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Trackable CEMB-Klean Cotton Transgenic Technology: Affordable Climate Neutral Agri-biotech Industrialization for Developing Countries

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Abstract

Background: Transgenic technology reflects the incorporation of novel useful traits in crop plants like cotton for economic benefits by overcoming the problems including insects' pests and weeds in special. The present study is the success story of the continuous effort of CEMB team started back in the 1990s.

Methods: This study includes characterization of a large number of *Bacillus thuringiensis* (Bt) strains taken from local soil and subjected to direct transformation of isolated BT genes into local cotton cultivars. Protocols for transformation into cotton plants were optimized and validated by the development of double gene codon optimized (Cry1Ac and Cry2A) transgenic cotton varieties.

Results: The resulting GMOs in the form of CEMB-33, CA-12, CEMB-66 have been approved by Punjab Seed Council in 2013 and 2016 respectively. Double Bt and weedicide resistant cotton harboring CEMB-Modified and codon optimized cp4EPSPS (GTGene). These varieties can tolerate glyphosate spray @ 1900ml per acre without the appearance of necrotic spots/shedding and complete removal of all surrounding weeds in the cotton field is a significant advance to boost cotton production without spending much on insecticides and herbicides.

Conclusion: In the current report, two unique sets of primers which amplify 1.1 Kb for CEMB-double Bt genes and 660 bp product for CEMB-Modified cp4EPSPS (GTGene) were tested. CEMB cotton variety CKC-01 is specially designed as low cost and easy to use by local farmer's technology has the potential to revolutionize the cotton growing culture of the country.



Introduction

Cotton crop is the main fiber source and an important cash crop of Pakistan that provides raw material to the textile industry [1]. It contributes up to 60% of the foreign exchange annually, with 8.2% of the value added in agriculture that counts about 2% to total GDP [2]. Pakistan is the 4th top producer of cotton in the world, yet in terms of yield, it is at the distant 10th position [3,4]. Biotic stress factors like an attack of insect pest, an infestation of the cotton leaf curl virus diseases and weeds are the keys factors responsible for the lower cotton production [5,6]. In Pakistan, approximately more than 150 species of chewing and sucking insect pests attack different stages of cotton and caused loses up to 2.5 million bales annually [3]. Cotton bollworm (*Helicoverpa armigera*), pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Earias insulana*), and armyworm (*Spodoptera littoralis*) fall in chewing category while whitefly (*Bemisia tabaci*), thrips (*Thrips tabaci*), jassid (*Amrasca devastans*), and aphids (*Aphis gossypii*) are mainly included in sucking category [7]. Traditionally the insect pest management mainly relies on the use of chemicals of worth \$8.1 billion per annum globally and out of these, nearly \$2.7 billion are spent only for cotton pest management. Non-judicious and frequent use of pesticide not only results in the development of the resistance in the targeted insect pest but also indirectly effects human health as well as environment.

Weeds infestation is another challenge for cotton crop production that accounts for 37% of yield loss annually [2,8,9]. Weeds not only compete with the cultivated plants for water, sunlight, and available nutrients but also provides shelter and food for plant pathogens. Mechanical and chemical methods including manual hoeing and herbicide spray are time-consuming, labor-intensive and expensive ways of weeds removing. Up until now, many conventional strategies have been devised to incorporate the blood from existing germplasm that all end up with little success due to the lack of resistant gene pool in available genotypes. The use of advanced technologies provides us an opportunity to genetically engineered the plant with enhanced protection, production and with greater nutritional value in a possibly short time [2,7,10,11]. These genetic modifications break the sexual incompatibilities barrier among the cross-species and

enormously increase the size of the available gene pool from the prokaryotic or viral origin [12].

