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Transformation and evaluation of Broad-Spectrum insect and weedicide resistant genes in *Gossypium arboreum* (Desi Cotton)

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ABSTRACT

Gossypium arboreum (Desi Cotton) holds a special place in cotton industry because of its inherent ability to withstand drought, salinity, and remarkable resistance to sucking pests and cotton leaf curl virus. However, it suffers yield losses due to weeds and bollworm infestation. Genetic modification of *G. arboreum* variety FBD-1 was attempted in the current study to combat insect and weedicide resistance by incorporating *cry1Ac, cry2A* and *cp4-EPSPS* genes under control of 35S promoter in two different cassettes using kanamycin and GUS as markers through *Agrobacterium*-mediated shoot apex cut method of cotton transformation. The efficiency of transformation was found to be 1.57%. Amplification of 1700 bp for *cry1Ac*, 167 bp for *cry2A* and 111 bp for *cp4-EPSPS* confirmed the presence of transgenes in cotton plants. The maximum mRNA expression of *cry1Ac* and *cp4-EPSPS* was observed in transgenic cotton line L3 while minimum in transgenic cotton line L1. The maximum protein concentrations of Cry1Ac, Cry2A and Cp4-EPSPS of 3.534 µg g^{-1}, 2.534 µg g^{-1} and 3.58 µg-g^{-1} respectively were observed for transgenic cotton line L3 as compared to control cotton line. On leaf-feed-based insect bioassay, almost 99% mortality was observed for *Helicoverpa armigera* on the transgenic cotton plant (L3). It completely survived the 1900 ml hectare^{-1} glyphosate spray assay as compared to non-transgenic cotton plants. The necrotic spots appeared on the third day, leading to the complete death of control plants on the fifth day of assay. The successful multiple gene-stacking in *G. arboreum* FBD-1 variety could be further used for qualitative improvement of cotton fiber through plant breeding techniques.

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1. Introduction

Gossypium arboreum, also called the Asiatic cotton (desi cotton), is one of the four cultivable cotton species around the world. It is a diploid cotton species (2 n), having absorbable, short and coarse fiber, highly favorable for the surgical industry. *Gossypium arboreum* is known to possess drought/salinity tolerance, Cotton Leaf Curl Virus (CLCuV) resistance,[1] nematode and root rot resistance.[2] However, *G. arboreum* is severely affected by bollworm, especially *Helicoverpa armigera*.[3] Due to the increasing attack of CLCuV on imported varieties of *Gossypium hirsutum*, the need for the hour is to put efforts toward *G. arboreum* to improve its fiber characteristics and resistance against chewing insects and herbicides by genetic manipulation of its genome.[4,5]

The most important pests are *Helicoverpa punctigera, H. armigera* (spotted bollworm), and *Tetranych us urticae* (spider mite).[6] Conventionally, insects are controlled by spraying insecticides, but this is very expensive.[7] In 2018–19, about 21,175 metric tons of pesticides were consumed in Pakistan's agriculture sector as per Economic Survey of Pakistan (2018–2019).[8] To cope with such challenges through biotechnology more than 100 *Bacillus thuringiensis* (Bt) crystal genes have been sequenced so far, which encode for Cry and Cyt proteins.[9] Such crystal proteins are toxic to the larvae of *Lepidoptera, Diptera* and *Coleoptera*. Several reports revealed the resistance against lepidopteron and dipterans insects in *G. hirsutum* when different *cry* genes (*cry1Ab, cry1Ac, cry1A, cry2A, cry1F* etc.) were transformed.[10] Cotton cultivars expressing both Cry2A and Cry1Ac endotoxins were more toxic to *Spodoptra frugiperda, H. zea* and *S. exigua*[11] and *H. armigera* as compared to those expressing single toxin.[12,13] Stacking different Cry proteins (Cry1Ac and Cry2A) can delay the development of resistance in Lepidopterans.[13] The Cry1Ac

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and Cry2A proteins do not share the high-affinity binding sites on brush border membrane vesicles in the insect midgut[12] and therefore stacking of *cry1Ac* and *cry2A* genes in the same crop has a potential advantage for resistance management.

Gossypium arboreum is highly vulnerable to weeds competition and 37% yield losses occur due to weeds.[14] Weeds can serve as hosts for diseases and pests, reduce the nutritional quality of soil and adversely affect cotton harvesting or its lint quality.[7] Weed removal through chemical herbicide sprays provided an alternative to manual weed management but chemical herbicides result in plant damage, especially the cotton field.[15] The glyphosate spray is broad-spectrum broad-leaf weedicide and inhibits the shikimate pathway enzyme.[16] Glyphosate (N-phosphonomethyl glycine) kills the plant by blocking an enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) – shikimate biosynthetic pathway enzyme. EPSPS converts phosphoenolpyruvate and shikimate-3-phosphate (S3P) to inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate. This pathway is involved in the production of amino acids, folic acids, phytoalexins, lignins, plastoquinones and many other secondary substances.[16] Blockage of EPSPS enzyme results in accumulating shikimic acid and hydroxybenzoic acid in leaves or nodes, leading to the plant's death.[16] *EPSPS*-encoding bacterial genes are being introduced to crop plants by stable genetic transformation to develop resistance from glyphosate herbicides in transgenic crops. A *Calotropis procera EPSPSII* gene transformation in upland cotton have been reported to successfully induce glyphosate tolerance.[11] In the current study design, we aim to transform the *cry* genes along with Glyphosate Tolerant Gene (*cp4-EPSPS*) or GTG in *G. arboreum* with a proposed resistance development in transgenic variety against pest attack and glyphosate, respectively.[14]

