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ORIGINAL ARTICLE

Enhancing the resilience of transgenic cotton for insect resistance

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Abstract

Background The efficacy of *Bt* crystal proteins has been compromised due to their extensive utilization in the field. The second-generation *Bt* vegetative insecticidal proteins could be the best-suited alternative to combat resistance build-up due to their broad range affinity with midgut receptors of insects.

Material and results The codon-optimized synthetic vegetative insecticidal proteins (Vip3Aa) gene under the control of CaMV35S promoter was transformed into a locally developed transgenic cotton variety (CKC-01) expressing *cry1Ac* and *cry2A* genes. Transformation efficiency of 1.63% was recorded. The highest Vip3Aa expression (51.98-fold) was found in MS3 transgenic cotton plant. Maximum Vip3Aa protein concentration (4.23 µg/mL) was calculated in transgenic cotton plant MS3 through ELISA. The transgenic cotton plant (MS3) showed one copy number on both chromatids in the homozygous form at chromosome 8 at the telophase stage. Almost 99% mortality of *H. armigera* was recorded in transgenic cotton plants expressing double crystal proteins pyramided with Vip3Aa gene as contrasted to transgenic cotton plant expressing only double crystal protein with 70% mortality.

Conclusions The results obtained during this study suggest that the combination of Bt *cry1Ac*, *cry2A*, and Vip3Aa toxins is the best possible alternative approach to combat chewing insects.

Keywords Cotton · Vegetative insecticidal proteins · Crystal proteins · Genetic improvement · Chewing insects

Introduction

Cotton is the major non-food cash crop of Pakistan, also well-renowned as "white gold", due to its 0.8% contribution to national GDP and 4.1% share in the agriculture sector. Pakistan is the 5th largest cotton cultivator; the 3rd major consumer and exporter in the world [1]. According to Pakistan economic survey for the FY 2019–20, cotton production was recorded to be 9.178 million bales which were 6.9% higher as compared to the previous year's yield of 9.861 million bales due to a significant increase in cultivated land. However, 618 kg/ha cotton yield was recorded for the year 2019–20 as compared to 707 kg/ha in the last year (Economic Survey of Pakistan, 2019–2020). The cotton plant is mainly affected by fifteen insect species that can be categorized as sucking and chewing insects and leads to poor fiber quality, low yield, and high production cost [2]. It has been projected that approximately 50-60% of losses are due to chewing insect infestations [3]. Different chemical insecticides have been extensively used to overcome these pests that not only damage the environment and human health but also create economical pressure on farmers. Moreover, these synthetics insecticides are non-specific and kill many beneficial insect species. More than 500 distinct insect kinds have been described in the literature to develop resistance to these chemical insecticides [4].

The genetic modification of crop plants with *Bt* insecticidal proteins has revolutionized agriculture biotechnology [5]. However, due to poor pest management strategies in the field, some insect species have developed resistance against these insecticidal crystal proteins. Some strains of *Bt* produce different vegetative insecticidal proteins (VIPs) in the culture medium during the vegetative growth phase [6]. The toxic effect of Vip3 protein is active against chewing insects [7] while SIPS toxin is effective against coleopteran insects [8]. The VIP toxins are categorised into four separate groups based on their amino acid sequence. The sequence homology

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of VIP toxins is completely different from any cry toxin [9]. The Vip1 and Vip2 are synergistically active against insects [10]. Some strains also secrete parasporal crystal protein which has non-specific targets referred to as parasporins [11]. Vip3 protein has no sequence homology with any other cry toxin and shows a broad spectrum of activity against lepidopterans [12]. The Vip3Aa have structurally different from any known crystal toxins [9]. These toxins have different receptor binding sites on the insect midgut epithelial cell line as compared to cry protein and show a broad spectrum of activity against many lepidopterans [13].

In Silico studies suggested that the Vip3 protein has α helices at the N-terminal region whereas β-helices and coils at the C-terminal region [14]. Under the electron microscope, the Vip3Ag4 structure looked like the protein may be folded together to form a tetrameric structure [15]. The N-terminal region of Vip3A protein contains some highly conserved amino acid sequence that is not processed during peptide secretion. These conserved sequences are responsible for the translocation of toxins into periplasmic space through the cell membrane. The N-terminal region of this protein is attached to membrane receptors and plays a critical role in protein folding [16]. The Vip3Aa proteins are highly target-specific due to the unpredictability in the C terminal region and the function of protein is lost due to the single mutation in the last few amino acids [17].

