. 2011. Bt cotton and farmer suicides in India: An evidence-based assessment. *The J. Dev. Stud.* 47:2:316-337.
- Khan, G., A. Bakhsh, M. Ghazanfar, S. Riazuddin and T. Husnain. 2013. Development of transgenic cotton pure lines harboring a pesticidal gene (cry1Ab). *Emirates J. Food Agri.* 25:434-442.
- Khan, M.A., A. Ali, M. Aslam, Z. Tahir, M.M. Khan and I. Nadeem. 2014. The role of morphological and chemical plant traits imparting resistance in Bt cotton genotypes against thrips, *Thrips tabaci* (Lind.). *Pak. J. Agri. Sci.* 51:725-731.
- Khan, N.U. and S.U. Khan. 2003. Integrated weed management in upland cotton. *Pak. J. Weed Sci. Res.* 9:185-192.
- Kiani, S., Ali, A., Bajwa, K.S., Muzaffar, A., Ashraf, M.A., Samiullah, T.R., Shahid, A.A., Husnain, T. 2013. Cloning and Chloroplast-targeted Expression Studies of Insect-resistant Gene with Ricin Fusion-Gene under Chloroplast Transit Peptide in Cotton. *Electronic journal of Biotechnology* Vol. 16:1-14.
- Lenin, K., M.A. Mariam and V. Udayasuriyan. 2001. Expression of a cry2Aa gene in an acrySTALLIFEROUS *Bacillus thuringiensis* strain and toxicity of Cry2Aa against *Helicoverpa armigera*. *World J. Microb. Biot.* 17:273-278.
- Monks, C.D., G. Wehtje, C. Burmester, A.J. Price, M.G. Patterson, D.P. Delaney, W. Faircloth and M.R. Woods. 2007. Glyphosate-resistant cotton response to glyphosate applied in irrigated and nonirrigated conditions. *Weed Technol.* 21:915-921.
- Muzaffar, A., S. Kiani, M.A.U. Khan, A.Q. Rao, A. Ali, M.F. Awanand and T. Husnain. 2015. Chloroplast localization of Cry1Ac and Cry2A protein-an alternative way of insect control in cotton. *Biol. Res.* 48:1-11.
- Rao, A.Q., A. Bakhsh, I.A. Nasir, S. Riazuddin and T. Husnain. 2013. Phytochrome B mRNA expression enhances biomass yield and physiology of cotton plants. *Afr. J. Biotechnol.* 10:1818-1826.
- Rao, A.Q., A. Bakhsh, S. Kiani, K. Shahzad, A.A. Shahid, T. Husnain and S. Riazuddin. 2009. The myth of plant transformation. *Biotechnol. Adv.* 27:753-763.
- Rao, A.Q., M. Irfan, Z. Saleem, I.A. Nasir, S. Riazuddin and T. Husnain. 2011. Overexpression of the phytochrome B gene from *Arabidopsis thaliana* increases plant growth and yield of cotton (*Gossypium hirsutum*). *J. Zhej. Univ. Sci. B.* 12:326-334.
- Shahid, M.R., J. Farooq, A. Mahmood, F. Ilahi, M. Riaz, A. Shakeel, I.V. Petrescu-Mag and A. Farooq. 2012. Seasonal occurrence of sucking insect pest in cotton ecosystem of Punjab, Pakistan. *Adv. Agri. Botany.* 4:26-30.
- Vogel, H.L. 2014. Entertainment industry economics: A guide for financial analysis. Cambridge University Press.
- Yamada, T., R.J. Kremer, P.R. de Camargo e Castro and B.W. Wood. 2009. Glyphosate interactions with physiology, nutrition, and diseases of plants: Threat to agricultural sustainability. *Eur. J. Agron.* 31:111-113.
- Zhao, T., C. Lin and Z. Shen. 2011. Development of transgenic glyphosate-resistant rice with G6 gene encoding 5-Enolpyruvylshikimate-3-Phosphate synthase. *Agri. Sci. China* 10: 1307-1312.