2. Materials and Methods

2.1. Field Setup and Sampling

This study was carried out at Center of Excellence in Molecular Biology, University of the Punjab, Lahore, during the years 2015–2016 (31°33′N, 74° 19′E) in which a completely randomized block design was set out in two consecutive growing seasons (2015–2016) of cotton in the same field.

2.2. Plasmid Construction and Transformation into Agrobacterium

Two Bt genes *cry1Ac+cry2A* of 1400bp were subcloned in pKHG4 at the *Hind*III site to produce recombinant pKHG4-*cry1Ac+ cry2A* vector under the expression of 35S promoter, (Fig. 1 A) hereafter referred to as cry construct. Likewise, a 1700bp *cp4-EPSPS* expressed under 35S promoter was cloned at *Nco*I and *Bgl*II sites of pCAMBIA1301 (Fig. 1 B). The two recombinant plasmids were separately transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation and confirmed through colony PCR by using gene-specific primers.

2.3. Plant Material and Transformation Procedure

About 5599 delinted seeds of *G. arboreum* were used for transformation experiments. Seeds were sterilized with a working concentration of 1% SDS and 1% $HgCl_2$ for 5 min and rinsed completely afterward with autoclaved water. These sterilized seeds were incubated in dark at 30°C overnight. Germination index of *G. arboreum* (Desi Cotton)

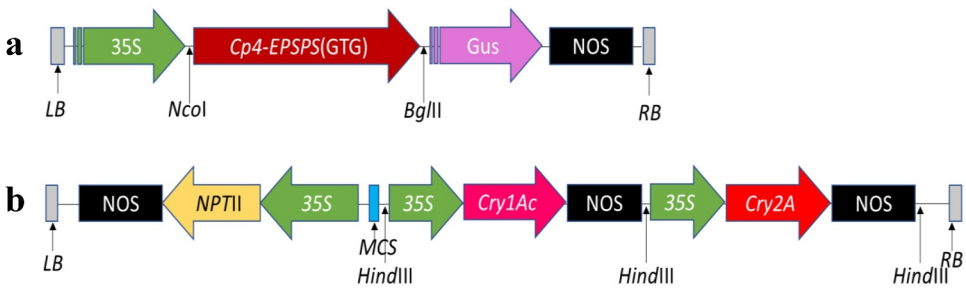

Figure 1. Physical map of gene constructs of (a) *cp4-EPSPS* and (b) *cry1Ac+cry2A*.

was determined by soaking thirty seeds in Petriplate at 30°C for 48 hours and determined by using the following formula:

Germination Index (%age) = (Germinated Seeds/Total Seeds) x 100.

Overnight grown cultures of *Agrobacterium* harboring *cry* and *cp4-EPSPS* constructs were mixed and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was decanted, and the pellets were dissolved in 10 ml of MS broth. Shoot apices of the 48 h germinated embryos were transformed according to Rao et al. (2011).[17] The co-cultivated embryos were embedded in MS plates (hypocotyl region downward in the MS phytagel and epicotyl facing upward above the MS phytagel) supplemented with B5 vitamins and cefotaxime (200 mg L^{-1}) and incubated at 28°C with a 16/8 h photoperiod (90 $\mu molm^{-2}s^{-1}$). After 3–5 days of co-cultivation, plantlets with healthy shoots and roots were transferred to a fresh MS tube containing 50 mg L^{-1} kanamycin, 200 mg L^{-1} of cefotaxime and B5 vitamins.

After 8–10 weeks of growth in MS tubes, six-inch -long plantlets were transplanted to pots having sterilized soil mixture (equal proportion of clay, sand and peat moss). The plants were covered with plastic bags, for first 15 days, to maintain proper humidity at a temperature of 25 ± 2°C with a photoperiod of 16:8 hours of light and dark cycle. The plants were acclimatized according to the method of Gul et al., (2020).[18] About 48 putative transgenic cotton plants were transplanted to cotton tunnels under controlled environmental conditions. Transgenic cotton lines were successfully self-pollinated to produce T1-generation and similarly for the T2-generation.