The mechanism of action of Vip3 protein is not discussed in literature due to the unidentified three-dimensional structures [18]. It is assumed that the attachment of Vip3 protein to its receptor and signalling mechanism across the membrane is entirely different from cry protein. The binding of the active Vip3Aa with *Ephestia kuehniella* receptor has been reported. However, the mechanism of action and sequence of Vip3Aa activation is the same as that of cry toxin i.e., the protein is activated by insect midgut protease that leads to the crossing of toxin across the peritrophic membrane, the toxin is attached to the membrane receptor on the surface of midgut epithelial cell line. The attachment of toxin to the receptor produces pours in the epithelial cells that ultimately lead to insect death [19]. The analysis of the gut cross-section of susceptible insects showed that Vip3Aa is responsible for the leakage of cellular material to the lumen and swollen epithelial cells [20].

The Vip3a gene has been successfully transformed in cotton and maize individually and with a combination of different fusion proteins like cry protein. These fusion proteins showed an improved defense mechanism of transgenic plants against insects [21]. The Vip3Aa19 was successfully individually transformed in cotton (COT102) and Vip3Aa20 protein in corn (MIR162) [22]. The combination of Vip3A has been transformed pyramided with cry1Fa (Agrisure Viptera= Vip3Aa + *cry1Ab* + *cry1Fa*) and (VipCot= Vip3Aa + *cry1Ac* + *cry1Fa*, and later with *cry1Ab* (Agrisure Viptera= Vip3Aa + *cry1Ab*) and (VipCot= Vip3Aa + c*ry1Ab*) that showed a broad spectrum of activity against lepidopterans [23]. The improved defense mechanism of combination studies help to design the current study (Vip3Aa + *cry1Ac, cry2A*, and GTG) against lepidopterans.

In this study, we have genetically transformed a codon-optimized synthetic *Bt* Vip3Aa gene cassette under constitutive CaMV35S promoter into cotton plants already expressing *cry1Ac* and *cry2A* toxins through *Agrobacterium*-mediated technology to compare the efficacy of double *Bt* toxins alone and in combination with Vip3Aa protein.

Materials and methods

Plant material

Gossypium hirsutum CEMB Klean Cotton (CKC-01) variety was selected for this study on account of its germination and heat tolerance in the field [24]. The seeds of the CKC-01 variety were provided by the seed biotechnology laboratory of the Centre of Excellence in Molecular Biology, the University of the Punjab on request.

Construction of recombinant vector

The full-length nucleotide sequences of Vip3Aa (JQ946639.1) were retrieved from the GenBank and its codon biases were optimized according to cotton (*Gossypium hirsutum*) genome through Gensmart software (https://www.genscript.com/gensmart-free-gene-codon-optimization.html). The full-length Vip3Aa gene cassette under CaMV35S promoter and *NOS* terminator with *XhoI* and *SacI* restriction sites were chemically synthesized by BioBasic Inc and provided in pUC57 cloning vector (https://www.biobasic.com/us/gene-splash-gene-in-vector/). The recombinant vector containing gene cassette was transformed into the competent cells of *E. coli* (strain Top 10) through the heat shock method. The DNA (500 ng) of recombinant plasmid was restricted with FastDigest *XhoI* and FastDigest *SacI* restriction enzymes (Cat# FD0694 & FD1134). The excised product was resolved on 0.8% agarose gel and purified through Thermo Scientific Gene JET Gel Extraction Kit (Cat# K0692). The Vip3Aa gene cassette was cloned into plant expression vector pCAMBIA-1301 by using *XhoI* and *SacI* restriction sites. The overhangs of the insert and vector were ligated through T4 DNA Ligase (Thermo Scientific Rapid DNA Ligation Kit, Cat# K1422) by following the instructions of the manufacturer. The map of the Vip3Aa gene into pCAMBIA-1301 is shown in Supp. Fig. 1.

