World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:9, No:1, 2015

Detection of Transgenes in Cotton (*Gossypium hirsutum* L.) by Using Biotechnology/Molecular Biological Techniques

Ahmad Ali Shahid, Muhammad Shakil Shaukat, Kamran Shehzad Bajwa, Abdul Qayyum Rao, Tayyab Husnain

Open Science Index, Biotechnology and Bioengineering Vol:9, No:1, 2015 publications.waset.org/10000681/pdf

Abstract**—**Agriculture is the backbone of economy of Pakistan and cotton is the major agricultural export and supreme source of raw fiber for our textile industry. To combat severe problems of insect and weed, combination of three genes namely Cry1Ac, Cry2A and *EPSP*S genes was transferred in locally cultivated cotton variety MNH-786 with the use of *Agrobacterium* mediated genetic transformation. The present study focused on the molecular screening of transgenic cotton plants at T_3 generation in order to confirm integration and expression of all three genes (*Cry1Ac, Cry2A* and *EPSP* synthase) into the cotton genome. Initially, glyphosate spray assay was used for screening of transgenic cotton plants containing *EPSP* synthase gene at T_3 generation. Transgenic cotton plants which were healthy and showed no damage on leaves were selected after 07 days of spray. For molecular analysis of transgenic cotton plants in the laboratory, the genomic DNA of these transgenic cotton plants were isolated and subjected to amplification of the three genes. Thus, seventeen out of twenty (*Cry*1*Ac* gene), ten out of twenty (*Cry2A* gene) and all twenty (*EPSP synthase* gene) were produced positive amplification. On the base of PCR amplification, ten transgenic plant samples were subjected to protein expression analysis through ELISA. The results showed that eight out of ten plants were actively expressing the three transgenes. Real-time PCR was also done to quantify the mRNA expression levels of *Cry1Ac* and *EPSP synthase* gene. Finally, eight plants were confirmed for the presence and active expression of all three genes at T_3 generation.

Keywords**—**Agriculture, Cotton, Transformation, *Cry* Genes, ELISA and PCR.

I. Introduction

COTTON is the chief source for raw fiber for textile industry as well as important oilseed crop in the world. The use of cotton fiber dates back to pre-historic times, and the modern day cotton being seen is the fruit of efforts of all the breeders and early farmers involved in the cultivation and breeding of cotton.

In 2013, share of agriculture in the GDP of Pakistan was 21.4% and cotton was the major shareholding crop in this percentage with 1.5% of GDP [19]. Beside its economic importance cotton faces many threats such as insect, weeds and viruses. Agricultural Research (ICAR, 2009), reported that weeds account for a major loss in Cotton crop as much as 47.5%. Cotton bollworms devastate the cotton crop resulting in significant losses and Deteriorate cotton quality [9].

There are more than 15 insects that are important to attack on cotton most of them belong to lepidopteran like American Boll Worm (*Heliothus armigera*), pink boll worm (*Pectinophora gossypiella*), spotted boll worm (*Earius insulanaivitella*), army boll worm (*Spodoptera lithura*). Nowadays agricultural system uses broad spectrum toxic agrochemicals for protection of crops from different pathogens, the application of these toxic chemicals resulted in environmental problems and human health issues, to cope with these problem efforts have been made on its biological control measures [4].

Transgenic cotton is included among those transgenic plants that were commercially adopted around the world. It was first brought into field in middle of 1990s, and has been rapidly adopted by farmers in several developed and developing countries. The economic, social and environmental benefits of transgenic cotton include increase of yield, reducing labor and cost, minimizing use of pesticides that result in decrease in environmental pollution. The widely used method for obtaining transgenic cotton is *Agrobacterium*-mediated genetic transformation. Although transgenic cotton plants with disease-resistance, abiotic stress tolerance, and improved fiber quality have been developed in the past decades, insect-resistant and herbicide-tolerant cotton are the two dominant transgenic cottons in the transgenic cotton market [6], [23].

The cost of pesticides used to control bollworms insects have been reduced by introducing *Bt* gene into cotton. Due to presence of toxin within plant *Bt* cotton provides an alternative to pesticides. Transgenic Bt cotton is effective in control of specific *Lepidopteran* species and its overall performance is better than conventional varieties [11], [14], [15].