2.4. Detection of Transgenes through Conventional PCR

Genomic DNA from putative transgenic cotton plants in T0 generation was isolated using G-spinII plant genomic DNA extraction kit (Cat# 17,271). Putative positive plants carrying *cp4-EPSPS, cry1Ac* and *cry2A* genes were confirmed by PCR from genomic DNA using gene-specific primers (Table 1). The PCR reaction consisted of; 2 µl genomic DNA, 2 µl of 10x pfu buffer ($MgSO_4$ added) (Fermentas cat# B34), 2 µl of 2 mM dNTPs, 2 µl of 10 pM forward and reverse primers each, 0.5 µl of 500 U Taq polymerase (Fermentas cat# EP0402) and nuclease-free water upto 20 µl. The PCR for all three genes was performed in three steps; the first step of one cycle of initial denaturation at 95°C for 5 min, second step of 40 cycles of each denaturation at 95°C for 45s, annealing at 60°C for 45s and amplification at 72°C for 1–2 min, and third step of final amplification at 72°C for 5 min and hold at 4°C.

2.5. Quantification of Proteins through Immunostrip Test Assay and ELISA

Immunostrip assay[19] and Enzyme-Linked Immuno sorbent Assay (ELISA) were performed to detect and quantify proteins, respectively, in T3 generation. For immunostrip assay, total crude protein was isolated from the fresh plant leaves. Immunostrip test assay was performed by placing strips about $1/4^{th}$ of an inch of the strip in the diluted protein samples for 30 minutes. Strips were observed for desired bands after 30 min. For ELISA, Envirologix Qualiplate ELISA kit for Cry1Ac (cat# AP003), Cp4-EPSPS (cat# AP010), and Quantiplate ELISA kit (cat# AP005) for Cry2A were used. The ELISA was performed as per manufacturer's instructions. The protein was quantified by measuring OD on microtiter ELISA plate reader at 450 nm absorbance.

2.6. Quantitative Real-Time Expression of Transgenic Cotton Plants

Young leaves of transgenic and non-transgenic control cotton plants in T1-generation were used to extract RNA according to the procedure performed as described by.[20] The cDNA was prepared as follows: total RNA (1pg- 5 µg), oligo $(dT)_{18}$ primers 1 µl; dNTPs mix(10 mM) 1 µl, nuclease-free water up to 14.5 µl, 5x RT buffer 4 µl, RiboLock RNase Inhibitor 0.5 µl, Maxima reverse transcriptase 1 µl and then incubated at 50°C for 30 min and the reaction was terminated at 85°C for 5 min. The cDNA was stored at −80 °C. The quantitative RT-PCR was carried out using gene-specific primers (Table 1). The reaction mix contained: Maxima SYBR Green/ROX qPCR Master Mix (2X) 12.5 µl, forward primer 0.5 µl, reverse primer 0.5 µl, cDNA 2 µl and nuclease-free water to 25 µl. The reaction mix was prepared in the dark to prevent light exposure of SYBR green. The cycling protocol of all qPCR

reactions was performed with the following conditions: one cycle at 94◦C for 10 min, 40 cycles at 94◦C for 45s, 52◦C for 45s and 72◦C for 30s. The endogenous expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as the internal control for the normalization of expression data.

2.7 Glyphosate Spray Assay

Herbicide resistance status of transgenic cotton plants was evaluated by spraying glyphosate on field plants in T1, T2 and T3 generation following method of.[14] Cotton was planted in the field and manual weeding was restrained. All the wild weeds could grow uncontrollably until the glyphosate solution (1900 ml hectare^{-1}) was sprayed on transgenic and control cotton lines. Vegetative lesions and complete killing of the plants were observed daily for 5–7 days.

2.8 Insect Mortality Assay

The mature leaves of the cotton plants in T3 generation were used for insect mortality assay. Detached leaves from the cotton plants were put on the moist filter paper in a petri plate. The age synchronized population of insects reared on non-transgenic plants was used in this study. The *H. armigera* larvae were released onto transgenic *G. arboreum* plants' detached leaves. Five leaves from five biological replicates of each transgenic plants were selected and to each biological replicate, five larvae were released. The petri plates were kept at 28°C ± 2°C in 16 hours light and 8 hours dark. The mortality data was recorded for 3–5 days of assay.

3. RESULTS

3.1. Transformation of cry1Ac+cry2A and cp4-EPSPS Genes in G. arboreum

The germination index was calculated to be 73%. The seeds germinated at 28°C after 2 days were transformed through shoot apex explant by *Agrobacterium*-mediated transformation (Fig. 1a). The *Agrobacterium* containing both *cry* and *cp4-EPSPS* constructs were combined as 1:1 ratio for cotton transformation. Healthy seedlings (3055), which developed roots and shoots (Fig. 2b), were transferred to MS medium culture tubes for selection with kanamycin. Only 262 plantlets (Fig. 2c) survived the selection pressure of antibiotics and transplanted to soiled pots. (Fig. 2d). The fungal contamination and acclimatization of the plants further reduced the number of surviving plants to 48, finally transplanted to the tunnel (Fig. 2e). The transformation efficiency as calculated from the data (Table 2) was found to be 1.57%. The frequency of transplantation at each developmental stage in the T1-generation seriously affected the plant survival regardless of the transformation efficiency.

Transformation Efficiency = (No. of positive plants shifted to tunnel/No. of embryos subjected to selection) x 100

Table 1. Gene sequences of the primer pairs used for PCR.