Transformation and confirmation of pCAMBIA_ Vip3Aa

The ligated product was transformed in the competent cells of *E. coli* (Top 10) through the heat shock method [25]. The transformed bacterial cells were spread on the LB agar plates supplemented with100 mg/mL Kanamycin and Tetracycline. The plasmid DNA from transformed colonies was isolated using Monarch Miniprep Plasmid Extraction Kit (NEB#T1010) and used as a template to confirm the positive clones through thermocycler (PCR). Gene-specific detection primers [Vip3Aa Forward 5′-ATCACAGAACGGAGATGA GG-3′ and Vip3Aa Reverse 5′-GTGTACCTCCCGATCTAG TAAC-3′] were used in the PCR reaction mixture to amplify the Vip3Aa gene in positive clones.

The recombinant plasmid DNA was also confirmed through restriction digestion using *XhoI* and *SacI* restriction enzymes and subsequently transformed into 100 µL competent cell of *Agrobacterium tumefaciens* strain LBA 4404 using BioRad Gene Pulser Electroporator (Model 165-2105). The transformed cells were spread on a YEP agar medium containing 100 µg/mL Rifampicin and 50 µg/mL kanamycin. The *Agrobacterium* transformed colonies were screened through colony PCR before proceeding further to cotton transformation experiments.

Agrobacterium-mediated genetic transformation of cotton

The foreign gene cassette was transformed into the cotton genome through the *Agrobacterium*-mediated shoot apex method as reported by [26] *Agrobacterium* cells containing gene cassette (Vip3Aa) were dissolved into 10 mL MS-zero broth [27] (Thermo Fisher Scientific cat #50–254-673). The injured cotton embryos were co-cultivated with MS-zero broth in a rotary shaker at 28 °C for 2 h and transferred on MS medium plates containing 250 µg/mL cefotaxime (Thermo Fisher Scientific cat # 64,485-93-4) to avoid bacterial contamination. The 3–4 days old plantlets were then transferred into MS glass tubes supplemented with IBA (1 mg/mL) and Vitamin B5 complex. The transgenic cotton plants were moved into pots filled with autoclave loamy soil containing an equal volume of peat moss, sand, and clay and wrapped with transparent bags to maintain the humidity [26]. The acclimatized cotton plants were shifted to tunnels for better growth and development.

Molecular analyses of putative transgenic cotton plants

Total genomic DNA was extracted from putative transgenic cotton plants following the method of [28]. The integration of the Vip3Aa gene in the cotton genome was detected through PCR using previously mentioned detection primers. Total RNA was isolated from cotton leaves following the modified protocol established by [29]. Random hexamers were used for the synthesis of complementary DNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622). The mRNA expression of the Vip3Aa gene in transgenic cotton plants was evaluated through quantitative Real-Time (qRT-PCR) using StepOne™ Real-Time PCR System (Applied Biosystems) with a 48-well plate. The Oligo (dt) random primers were used to reverse transcribe the mRNA into complementary DNA. Gene-specific qRT-PCR primers [R-Vip3Aa Forward 5′-CGGCTCCCT TAATGATTTGA-3′] and [R-Vip3Aa Reverse 5′-CGATCT GCAATGAAAGAGCA-3′] were used in reaction mixture to amplify Vip3Aa gene in transgenic cotton plants. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as a comparative reference to analyse the data. The Vip3Aa protein in transgenic cotton plants was detected and quantified through Enzyme-Linked Immunosorbent Assay (ELISA) using Agdia Vip3Aa ELISA kit (Cat# PSP 83,500). The crude protein isolated from transgenic and non-transgenic cotton leaves was used as an antigen against the Vip3Aa antibody coated in the ELISA well plate. The sample O.D was taken at 650 nm through an ELISA plate reader.

Detection of transgenes

The foreign gene copy number and location in transgenic cotton plants were examined through Fluorescence *in situ* Hybridization (FISH). The fluorescent signal was detected by fluorescent microscope (Olympus Model BX61) using the blue filter for 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) dye. The fluorescent signal was detected using a CCD camera connected with the microscope and analyzed with Adobe Photoshop 7.0.