The objectives of the present study were molecular characterization of triple gene transformed transgenic cotton for T_3 generation plants which were grown under tunnel conditions. The transgenic cotton plants were transformed with *Cry*1*Ac*, *Cry*2*A* and *EPSP* synthase genes and through continuous selection process T_3 plants were sown under tunnel environment. These plants were grown for screening purpose, to select putative plants for raising T_4 generation.

Ahmad Ali Shahid, Muhammad Shakil Shaukat, Kamran Shehzad Bajwa, Abdul Qayyum Rao and Tayyab Husnain are with Centre of Excellence in Molecular Biology, University of the Punjab, Thoker Niaz Baig, Lahore, Pakistan (Corresponding Author; Phone: +92-42-35293141-45 ext 144; Fax: +92-4235293149; e-mail: ahmadali.shahid@gmail.com).

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II. Materials And Methods

A. Sowing of Transgenic Cotton Plants at T_3 Generation

Seeds of transgenic cotton plants of variety MNH-786 were selected at T_2 generation and subjected to further molecular analysis at T_3 generation. Seeds of transgenic cotton plant were sowed in tunnel under field conditions.

B. Glyphosate Spray Assay

After 45 days of sowing, transgenic cotton plants were subjected to glyphosate spray assay. The transgenic cotton plants at T_3 generation were selected for screening using the spray test. Roundup-Ready glyphosate spray was used at the rate of 1600 ml/ha (field equivalent rate) on transformed (Cry1Ac, Cry2A and *EPSP*) cotton plants and non-transformed cotton plants (control) in order to know which plant possess greatest degree of tolerance. The vegetative injury level of both transformed and a control plant was recorded. The most tolerant transgenic lines were utilized for tolerance comparisons.

C. Molecular Analysis of Transgenic Cotton Plants

Transgenic cotton (*Gossypium hirsutum* L.) plants of MNH-786 belonged to T_3 generation were used for the molecular analysis. The plants had been transformed to express stacked genes namely, *Cry*1*Ac*, *Cry*2*A* and *EPSP* synthase gene. The plants were grown in tunnel under field condition. Different molecular techniques were used for confirmation of transgenic cotton such as PCR, Real Time PCR, Bradford Assay, ELISA and glyphosate spray assay.

D. Genomic DNA Isolation from Transgenic Cotton Plants

Leaf samples of transgenic cotton plants which were collected from the tunnel of Centre of Excellence in Molecular Biology (CEMB), university of the Punjab, Lahore, Pakistan. The leaves were crushed to fine powder by using liquid nitrogen with the help of pestle and mortar described by [25] with some modifications.

E. Amplification of Cry1Ac, Cry2A and EPSP Synthase Genes

Amplification of *Cry*1*Ac* and *Cry*2*A* genes into the cotton genome of transgenic cotton plants was performed using polymerase chain reaction. Genomic DNA of transgenic cotton plants were used for amplification of Bt genes, genomic DNA of non-transgenic plant was used as a negative control and plasmid *pk*2*Ac* as positive control. The PCR reaction was carried out at initial denaturation 94^{o}C for 3 minutes and subjected to 35 cycles as follows; at 94^{o}C for 45 seconds, at 56^{o}C for 45 seconds (Cry1Ac gene), 55^{o}C for 45 seconds (Cry2A gene) and 60^{o}C for 45 seconds (*EPSP* synthase gene) and at 72^{o}C for 45 seconds. The final extension phase was prolonged to 10 minutes at 72^{o}C. The amplified PCR products of Cry1Ac and *EPSP*S genes were run on 2% agarose gel along with 100bp ladder and amplified PCR product of Cry2A gene was run on 1.5% gel along with 1Kb DNA marker. All three gels were visualized under UV light [7].

F. Protein Expression Analysis Of Transgenic Cotton Plants

1. Bradford Assay

Quantification of total and crude protein was done by Bradford assay [5]. Fresh leaves of PCR positive plants were collected in liquid nitrogen and crushed by using pestle and mortar. The fine powder was then transferred to 1.5 ml tube and 700 µl of protein extraction buffer was added and eppendroff tube was incubated at 4^{o}C for overnight. After incubation, centrifugation was performed and supernatant was taken in fresh 1.5 ml tube.780 µl 1X PBS was added in supernatant and then 20 µl of protein solution was added in corresponding tubes. The tubes were kept at room temperature for 10 minutes. The value of each sample was found with the help of spectrophotometer [5].