Primer name	Sequence (5'to 3')
Cry1Ac-F	ACAGAAGACCCTTCAATATC
Cry1Ac-R	GTTACCGAGTGAAGTGTTAA
Cry2A-F	CCGCTCCATTACAACCAGAT
Cry2A-R	ATGGTGAAGCCGGTGTAGTC
Cp4-EPSPS-F	*TATGGCTTCCGCTCAGGT*
Cp4-EPSPS-R	*AGCATCTTCTCAGTGTGGTCTCT*

3.2. Molecular Analysis of Putative Transgenic Cotton Plants

Putative transgenic cotton plants harboring *cry1Ac, cry2A* and *cp4-EPSPS* genes were analyzed through high throughput molecular techniques. These include PCR, ELISA, DIPSTICK test and Real-Time (RT) PCR. PCR amplification from genomic DNA of transgenic cotton plants with gene-specific primers revealed a 1700bp fragment of *cry1Ac* (Fig. 3 A) and 167bp of *cry2A* (Fig. 3 B) while 111bp for *cp4-EPSPS* (Fig. 3 C).

3.3 Gene Expression Analysis of cp4-EPSPS through Quantitative Real-Time PCR

Quantitative real-time mRNA expression analysis was conducted to determine the relative expression of transgene *cp4-EPSPS* in cotton plants. The expression determination of three genes revealed a several-fold increase of mRNA in different transgenic cotton lines (represented by L1, L2, L3, L4 throughout the rest of the analysis). The highest mRNA expression (5.6-fold) of *cry1Ac* was found to be in L3 (Fig. 4 A), while *cry2A* expression was highest in transgenic line L4 up to 79.9-fold (Fig. 4 B). The maximum mRNA expression of *cp4-*

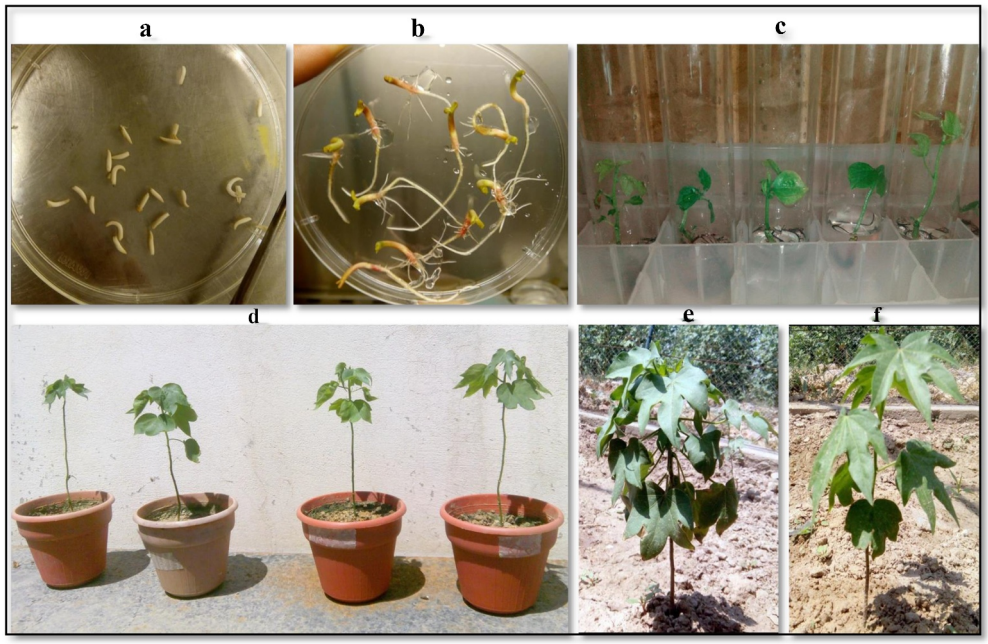

Figure 2. Process of cotton transformation. (a) Embryos isolated from germinated seeds of cotton. (b) Transplantation of five days old cotton plantlets into culture tubes. (c) Twenty days old plantlets in glass tubes supplemented with kanamycin (50 mg L^{-1}), cefotaxime (200 mg L^{-1}), IBA and kinetin (100 mg L^{-1}). (d) Shifting of putative transgenic cotton plants to plastic pots and tunnel. (e) Two months old putative transgenic cotton plants in the tunnel. (f) Two months old non-transgenic cotton plant in the tunnel.

Table 2. Numerical data of transformation experiments and transformation efficiency.

Embryos processed	Embryos shifted to MS tubes for selection	Survived plants transferred to soil pots	Plants contaminated with fungus in pots	Plants shifted to tunnel	Transgenic plants survived in tunnel
5599	3055	262	210	48	4

EPSPS in transgenic plant L3 was found to be 66.47 (**Fig. 4 C**).