Bacillus thuringiensis is a Gram-positive spore-forming bacterium with entomopathogenic properties and a long history of safe use as a sprayable biopesticide [13]. Parasporally formed crystals are predominantly composed of one or more proteins, which penetrate epithelial cells of the insect midgut by inserting pores into the plasma membrane. These proteins also are known as Bt toxin which is harmful to the larvae of moths, butterflies, beetles, and flies. When insects feed on the plant, the toxin enters the body and binds to the insect's gut. Hence, it disrupts its feeding and digestion process and eventually leads to the death of the insect. Various studies and field trials on the crop plants that harbor the Cry portions in their leaves from *Bacillus thuringiensis* have shown the effectiveness as an alternative to the synthetic insecticides [9,10,14]. Glyphosate (N-phosphono methyl glycine), is a non-selective potent herbicide which interferes in the shikimate metabolic pathway by inhibiting the synthesis of 5-enolpyruvyl- 3-phosphoshikimate (EPSPS). This disruption prevents the synthesis of the three important aromatic amino acids, phenylalanine, tryptophan, and tyrosine and is subsequently leads to plant death (Herrmann and Weaver, 1999). Stable transformation of the bacterial gene encoding EPSPS protein in the plant can make the plant resistant against glyphosate weedicide spray. Currently, the most widely accepted genetically modified traits in GM crops are herbicide tolerance (HT) and insect resistance (IR). GM soybean, maize, canola, and cotton are the most common examples of these crops in the market [15,16]. Currently, USA is on the top of the list of world GMO crop producer with 70.9 million ha of cropland followed by Brazil (44.2 million hectares), Argentina 24.3 million ha and India 11.6 million and Canada 11.0 million ha respectively. The use of GM cotton plant in India has registered phenomenal growth in cotton production and topped the world with 95% resilient adoption rate [7].

Keeping in view the potential of above-mentioned genetic resources, for the first time in Pakistan a transformation of Cry1Ac and Cry2A in cotton was started in early 2000. Although the efficiency and characterization of these Cry proteins had studied been studied and confirmed by the CEMB researchers in the early 90s [17-20]. A single construct harboring the CEMB Cry1Ac+Cry2A driven by *CaMV35S* promoter,

and *nptII* gene was transformed in local cultivar CIM-482 of *Gossypium hirsutum*. Later, Rashid *et al.*, reported the stable advanced event of this transformation and name as CEMB-02, which got NBC approval in 2009 and subsequent commercialization license in 2016. Azam *et al.*, [21] reported the dissemination of CEMB-02 derived Bt cultivar on all-over the cotton growing belt of Pakistan that raised the local farmer's profit mostly by low or no seed cost decreasing the pesticides expenditures to control the pest attack [22]. CEMB has also been continuously conducting risk assessment studies since 2000 of the CEMB 02 event harboring the double gene Bt on different organisms including Mice, Fish, Earthworm, Chicks and Rabbits along with gene flow studies including horizontal and vertical gene flow which results in delaying their efforts to be brought in front of public [21].

CEMB started work on the development of herbicide tolerance in *Gossypium hirsutum* to save the yield losses by weed infestation. [8] reported the stable transformation of synthetic codon-optimized 5-enolpyruvylshikimate-3-phosphate synthase gene cloned into pCAMBIA 1301 vector under a 35S promoter with *Agrobacterium tumefaciens* in CEMB-02 event having the Cry1Ac+Cry2A genes in a single cassette. They also further evaluated the transgenic line harboring the Cp4 EPSP transgene line by glyphosate spray at the concentration of 1900ml/acre. one of the advance lines that pass the spray and insect bioassay with stable transgene expression was named as CEMB-Klean Cotton. In this study, to address the regulatory and effective labeling issues, we reported the PCR based detection of the CEMB-Klean cotton event by using the two set of unique and specific primers pairs and also confirmed the through the ELISA for protein quantification of the transgene.

Methods

Testing Material and Tissue Sampling

The 40 days old grown homozygous lines of CEMB-Klean cotton transgenic plants as testing material were obtained from CEMB cotton research substation Multan for molecular analysis. leaf samples; newly emerged leaf from the top; mature leaf from the middle; and older leaf from the bottom of six plants were

cleaned with ddH₂O, harvested with a sterile scissor from each plant separately and immersed into liquid nitrogen. The leaves of non-transgenic cultivar CIM-482 were used as control samples. These samples were then ground well into a very fine powder and stored at -80C till further molecular investigations.