G. ELISA of Transgenic Cotton Plants (Cry1Ac, Cry2A and EPSP Synthase Proteins)

Expression of *Cry*1*Ac*, *Cry*2*A* and *EPSP synthase* (GTGene) was determined by ELISA using Envirologix kit (Cat. # 051) temporarily as well as spatially plant samples were brought to laboratory, ground in liquid nitrogen and one-third of crushed leaves and other plant parts sample, 700 µl protein extraction buffers was added in each tube. After 1 hour of incubation on ice, centrifugation was performed at 13000 rpm for 25 minutes and supernatant removed and used for further analysis.

ELISA was performed according to kit procedure and endotoxins quantification was done by plotting absorbance values of *Cry*1*Ac*, *Cry*2*A* and *EPSP synthase* (GTGene test samples on standard curve generated with purified *Cry*1*Ac* and *Cry*2*A* standards for respective kits on each ELISA plate and expresses as µg/g of fresh tissue weight.

H. mRNA Expression Analysis of Transgenic Cotton Plants

1. Total RNA Extraction

Total RNA was extracted from transgenic cotton leaves according to the method described by [17]. Newly emerging leaves of transgenic cotton were ground in liquid nitrogen using pestle and mortar. Fine powdered was shifted into two 1.5 ml microcentrifuge tubes having 750 µl of preheated (65^{o}C) extraction buffer-I. Tubes were vortexed briefly and incubated at 65^{o}C for 15 minutes with occasional shaking. Equal volume of chloroform isoamylalcohol, vortexed was added and centrifuged at 13000rpm for 10 minutes at room temperature and supernatant was transferred to new microcentrifuge tube. Repeat the last step and 750 µl of extraction buffer-II was added to the supernatant and vortexed a briefly. Added 200µl of chloroform into the tube, vortexed and incubated for 10 minutes at room temperature. Later, samples were centrifuged at 13000 rpm for 10 minutes at 4^{o}C. Top aqueous layer was taken in a new tube carefully; isopropanol was added and vortexed briefly and incubated at room temperature for 10 minutes. Centrifuged at 13000 rpm for 10 minutes at 4^{o}C and decanted the supernatant. RNA

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pellet was washed with 70% ethanol and air dried. Pellet was dissolved in DEPC treated autoclaved water and stored at -70°C for future use.

I. Quantitative Real Time PCR

The expression of selected genes was measured by real-time PCR (quantitative mRNA expression) with very high accuracy. The quantitative mRNA expression of these genes was measured by copying it to cDNA through reverse transcriptions using Fermentas cDNA synthesis kit.

J. qRT-PCR

Pairs of primers used for the quantitative real time PCR of the transgenic plants was as follows;

EPSP synthase:

Forward Primer: 3' TATGGCTTCCGCTCACGT 5'

Reverse Primer: 3'AGCATCTTCTCAGTGTGGTCTCT 5'

*Cry*1*Ac:*

Forward Primer: 5'-GAAGGAGTGGATGGAGTGGA-3'

Reverse Primer: 5'-GCGGTCTGGTAGGTGTTGAT-3'

Real-time PCR reactions were performed in iQ5 cycler (Bio-RAD) with 96 well plate (Bio-RAD) using IQTM SYBER-Green Super mix (Bio-RAD). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control and 50 ng of cDNA was used in each reaction.

The reaction conditions for *EPSP synthase* were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s and final elongation step at 72 °C for 10 min. A melting curves analysis were carried out by continuously monitoring fluorescence between 57.5°C to 95°C with 0.5°C increments every 45s.

Statistical analysis of results was performed with the help of iQ5 software (Bio-RAD) version 1.0 on bases of CT values of gene in different samples converted to their linear form normalized with GAPDH gene. For variance ANOVA was performed to analyze significant difference in transcript expression in leaves of control and experimental cotton plants.