Insect mortality bioassays

The transgenic cotton plants of T_0 generation were exposed to leaf-detach insect bioassays to evaluate the efficacy of insecticidal toxins individually and in combination with Vip3Aa against cotton bollworm (*Helicoverpa armigera*). Two PCR positive transgenic cotton plants harbouring double crystal and Vip3Aa gene were selected and one transgenic plant expressing only double crystal gene as a positive control and non-transgenic cotton plant as a negative control was also used in this insect bioassay. One fresh leaf of non-transgenic and transgenic cotton plants was placed in petri dishes containing wet filter papers. Three 2nd instar larvae f cotton bollworm (*Helicoverpa armigera*) were released on cotton leaves. The sealed petri plates were put in a culture room at 25 ± 2 °C and 16 h light: 8 h

of the dark cycle along with ≈ 60% humidity. The percent mortality was calculated on the third day of infestation as reported by [26, 30].

Results

Confirmation and transformation of pCAMBIA_ Vip3Aa vector

Plant expression vector (pCAMBIA-1301) containing Vip3Aa gene cassette in positive clones was verified through PCR amplification using gene-specific detection primers. The PCR amplification of the 682 bp gene fragment shows the successful transformation of the gene cassette in the bacterial host (Supp. Fig. 2). The plasmid was further immobilized into *Agrobacterium tumefaciens* strain.

Molecular analyses of putative transgenic cotton plants

The incorporation of transgene cassette in the putative transgenic cotton plants was verified through polymerase chain reaction using Vip3Aa specific detection primers. The plasmid DNA isolated from positive clones was used as positive control while DNA extracted from non-transgenic cotton plants was used as a negative control. The PCR product was run on 1.5% agarose gel and visualized under ultraviolet light (Supp. Fig. 3). Total 65 acclimatized transgenic cotton plants were shifted to the tunnel, out of which 35 plants were confirmed through PCR with a transformation efficiency of 1.63%.

Quantification of mRNA expression in transgenic cotton plants

The mRNA expression of Vip3Aa in cotton plants was studied through quantitative real-time (qRT-PCR). The complementary DNA of transgenic cotton plants (MS1, MS2, MS3, MS4, MS5, MS7, MS8) were exponentially amplified using gene-specific real-time primers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as a comparative reference for the normalization of data. The highest Vip3Aa expression (~52-fold) was calculated in MS3, whereas no transgene expression was seen in non-transgenic control cotton plant (Fig. 1). The Vip3Aa protein in transgenic cotton plants was detected and quantified through ELISA. The maximum protein concentration (4.23 µg/mL) in transgenic cotton plant MS3 and minimum protein concentration (1.19 µg/mL) was detected in MS1 through ELISA (Fig. 2).

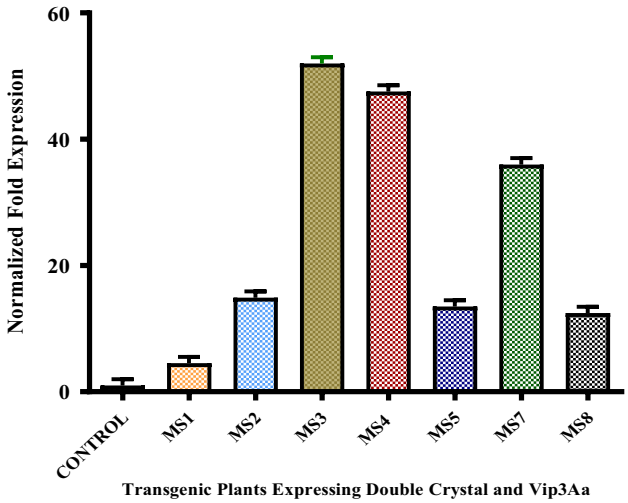

Fig. 1 mRNA expression of Vip3Aa gene through qRT-PCR. The results showed enhanced transcript levels of Vip3Aa in MS3 and MS4 lines followed by MS7, MS2, MS8 and MS1