Isolation of gDNA and PCR Analysis of CEMB-Klean Cotton Plants

In 2019 it does not make much sense to write protocols in such a detail naming the protocol would be enough. In my opinion, the DNA isolation and PCR analysis should be combined and shortened significantly.

Modified CTAB based DNA isolation method was used with slight modification as mentioned by the Shah *et al.* Briefly as, the grounded samples were mixed with pre-heated CTAB buffer having freshly added 1% in ratio Mercaptoethanol, incubated at 65 °C for thirty min with vigorous shaking on vortex mixer each after 5 min. A pre-chilled equal volume of Chloroform-Isoamyl alcohol (24:1) (sigma#C0549-1QT) was added to the incubated samples and mixed thoroughly by inverting the tubes. For supernatant separation centrifugation of the mixed samples was carried out at 10K rpm for 30 min at 4°C. The obtained supernatant was mixed with double volume of pre-chilled ethanol (Merk # 64-17-5) and 1/10 volume of 3M sodium acetate (pH:5.2) (sigma# S7899-100ML), incubated at -70°C for 5 h for genomic DNA precipitation. For pellet formation, the incubated samples were further centrifugated at 12k rpm for 30 min at 4 °C. gDNA pellet washing was carried out by 70% ethanol and purified by the genomic DNA purification kit (Cat#: K0512) by Thermo Scientific as per manufacturer protocol. Total RNA contamination from the extracted DNA was removed by using the RNase A solution by the Thermo Scientific (Cat# EN0531) as per manufacturer protocol. The DNA integrity was accessed by the gel electrophoresis while Quantity and purity were accessed by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc) by measuring the absorbance at 260nm (Table-1).

The specific amplification of the CEMB CryAc+Cry2A and GTG genes from the CEMB-Klean cotton plants were carried out by using the specifically designed primers. Table 2 shown the primer sequences with their expected amplicon size. These primers were designed by using the Primer Premier 6.0 primer

designing software and synthesized from the Eurofins Oligo synthesis facility Pvt. Ltd. The total 25 μ l reaction volume was prepared for a single conventional PCR reaction using the 10XPfu+MgSO₄ PCR reaction buffer (Thermo Scientific™ cat# 10202830). Veriti™ 96-well Thermal Cycler (Thermo Scientific Cat#4375786) was programmed as; initial denaturation for 3min at 95 °C, followed by the 35 cycles with cyclic condition includes denaturation 95 °C for 30 sec, annealing at 52 °C for CEMB-02 primer and 51°C for GTG primer for 30 sec, followed by the cyclic extension at 72°C for 30 sec. At the last stage, a final step of the final extension for 10 min at 72°C was added. The amplified products were further resolved on 1.5% agarose gel prepared in the 1x TAE buffer, stained with “ethidium bromide” and visualized by gel documentation system (UVP GelTower: Analytik Jena the US).

Sample ID	Concentration (ng μ l ⁻¹)	260/280	260/230
Plant 1	700	2.1	2.01
Plant 2	650	2.2	2.00
Plant 3	720	1.89	1.88
Plant 4	800	1.85	2.01
Plant 5	515	1.88	1.88
Plant 6	650	1.89	1.89

Table 1: Concentration and purity of the gDNA extracted from the CEMB-Klean cotton leaf tissue.

Primer ID	Sequence (5'----3')	Amplicon Size
Cry1Ac	F: TTCTCTTCAACGGTTCTGTCAT	382 bp
	R: ACTGGAATGAACTCGAATCTGT	
Cry2A	F: CCAGGCTCCATGACATGCTCGA	730 bp
	R: TCGTTGGCGGCGTAGATGTTGT	
CEMB-02	F: GTTCCAGCTACAGCTACCTCCT	1.1kb
	R: GTCGGTCTCTTCCACTCCATC	
CEMB-GTG	F: CAGCCTGAATGGCGAATG	660 bp
	R: TTCTAGCAGTAGCAGGTCTG	

Table 2: Primer sequences with their respective amplicon size used for detection of Cry1Ac, Cry2A, Cry1Ac+Cry2A and GTG genes by PCR analysis from transgenic CEMB-Klean cotton and non-transgenic plants.