3.4 Quantification of Cry1Ac, Cry2A and Cp4-EPSPS Proteins through ELISA

Crude protein was freshly extracted from fresh leaves of cotton plants for dipstick assay. Protein detection through dipstick assay revealed red bands for Cry1Ac and Cry2A in all four transgenic cotton plants. The appearance of a band in standard control confirmed the presence of respective proteins (Fig. 5 A & B). For ELISA, the sample was taken from three different parts of the plant i.e., upper, middle and lower canopy. ELISA assay was conducted to quantify the protein concentration in transgenic cotton plants. The assay results showed that maximum concentration of Cry1Ac and Cry2A was found to be 3.534 μg g^{-1} and 2.534 μg g^{-1} respectively, in fresh leaves of transgenic cotton plants. The Cry1Ac protein concentration in *G. arboretum* (diploid) is very much improved (**Fig. 6A**) than the similar protein's expression in the *G. hirsutum* (tetraploid) as reported by previous studies (Fig. 6B).[11,14] The quantification of Cp4-EPSPS protein revealed maximum concentration to be 3.870 μg g^{-1} in transgenic cotton line L4 (**Fig. 6A**)) which is also much higher than tetraploid version cotton reported in previous studies (**Fig. 6B**).[11,14]

3.5 Insect Resistance and Herbicide-Tolerance Status of Transgenic Cotton

The leaves of cotton plants were fed to *H. armigera* larvae. The efficacy of *Bt* proteins on larvae was evaluated by amount of leaf consumed, weight gain of larvae, and larvae's death. After 3 days of the assay, complete mortality was observed in L3 and L4 transgenic cotton lines while the amount of plant leaf consumed in L3 transgenic cotton line was more than L4. In the non-transgenic control cotton plants, 90% larvae survived, while in and L1 and L2

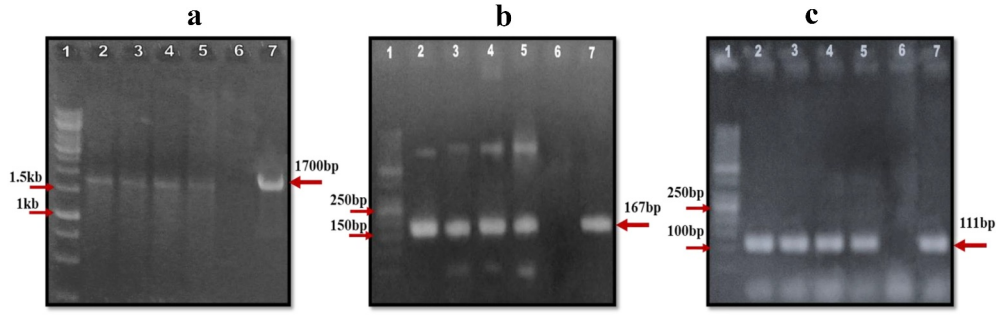

Figure 3. PCR detection of transgenic cotton plants. (a) PCR amplification of *cry1Ac*. Lane 1: 1kb DNA Ladder; Lane 2–5: putative transgenic cotton plants showing amplification of 1700bp PCR product of *cry1Ac*; Lane 6: Negative control; Lane 7: Positive control. (b) PCR amplification of *cry2A*. Lane 1: 50bp DNA Ladder; Lane 2–5: putative transgenic cotton plants showing amplification of 167bp PCR product of *cry2A*; Lane 6: Negative control; Lane 7: Positive control. (c) PCR amplification of *cp4-EPSPS*. Lane 1: 50bp DNA Ladder; Lane 2–5: putative transgenic cotton plants showing amplification of 111bp PCR product of *cp4-EPSPS*; Lane 6: Negative control; Lane 7: Positive control.

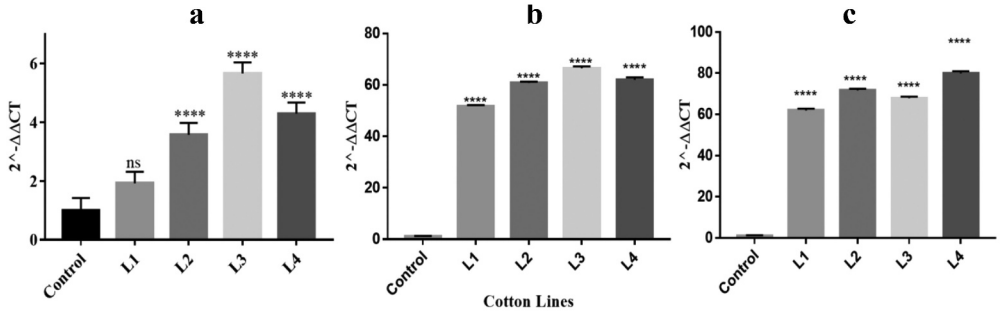

Figure 4. Relative quantification of mRNA expression through quantitative real time PCR. Non-transgenic wild type plant is represented by Control, and transgenic cotton plants as L1 to L4. (a) mRNA expression of *cry1Ac* gene. (b) mRNA expression of *cry2A* gene. (c) mRNA expression of *cp4-EPSPS*. Significant difference at $p < .0001$ is represented by **** as calculated by Dunnett's multiple comparison test and one-way ANOVA.

transgenic cotton lines, 60% and 90% survival were observed, respectively, along with maximum consumption of the plant leaf as shown in Fig. 7A.