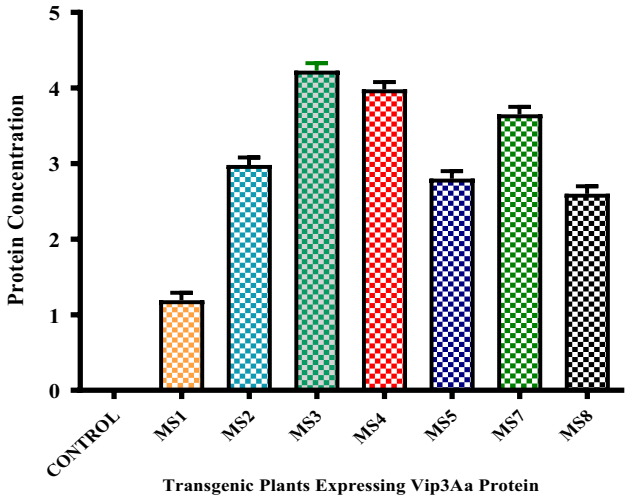

Fig. 2 Quantification of Vip3Aa protein through ELISA. The analysis revealed accumulated levels of Vip3Aa protein in MS3 lines followed by MS4, MS7, MS7, MS8, MS2 and MS1

Determination of transgene location and copy number

The exact location and number of transgene copies in transgenic cotton plants were examined through FISH using a Vip3Aa specific probe. The transgenic cotton plant (MS3) showing the highest Vip3Aa expression in qRT-PCR was selected for the analysis. One copy of the transgene on both the chromatids of chromosome number 8 in homozygous form was detected in the telophase stage. The non-transgenic plant showed no signal of transgene integration (Fig. 3A, B).