ELISA Immunoassay of CEMB-Klean Cotton Plants

Enzyme-linked immunosorbent assay (ELISA) was carried out to determine the expression of CEMB Cry1Ac+Cry2A and GTG genes at the protein level. Young, mature and old leaves tissues from the top, middle, and bottom of CEMB-Klean cotton plants were harvested, crushed well in liquid nitrogen and incubated on ice for 1 hour in 1ml protein extraction buffer [1].

Extracted crude protein was harvested by the centrifugation of the incubated samples at 13k rpm for min at 4 °C. Gene-specific protein quantification was carried out by using the Envirologix ELISA kit (Cat. # 051) by following the manufacturer's protocol.

Results

Genomic DNA Extraction and Quality Evaluation

The Modified (Cetyltrimethylammonium-bromide) CTAB method followed by the column-based purification was used to extract the good quality of DNA from the well ground stored samples. The extracted yield of DNA from all the samples was in the range of 520 to 800 ng/ μ l. Qualitative 260/280 and 260/230 ratios for all the samples were in the range of 1.85 to 2.01 (Table 1). The integrity and quality of the extracted DNA with intact bands are shown in Figure 1.

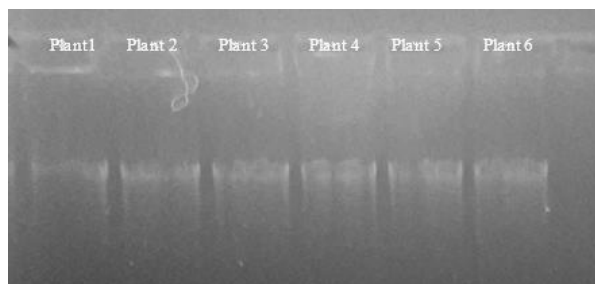


Figure 1: Isolated Genomic DNA from the leaf samples of CEMB-Klean Cotton transgenic plants.

PCR Based Detection of CEMB Cry1Ac+Cry2A and GTG Genes with Specific Primers

The CEMB-Klean cotton transgenic plants contain locally isolated Cry1Ac and Cry2A genes in a single construct [14] and GTG gene as a separate event in a different construct that was transformed and evaluated previously [8]. In this study, we targeted the CEMB-Klean Cotton transgenic plants and CIM-482 non-transgenic plants to check the integration of the stable transgene at the DNA level by using specific primers. Initially, an amplification of the Cry1Ac and Cry2A genes was carried out by gene-specific primers. The Cry1Ac specific primer for Cry1Ac Bt gene and Cry2A specific primer for Cry2A Bt gene amplified both genes independently and recorded the 382 bp and 730 bp amplification respectively (Figure 2). The CEMB-02 primer generated the 1.1kb specific product from all tested samples. The CEMB-GTG primer amplified the 660 bp band from the transgenic plants (Figure 3). Both these primers gave the unique amplification for the

CEMB Klean-cotton harboring transgene while no amplification was observed from the control plants. Therefore, both these primer (CEMB-02 and CEMB-GTG) could be used to screen out the CEMB-Klean cotton transgenic plants from mixed populations. No non-specific amplification was observed by these primers on the gel (Figure 2-3).