III. Results

A. Glyphosate Spray Assay

The transgenic plants of cotton with stacked genes were sprayed with roundup ready glyphosate at the rate of 1600ml/ha. The plants were thus screened initially on the presence and absence of glyphosate tolerant gene (GTGene/*EPSP synthase*gene). Fig. 1 indicated results of weeds and transgenic cotton plants before and after roundup ready glyphosate spray assay. Before spray weeds and transgenic cotton plants are survive but after 7 days of spray, weeds were killed with the effect of spray in the tunnel trail.

B. Genomic DNA Isolation from Leaves

The DNA isolation from the leaves samples from the T_3 generation of triple gene transgenic cotton was carried out with CTAB method and the DNA, thus isolated was run on 0.8% agarose gel with λ/Hin*d*III ladder. Fig. 2 shows the presence of DNA in the samples and the concentration of DNA in each samples was determined by comparing with the λ/Hin*d*III ladder. This isolated DNA was used for carrying out further analyses of gene amplification.



Fig. 1 Condition of transgenic cotton plants after 07 days of Round-up Ready Glyphosate spray (at the rate of 1600ml/ha) in the tunnel trails at T_3 generation Arrow indicated transgenic cotton plants survive after glyphosate spray assay

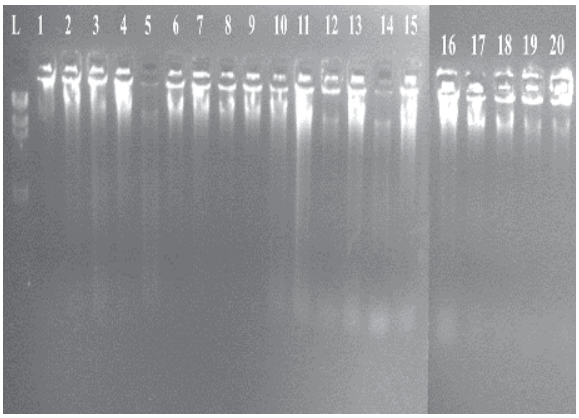


Fig. 2 Isolation of genomic DNA from the leaf samples of the triple gene transgenic cotton plants: Lane L: λ/Hin*d*III ladder; Lanes 1-20: Plant samples P-01 to P-20 in sequence, DNA was present in all the samples with varying concentrations

C. Amplification of Cry1Ac

The polymerase chain reaction was performed with the objective of confirmation of transformation and presence of *Cry*1*Ac* gene. Orientation primers were designed and polymerase chain reaction with the *Cry*1*Ac* primers amplified international fragment of the *Cry*1*Ac* gene in 17 out of 20 samples. The genomic DNA isolated in previous step in methodology was used for this PCR. The bands were clearly visible at the product size of 162 bp and the samples were run with 100 bp ladder on 2% agarose gel. The result showed negation for presence of *Cry*1*Ac* gene in samples no. 01, 17 and 18 as shown in Fig. 3.

D. Amplification of EPSP Synthase

In the polymerase chain reaction for the purpose of amplification of *EPSP* synthase (GTGene) in the transformed cotton sample, orientation primers were designed and deployed for the amplification of internal segment of that gene. The polymerase chain reaction with the DNA isolated

previously showed positive bands at 230 bp when run with 100 bp ladder in 2% agarose gel as shown in Fig. 4.

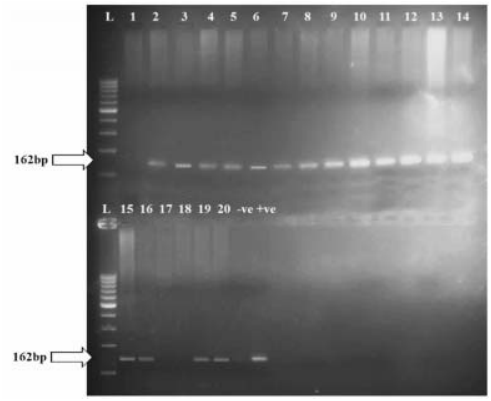


Fig. 3 Amplification of the DNA isolated from the transformed cotton with *Cry*1*Ac* primers showing bands at ~162 bp when run with 100 bp Ladder: Lane L= 100 bp ladder; Lanes 1 through 20 = plant samples P-01 through P-20. *Pk*2*Ac* containing plasmid was +ve control