Resistance to *cp4-EPSPS* gene was evaluated by spraying glyphosate on cotton plants. All the transgenic cotton lines survived when observed after five and ten days of spray while all type of weeds were dried up after glyphosate spray in all transgenic cotton lines (Fig. 7B).

4. Discussion

Gossypium arboreum is an Asiatic cotton or "Old World" cotton, originating from Indian Subcontinent. This cotton is cultivated in Pakistan due to its disease and insect resistance,[24] early maturity, drought and stress tolerance[25] and fiber characteristics.[26] *G. arboreum* is naturally resistant to *Bemisia tabaci* which is the biological vector of CLCuV transmission. This results in a loss of millions of dollars on CLCuV management.[18] Because of the natural resistance of *G. arboreum* to *B. tabaci* and CLCuV disease,[27] *Bt* genes integration could further enhance broad-spectrum resistance. Glyphosate – commercially known as "Roundup" – is a general weed killer and capable of suppressing the growth of broad leaved weeds along with crop plants.[28] We wanted to indirectly improve the yield and quality of fiber from *G. arboreum* by stacking herbicide resistance genes and insect resistance genes. The current study was proposed to control chewing insect pests and weeds through genetic modification of *G. arboreum* with a codon-optimized *cry1Ac* and *cry2A* gene construct for resistance against chewing insects and the *cp4-EPSPS* gene for tolerance against glyphosate herbicide.

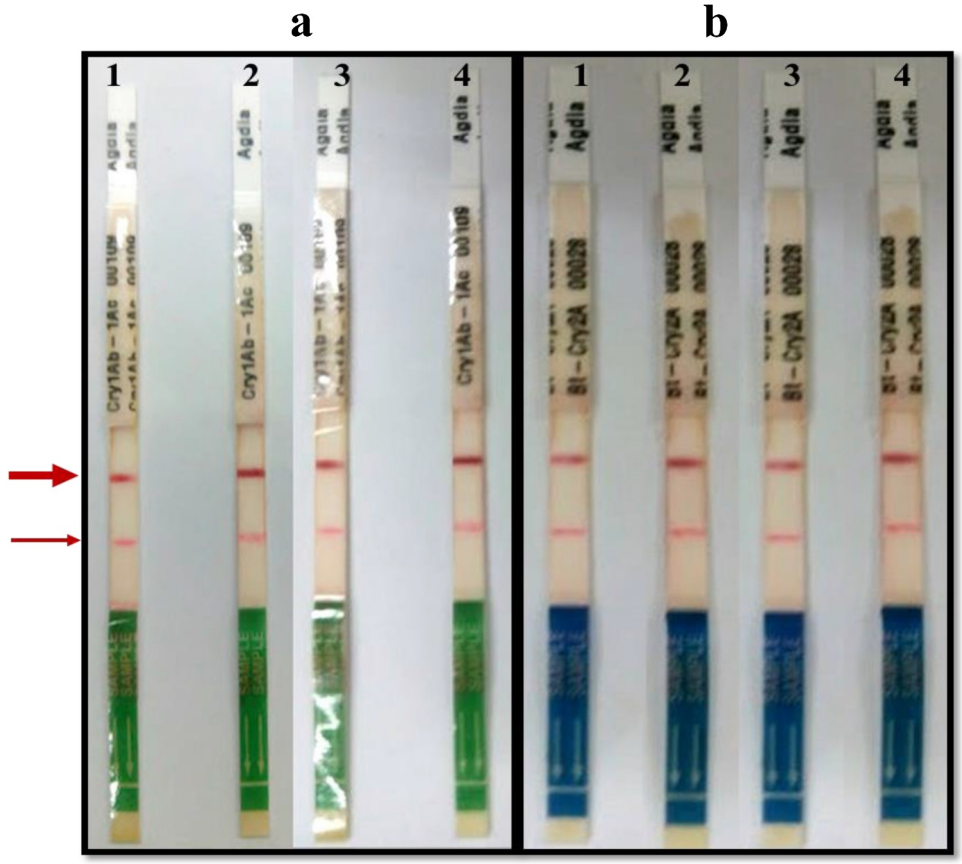

Figure 5. Immunostrip or dipstick assay. (a)Detection of Cry1Ac protein. The upper dark red line is the control line; Lower faint line shows a positive test line. (b) Detection of Cry2A protein. The upper dark red line is the control line; Lower faint line shows a positive test line.