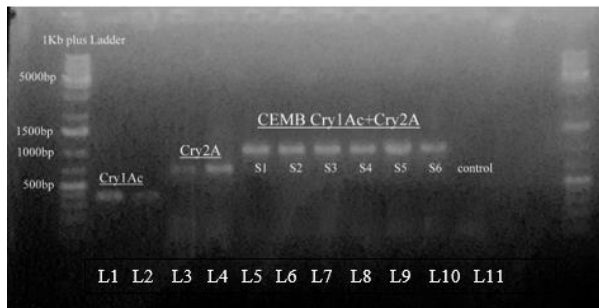


Figure 2: PCR amplification of the transgenes harboring CEMB-Klean Cotton plants. Lane 1 and Lane 2 represent the amplification of the Cry1Ac Bt genes (382 bp) by using Cry1Ac primer. Lane 3 and Lane 4 represents the amplification of the Cry2A Bt gene at 730 bp by using the Cry2A primer. Lane 5 to Lane 10 represents the specific amplification of the CEMB Cry1Ac+Cry2A gene from the CEMB-Klean Cotton transgenic plants by Specific primers (Table1). No amplification was observed in the control plants (L11). 1Kb plus ladder was used to measure the amplified products.

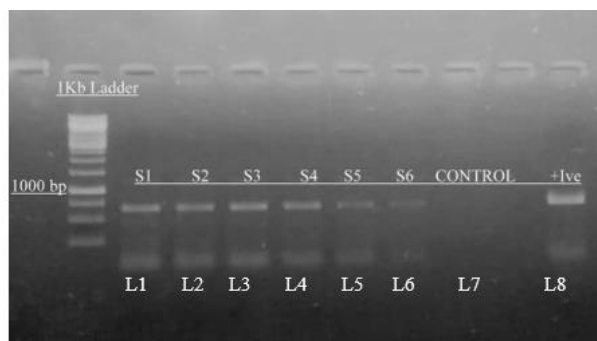


Figure 3: GTG gene amplification by CEMB-GTG primer from the isolated DNA of the CEMB-Klean Cotton Transgenic plants showing amplification of 660 bp of the product (Lane 1 to Lane 6). No amplification was recorded in control plants (Lane 7). Lane 8 represents the amplification from the +ve plasmid DNA. 1Kb ladder was used to measure the amplified products.

Transgenes Protein Estimation Through ELISA

Enzyme-linked immunosorbent assay (ELISA) procedure was used to detect the protein concentration of the Cry1Ac, Cry2A, cp4EPSP transgene in the transgenic plants. The quantification of the proteins encoded by the studied transgenes are shown in Figure 4. The concentration of Cry1Ac ranged from 2-95 to 3.5 ng g⁻¹ total soluble protein, the concentration of cry2A

ranged from 2.8 to 3.8 ng g⁻¹ total soluble protein, while the concentration of cp4EPSP ranged from 2.8 to 3.2 ng g⁻¹ total soluble protein in different transgenic lines (Figure 4).

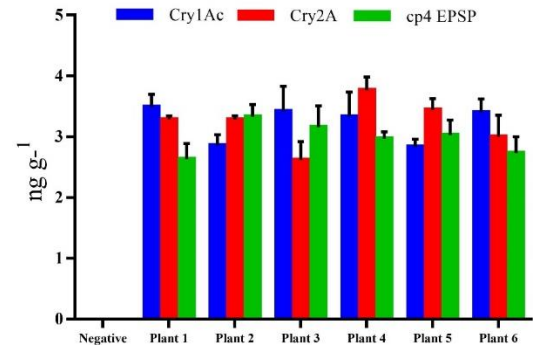


Figure 4: Protein concentration determination (ng g⁻¹ of total soluble protein) of the Cry1Ac, Cry2A, cp4EPSP (GTGene) in the leaf tissue of the CEMB-Klean Cotton transgenic plants by ELISA.