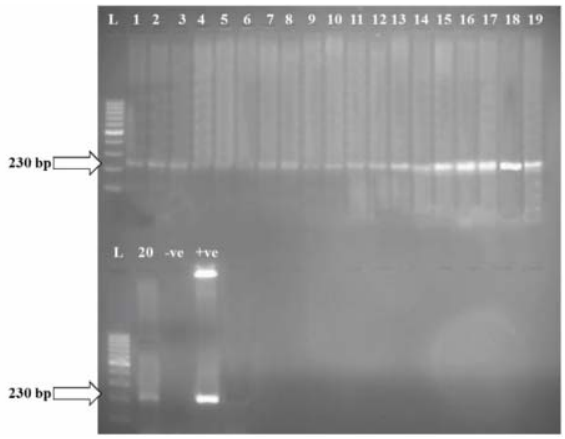


Fig. 4 Amplification of the *EPSP synthase* gene from the DNA isolated from transformed cotton showing bands at ~230 bp when run with 100 bp ladder: Lane L= 100 bp ladder; Lanes 1 through 20 = plant samples P-01 through P-20, All samples were positive, having *EPSP synthase* gene and shown bands at ~230 bp fragment size whereas, –ve control did not show any band. A plasmid with *EPSP* synthase gene was used as +ve control

E. Amplification Chain Reaction of Cry2A

The polymerase chain reaction for the confirmation of presence of *Cry*2*A* gene in the plant samples was carried out with the orientation primers designed for the amplification of internal segment of size 580 bp. The DNA isolated in the methodology section was used for the amplification of the DNA segment. The product of reaction was run with 1 kb ladder. Only 10 out of 20 samples were detected positive with amplification of *Cry*2*A* gene segment as shown in Fig. 5.

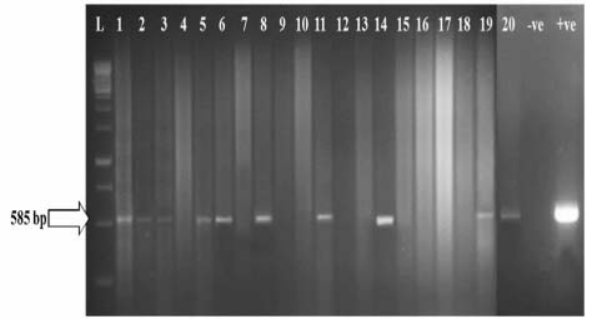


Fig. 5 Amplification of the *Cry*2*A* gene from the DNA isolated from transformed cotton showing bands at ~580 bp when run with 1kb ladder: Lane L= 1kb ladder; Lanes 1 through 20 = plant samples P-01 through P-20. 10 out 20 plant samples were confirmed positive for presence of *Cry*2*A*.*Pk*2*Ac* containing plasmid was +ve control

TABLE I
SUMMARY OF SELECTED TRANSGENIC COTTON PLANTS ON THE BASE MOLECULAR ANALYSIS FOR FURTHER EXPRESSION ANALYSIS

Sr. No.	Sample No.	Amplification With			Remarks
		*Cry*1*Ac*	*EPSP Synthase*	*Cry*2*A*	
1.	P-01	-	+	+	selected for further analyses
2.	P-02	+	+	+	selected for further analyses
3.	P-03	+	+	+	selected for further analyses
4.	P-04	+	+	-	not selected
5.	P-05	+	+	+	selected for further analyses
6.	P-06	+	+	+	selected for further analyses
7.	P-07	+	+	-	not selected
8.	P-08	+	+	+	selected for further analyses
9.	P-09	+	+	-	not selected
10.	P-10	+	+	-	not selected
11.	P-11	+	+	+	selected for further analyses
12.	P-12	+	+	-	not selected
13.	P-13	+	+	-	not selected
14.	P-14	+	+	+	selected for further analyses
15.	P-15	+	+	+	not selected
16.	P-16	+	+	-	not selected
17.	P-17	-	+	-	not selected
18.	P-18	-	+	-	not selected
19.	P-19	+	+	+	selected for further analyses
20.	P-20	+	+	+	selected for further analyses

F. Enzyme Linked Immuno-Sorbent Assay

ELISA was performed for the confirmation of gene expression of the transformed genes. The quantification of *Cry*1*AC*, *EPSP* and Cry2A protein is shown in Fig. 6.