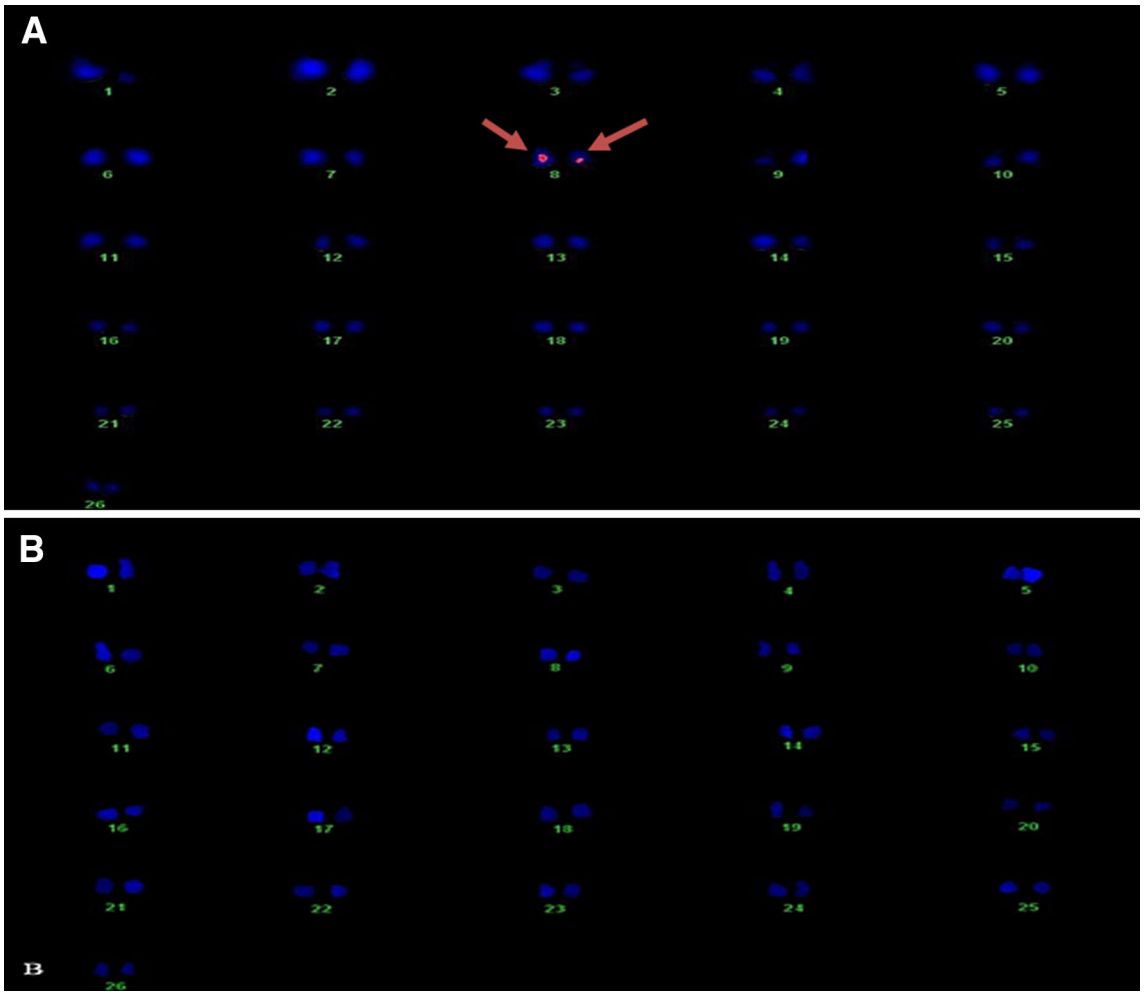

Fig. 3 Determination of copy number and transgene location through FISH. **A** The karyogram of transgenic cotton plant (MS3) showing one copy number on both chromatids in homozygous form at chromosomes 8. **B** The karyogram of non-transgenic plants showed no signal of transgene integration

Insect mortality bioassay with cotton bollworm (*Helicoverpa armigera*)

The transgenic cotton plants expressing first-generation *Bt* crystal toxins (*cry1Ac* and *cry2Ab*) and second-generation *Bt* Vip3Aa protein in T_0 generation were subjected to leaf-detach insect bioassays for determination of the transgene efficiency against cotton bollworm (*Heliothis armigera*) in laboratory conditions. The data was collected as mortality percentage on the third day of infestation. Transgenic cotton plants expressing double crystal and Vip3Aa protein were more resistant to insects' damages showing almost 99% mortality as compared to the transgenic cotton plants harbouring double crystal proteins with 70% insect mortality. Insects remained alive and completely damaged the leaves of non-transgenic cotton plants (Fig. 4). The insect mortality percentages graph between control, transgenic cotton plants expressing double crystals and along with Vip3Aa protein have been shown in Fig. 5.

Discussion

Cotton is the major cash crop of Pakistan that holds a significant contribution to the national economy [31]. Among all the cotton-producing countries, the cotton yield in Pakistan is comparatively low [32]. Many factors affect the cotton crop in Pakistan like insects, viruses, and weeds that lead to poor fibre quality and low cotton yield [33]. Approximately, 50% yield losses are estimated due to insect infestation [3]. Breeding techniques alone are not enough to get desired results and a lot of time is required for the achievement of specific traits. Therefore, recombinant DNA technology could be the best possible alternative to conventional