Shoot apex transformation method has already been proved successful for recalcitrant crops like cotton. The transformation efficiency for *G. arboreum* was found to be 1.59%, though promising but less than other reports[3,18,29] and still holds the potential for improvement. PCR amplification confirmed the successful integration of all genes, *cry1Ac, cry2A* and *cp4-EPSPS* genes in *G. arboreum*. The transcript accumulation analysis by qRT-PCR revealed 51- (minimum) to 66.47-fold (maximum) *cp4-EPSPS* transcripts in transgenic event L1 and L3, respectively. Increase in expression in transgenic cotton leaves demonstrates the successful constitutive expression of CaMV35S promoter in *G. arboreum*. This study's result is consistent with the study by[30] who obtained upto 12-fold expression. For *cry1Ac*, about 5.66-fold increase in L3 and 4.29-fold increase in L4 was observed compared to the non-transgenic control line. Similarly, *cry2A* transcript level ranged between 61-fold to 79-fold in L1 and L3 transgenic cotton lines, respectively. Qualitative analysis with dipstick immunostrip assay confirmed Cry proteins' presence (Cry1Ac and Cry2A) in transgenic cotton plant leaves in accordance with the study by.[31,32] The quantitative analysis of Cry1Ac, Cry2A and Cp4-EPSPS protein with ELISA from fresh leaves of transgenic cotton plants showed 3.534 µg g^{-1} and 2.534 µgg^{-1} and 3.8 µg g^{-1} proteins, respectively. Comparison with other studies revealed that *G. arboreum* has more expression than *G. hirsutum*.[11,21] In this study, Cry1Ac protein concentration is well above (3.534µg g^{-1}) the LD95 concentration of 2.20 µg g^{-1} as determined by.[33] and 159 µg g^{-1} as determined by.[34] For a toxin to be effective, a higher LD95 value is a prerequisite for effective control of insects. The evolving reports of *H. armigera* resistance suggest that Bt-protein's sub-lethal expression level is one of the key factors in resistance development in Pakistan among many other management-related issues.[33] There was also maximum concentration of Cp4-EPSPS protein in the transgenic line L4 (3.84ug g^{-1}) as compared to control cotton plant which is

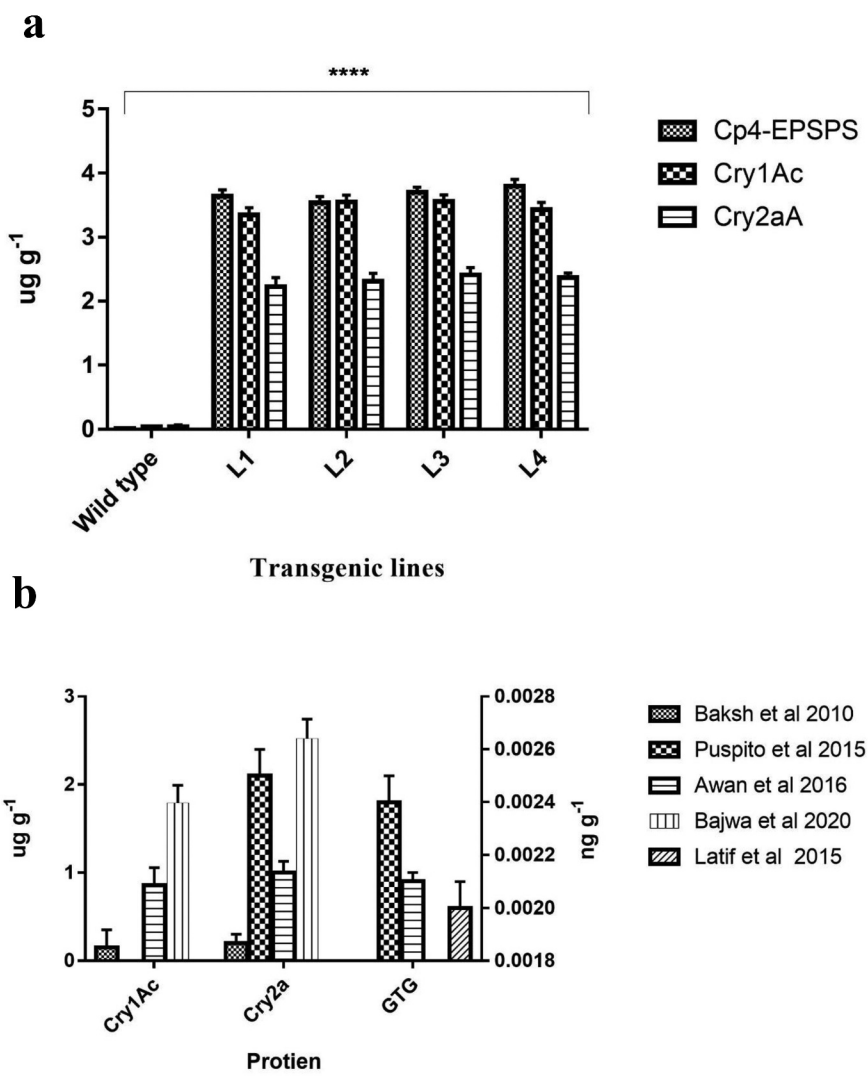

Figure 6. Comparison of transgene protein expression in diploid and tetraploid cotton. (a) shows the obtained concentration of GTG (Cp4-EPSPS) in diploid transgenic lines (*Gossypium arboretum*). (b) depicts the obtained concentration of Cp4-EPSPS, Cry1Ac and Cry2a in tetraploid cotton (*Gossypium hirsutum*) as determined in different studies.[11,14,21–23]

consistent with the study by.[30] Also higher Cp4-EPSPS protein concentration was evident in *G. arboreum* as compared to *G. hirsutum* previously reported by[11,14,21] which may be due to the fact that diploid cotton can have higher expression of traits which make it tolerant to CLCuV and other insects pests as well. Uncontrolled weed growth, alongside crop plants, compete for soil nutrition and harbor insect pests and viruses.[35] This escalates the investment cost for farmer and poses economic burden. Stacking insect-resistant genes and herbicide-tolerance genes in the same transgenic plants can substantially enhance yields, relief from insect infestation as well as less consumption of pesticides.[11,14]