Discussion

The agriculture sector is the main contributor to the GDP of Pakistan. Cotton is one of the main cash crops, provides raw material to the textile and edible oil industry and considered as a backbone of the economy [11]. Farmers bear the major losses in the production of cotton in recent past due to biotic and abiotic factors like an attack of insect pests, weeds infestation and viruses attack [4]. The use of genetic engineering able the researcher to establish the novel environment-friendly agricultural practices to manage the yield affecting factors [7]. The effective way is to develop the genetically engineered crop plants with improved insect resistance and herbicide tolerance characteristics to reduce the yield losses by an insect pest, labor cost and chemical application [1,2,9-11]. Isolation and characterization of the most efficient gene is prerequisite for crop improvement through genetic engineering. Cry proteins are endotoxin in nature encodes by the soil bacterium *Bacillus thuringiensis* and used to provide resistance against the chewing insect pest [24]. [20,25] carried out the intensive characterization of the Cry proteins from locally isolated strains, record their effectiveness against the insect pests and established an efficient protocol to transform the transgenes via Agrobacterium-mediated transformation method in crop plants in the early 1990s at Center of excellence in molecular biology (CEMB), University of the Punjab, Lahore, Pakistan. Based on

these studies Cry1Ac and Cry2A genes were locally isolated cloned in a single cassette and transformed in the *Gossypium hirsutum* plants in the early 2000s. The stable expression of these genes in the transgenic cotton plants has shown the high mortality rate of chewing insect in homozygous lines due to Synergist effects of Cry1Ac+Cry2A [14]. The combined effect of the endotoxin encoding by more than one gene not only provides the effective protection layer against the insects but also diminishes the chance of resistance development towards the toxin among the insect population [33].

Weeds are the unwanted plants that cause 30% losses in cotton plants by consuming the available moisture, light, and nutrients [8]. These also harbor and provide shelter to insect pest on off season [11]. The conventional way of weeds management is expensive and laborious. Glyphosate (N-phosphonomethylglycine) is a broad-spectrum herbicide that inhibits the 5-enolpyruvyls-Shikimate-3-phosphate (EPSP) synthase pathway. This inhibition ceases the production of aromatic amino acids like phenylalanine, tryptophan, and tyrosine and halts plants growth [2]. The integration and overexpression of bacterial origin glyphosate tolerant gene (GTGene) able the plants to resist the glyphosate spray by encoding the 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) enzyme. [8] initially reported the stable transformation of GTGene in the *Gossypium hirsutum* transgenic cultivar (CEMB-02) at CEMB and evaluated under glyphosate spray effectively. Later on [2,10] also shown the GTGene efficiency for herbicide tolerance in transgenic cotton plants. The efficiently engineered crop plants should contain a single copy of the transgene in each cell [7]. The detection of the transgene(s) and its quantification in the genetically engineered organism is critical for acceptance and commercialization [34]. The proper detection system to trace the GM contents in the crop plants, molecular characterization of transgene, point of insertion and localization information are the mandatory requirements for their release in many countries like members of EU, India, Australia, China, Japan, Korea, Russia, Taiwan and other before commercialization [35]. The strict implementation by the Cartagena protocol and Convention on Biological diversity also demand specific detection system and adequate level of protection layers before the handing and transfer of the GM materials [35-37]. With an

increasing number of product types and possible chance of mixing of GMOs, products have created the demand for the development of specific detection methods. To address the requirements of the regulatory bodies and commissions related to GM crops, DNA based detection methods are applied [7]. Detection of the transgene in the genetically modified plant by polymerase chain reaction is considered as a gold standard due to its sensitivity, cost-effectiveness and user-friendly ability [38]. Even a traceable amount of transgene can be detected by this approach. A number of reports cited the effectiveness of PCR approach to detect the transgene in cotton [11], maize, soybean, canola [39], rice [40] and GM sugar beet [41]. Here in this study, we establish a PCR protocol to detect the CEMB-Klean Cotton transgenic plants by using specific primers. The practice of this protocol with testified primers will support to fulfill the regulatory framework and policies before commercialization.

Here in this study we developed a protocol and reported the two unique set of primers, that detect the CEMB-Klean Cotton transgenic plants specifically by using the conventional polymerase chain reaction. This easy screening method could distinguish CEMB-02/CEMB-Klean Cotton and would be useful to monitor the spread of gene in mixed populations. Monitoring the protein concentration would help researchers to tackle silencing issues if any in proceeding generations. The CEMB-Klean Cotton will help cotton growers to get the benefit of locally developed low-cost technology.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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