G. Quantitative Real-Time PCR

Presently, the standard protocol was used for mRNA expression analysis that offered best sensitivity dynamic range and reproducible of any standard technique was quantitative Real Time PCR. In qRT PCR mRNA transcripts were reverse transcribed into cDNA using oligo (dt) random oligomer in the first step. The cDNA of EPSP synthase and Cry1Ac was then exponentially amplified through PCR using gene specific PCR. The concentration of amplicon in reaction was monitored with cyber green dye. cDNA of ten transgenic plants with EPSP synthase and nine samples of Cry1Ac(out of ten selected samples which were confirmed positive via

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amplification with gene specific primers) were quantification by real time PCR. As shown in Figs. 7 and 8, the transgenic plants have higher expression level as compared to the control.

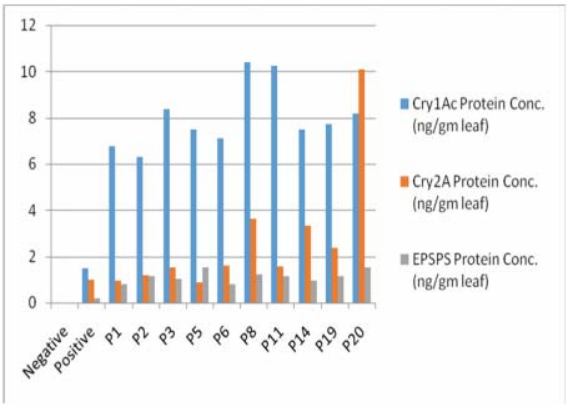


Fig. 6 Comparison of protein expression of transgenic cotton plants containing all three genes Cry1Ac, Cry2A and *EPSP*S genes

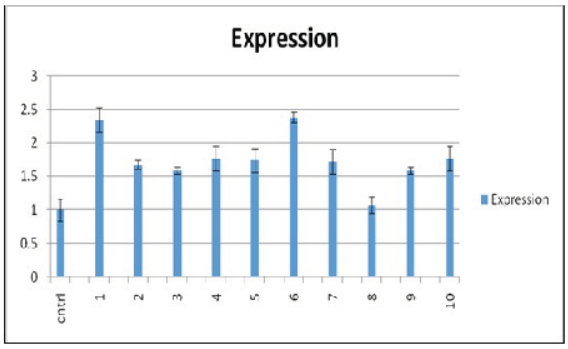


Fig. 7 Quantitative Real Time PCR of mRNA isolated from leaves of transgenic cotton plants (*EPSP synthase* gene): Control lane represents the expression level of control plant samples (mRNA of untransformed cotton) and lanes 1 through 10 describe the different expression levels of transgenic mRNA samples, indicating the expression of putative transgenic plants (mRNA of transgenic cotton), GAPDH was used as an internal control for normalization

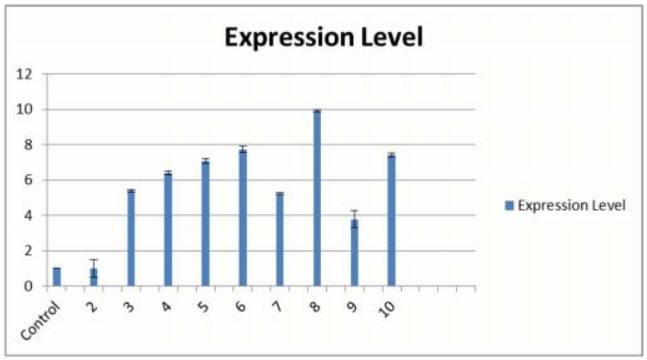


Fig. 8 Quantitative Real Time PCR of mRNA isolated from leaves of transgenic cotton plants (*Cry*1*Ac* gene): Control lane represents the expression level of control plant samples (mRNA of untransformed cotton) and lanes 2 through 10 describe the different expression levels of transgenic mRNA samples, indicating the expression of putative transgenic plants (mRNA of transgenic cotton), GAPDH was used as an internal control for normalization