The transgenic cotton plants' insect bioassay revealed 100% mortality of *H. armigera* larvae in transgenic cotton lines L3 and L4 (Fig. 7A). The insect bioassay result was consistent with the study by.[11] and[21] Pyramiding the cotton plant with *cry* genes and *cp4-EPSPS* gene in *G. hirsutum* also showed success by killing 100% insects and tolerance from glyphosate[11,21] this study for *G. arboreum*. However, this study's uniqueness is that the data for insect mortality and glyphosate tolerance was analyzed in the T3 generation of *G. arboreum* transgenic plants, depicting stable gene expression in highly selective transgenic lines.

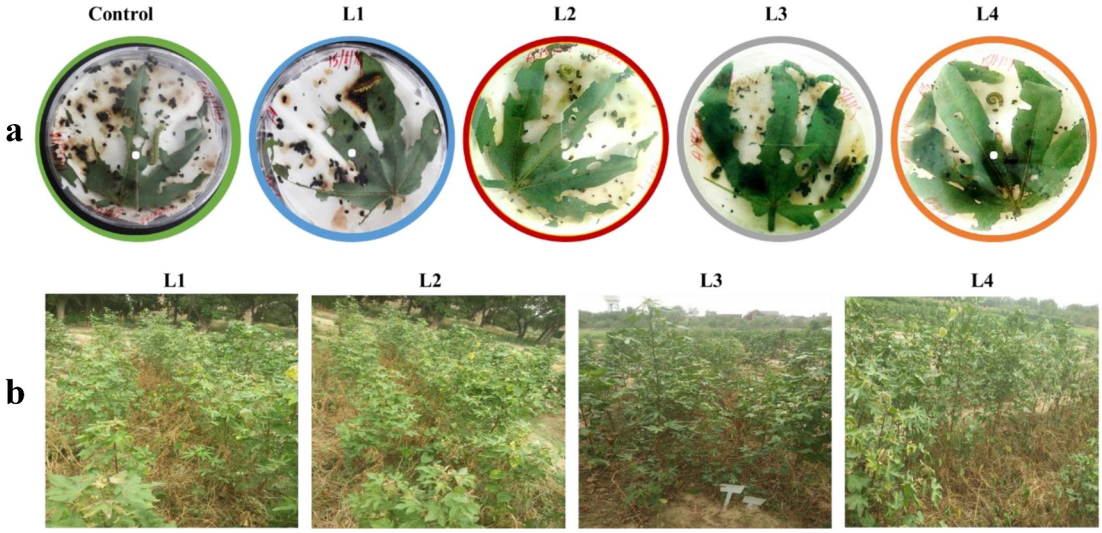

Figure 7. Resistance evaluation of transgenic cotton lines (*cp4-EPSPS*). (a) Insect mortality assay on detached leaves of representative cotton lines. (b) Glyphosate spray assay conducted in field to determine the herbicide resistance status of transgenic cotton plants.

The transgenic cotton plants' efficacy against glyphosate challenge assay demonstrated the survival of the transgenic cotton plants in the field and successful removal of all type of weeds (Fig. 7B). There have already been reports about the introgression of *cp4-EPSPS* in *G. hirsutum* from our lab[11,14,21] and other labs.[30] Still, to our knowledge, this is the first report of *in-planta* transformation of these genes into *G. arboreum* with a higher transformation efficiency and stable gene expression. Improvement of *G. arboreum* fiber yield, which is already resistant for CLCuV,[36] is a great asset for cotton germplasm and cost-efficient cotton production by farmers. The insect-resistant and herbicide-resistant *G. arboreum* variety can serve as a resource for fiber and yield improvement in the future.

5. Conclusion

Cotton farmers in Pakistan suffer huge economic burden due to insects, weeds, fiber quality and droughts/floods. In this study, the Gene pyramiding approach demonstrates the successful transformation and expression of Cry1Ac and Cry2A toxins and Cp4-EPSPS protein in a local variety of cotton (FBD-1), which is already resistant to the devastating insect *B. tabaci*. This approach would help farmers increase cotton yields, reduce insecticidal spray and manual weeding of cotton and decrease soil quality through excessive weedicides and insecticides. This improved variety of *G. arboreum* will provide opportunity to utilize this material further to improve fiber traits and its yield through breeding in the future.

Acknowledgments

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Author's Contribution

AQR came up with the idea and approval of this research project. MST carried out all the research work. AL supervised the plant transformation experiments. NS supervised the protein quantification assays. SB and MAUK supervised the insect bioassay. AG prepared the initial manuscript and final editing and formatting. AAS and TH evaluated the material for field experiments. All authors reviewed and approved the article for submission.

Disclosure statement

The authors declare that they have no competing interests.

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