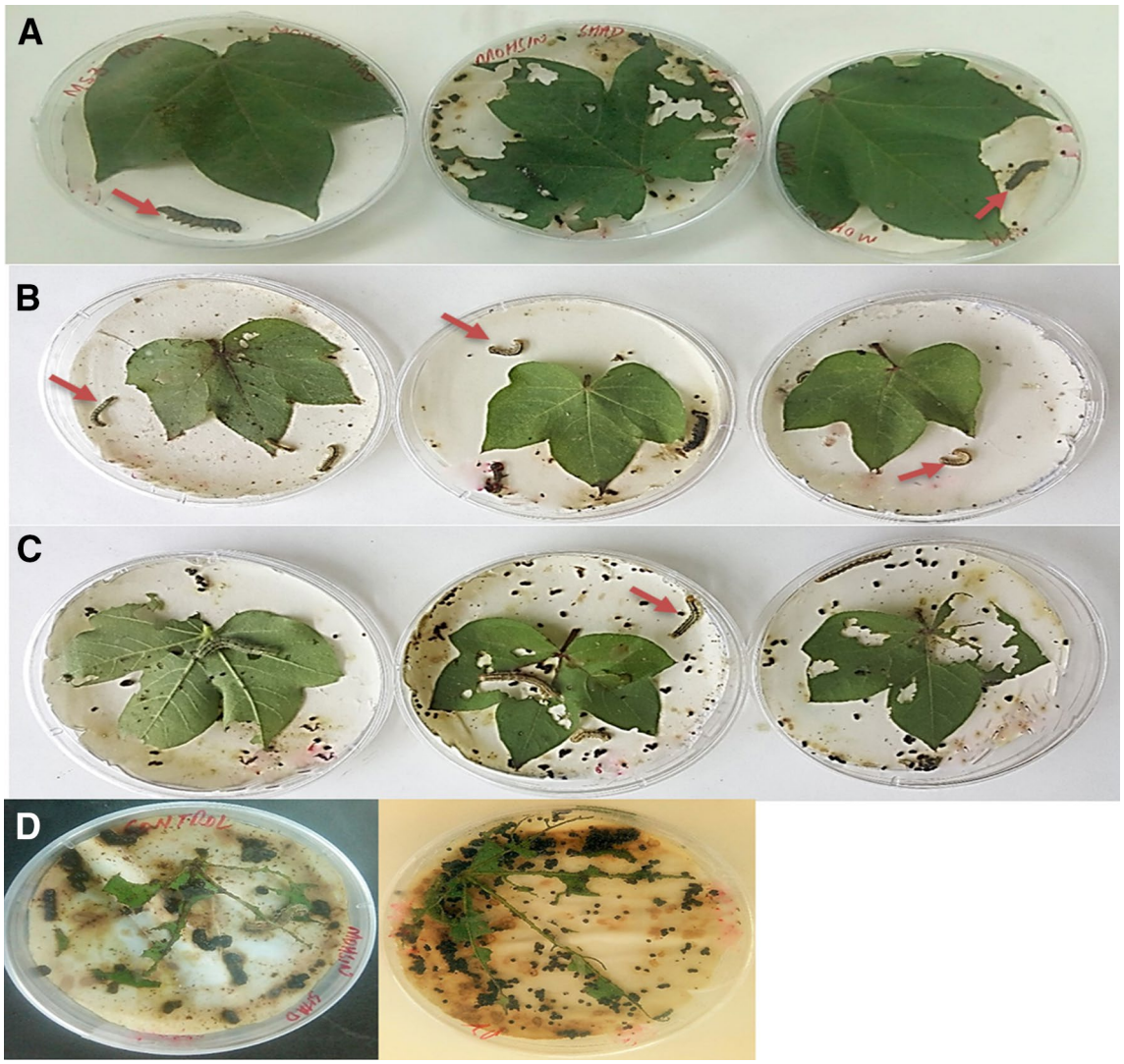

Fig. 4 Insect bioassays with *Helicoverpa armigera*. **A**, **B** MS3 and MS4 Transgenic cotton plants containing double Cry and Vip3Aa toxins. **C** CKC Transgenic cotton plants containing double crystal toxins. **D** Non-transgenic cotton plants without any toxin

breeding as used in the current study by introducing insecticidal toxins to achieve maximum resistance against cotton pests [34]. To combat the resistance developed in insects against the already used delta endotoxins, a new version of the *Bt* toxin (VIP) was pyramided with crystal proteins for an enhanced and broad spectrum of insect resistance [26, 35].

In this study, the codon-optimized synthetic Vip3Aa gene under CaMV35S promoter was transformed into a locally developed cotton variety (CKC-01) expressing *cry1Ac* and *cry2A* genes. The CKC-01 variety was selected for transformation due to its better performance and germination in the field as was done by Puspito et al. [30]. CaMV35S is a constitutive promoter that is expressed all the time in each cell but at some times its expression is comparatively low in phloem cells [36]. The Vip3Aa was processed to be cloned under 35 S promoter and *Nos* terminator in pCAMBIA_1301 vector with the help of *XhoI* and *SacI* restriction sites as done by Din et al. [26]. The heat shock method was adapted for the transformation of gene cassette into the competent cell of *E. coli*. The plasmids were confirmed through restriction digestion and polymerase chain reaction. The recombinant pCAMBIA 1301 vector containing gene cassette was transformed into the *Agrobacterium* competent cells through electroporation and confirmed through restriction digestion and polymerase chain reaction [37, 38].