IV. Discussion

The objectives of the research were to produce such improved transgenic cotton with stacked genes which is not only resistant to the attack of a broad range of lepidopteran insects but it should also have resistance against the commonly used broad-leaf herbicide spray (i.e. Roundup). For this purpose, the local cotton variety MNH-786 was transformed with Cry1Ac, Cry2A and EPSP synthase gene. Then successful transformed plants were screened and selected in the laboratory conditions and later under tunnel conditions (Fig. 1). The study revolved around molecular characterization of the third generation (T3) of the progeny of these plants grown under tunnel conditions.

The plants in the tunnel were firstly sprayed with glyphosate spray which screened the plants which were positive for EPSP synthase and the leaves of such plants showed no damage, the results and the study was in accordance with the work of [23]. Resistance against glyphosate spray is a crucial trait for the survival of the cotton crops since broad-leaf herbicide sprays are very common in the production technology for cotton. The crops transformed with EPSP synthase gene (Fig. 1) are more successful in terms of yield per acre and quality of yield. Hence, glyphosate tolerance has become an essential part of modern genetic engineering projects involving improvement in cotton genome [2], [18], [21]. Twenty plants were selected after 07 days of glyphosate spray assay on the morphological basis. The plants without any kind of leaf burn due to spray were selected and passed through molecular characterization for confirmation of presence and expression of the three aforementioned genes. DNA based method for detection of transgenes was followed for the confirmation of presence of foreign DNA in the cotton genome [24].

The DNA was isolated from the young and fresh emerging leaves of these plants and then amplified with the internal primers for all these three genes (Fig. 2). EPSP synthase was present invariably in all of the plant samples (Fig. 4) which confirmed our initial observation of resistance to glyphosate spray [10]. In the amplification of Cry1Ac gene, seventeen out of twenty plants samples were confirmed positive (Fig. 3) but in the amplification of Cry2A gene (Fig. 5), only ten out of twenty plants samples were confirmed positive for the presence of Cry2A gene. And these ten plant samples (which were confirmed positive with Cry2A; since the transgenic plants with Cry2A were least commonly) were further selected and subjected to protein expression analysis (Table I). Since, insect resistance against common insects is strengthened with introduction of more than one Cry genes in the cotton genome. Hence, the thought of transforming cotton with multiple Cry proteins caught ground and Cry2A was a novel choice for this purpose [4], [8], [12], [15], [20]. This pyramided cotton trait has also been sought with other combinations of insecticidal genes with Cry1Ac such as Vip3A was important combination according to the work by [13] and this had improved the overall efficacy against the target insects and widened the target insects' range [1].

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The presence of gene through amplification of the gene was further confirmed through protein based analysis viz., enzyme linked immune-sorbent assay (ELISA) for the three aforementioned genes (Fig. 6). The proteins were extracted and analyzed with UV spectrophotometer at OD 450nm. The results of ELISA (Fig. 6) were in accordance with the results of amplification through polymerase chain reaction indicating the presence as well as expression of the three genes in the screened plant samples. These results were in accordance with [4]. The results of quantitative real time PCR for EPSP synthase and Cry1Ac genes are also strong indicative of elevated expression levels of these gene in all of the transgenic plant samples (Figs. 7, 8) and these results were in accordance with the results obtained from the real time quantitative evaluation of performance of Cry1Ac by [3] and for the glyphosate tolerance in Mon1445 and Mon531 by [22].

Hence, through the overall molecular characterization enables to screen the plants for collection of seeds and raising of next generation from these plants (namely P-02, P-03, P-06, P-08, P-11, P-14, P-19 and P-20), which have confirmed presence and expression of the Cry1Ac, EPSP and Cry2A genes (Table I). The performance of these selected plants was also satisfactory under tunnel conditions and they showed good morphological traits and their molecular analyses have also been documented. In the light of the previous studies and the present work, it can be concluded that transgenic lines having three stacked gene are superior to plants having only one gene, these plants screened are The most suitable choice for raising the next generation which would have putative genes and significant expression level of these genes to raise a uniform generation of plants which could be realized into a new cotton variety in the due time [2], [15], [16].

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