The transformation of foreign gene cassette (pCAMBIA_Vip3Aa) into the host genome was done through *the Agrobacterium*-mediated shoot apex technique [26, 39]. Approximately, 4000 mature cotton embryos were excised and infected with *Agrobacterium* transformed cells. The 65 acclimatized transgenic cotton plants were shifted to a tunnel

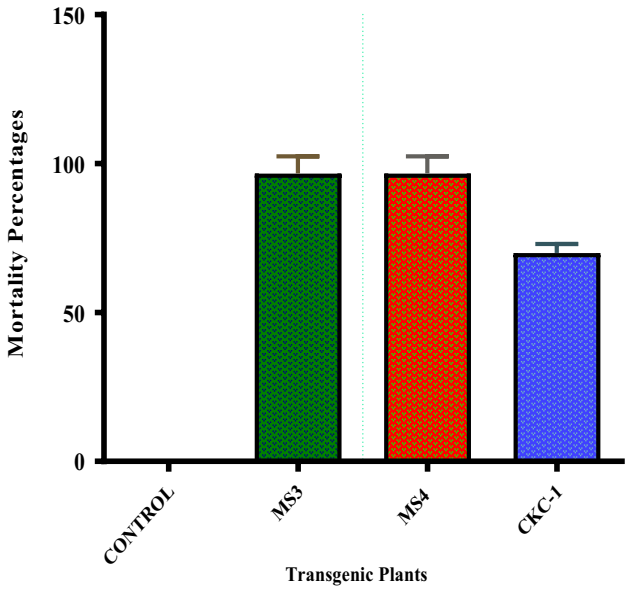

Fig. 5 Mean percent mortality of cotton bollworm (*Helicoverpa armigera*) larvae. Control: mon transgenic cotton plant, MS3 and MS4: transgenic cotton plants containing double Cry and Vip3Aa toxins, CKC-I: transgenic cotton plants containing double crystal toxins

out of which 35 plants were amplified through PC showing amplification of 682 bp Vip3Aa. The transformation efficiency was found to be 1.63%. The transformation results were in accordance with Bajwa et al. [37] and Sufyan Tahir et al. [40] .The transformation efficiencies of cotton were reported about 1–1.2% by using the same techniques [26, 30, 40, 41].

Different molecular biology techniques were used to check the successful transformation of the foreign gene in the transgenic cotton plants. qRT-PCR was used to determine the mRNA level of an insecticidal gene in transgenic cotton plants. The C_t values obtained from the qRT-PCR machine were used to calculate the relative expression of the Vip3Aa gene in transgenic cotton plants. The highest Vip3Aa expression of (51.12-fold) was calculated in the MS3 transgenic cotton plant (Fig. 1) while the lowest was observed MS1 plant, respectively. The results are in accordance with Ahmed et al. [39]. The quantification and detection of Vip3Aa protein in transgenic cotton plants were performed through ELISA. The maximum quantity of Vip3Aa protein concentration (4.23 µg/mL) in transgenic cotton plant MS3 and a minimum of Vip3Aa protein concentration (1.19 µg/mL) transgenic cotton plant MS1 was obtained through ELISA (Fig. 2).

The foreign gene copy number and location in transgenic plants were determined using FISH. MS3 plant showed the highest Vip3Aa gene expression in qRT-PCR was selected for FISH. The PCR product of the Vip3Aa specific gene was used in probe labelling. The transgenic plant (MS3) showed one copy number on both chromatids in the homozygous form at chromosome 8 in the telophase stage. Non-transgenic plants showed no signal of chromosomes integration as visualized in Fig. 3A, B). The results are in accordance with Ali et al. [4] and Yaqoob et al. [38]. The main aim of the study is to transform the second generation *Bt* toxin that has a broad spectrum of activity against cotton bollworm *(H. armigera).* Almost 99% mortality of *H. armigera* was recorded in transgenic cotton plants expressing double crystal proteins pyramided with Vip3Aa gene as contrasted to transgenic cotton plant expressing only double crystal protein with 70% mortality. The success story of this research is to develop a transgenic cotton plant expressing Vip3Aa along with double crystals (*cry1Ac, cry2A*) protein showed significant resistance i.e., 99% mortality against 2nd instar larvae of chewing insects.

Conclusions

The current research work was proposed to evaluate the combined insecticidal potential of double crystal (*cry1A*c and *cry2A*) endotoxins and Vip3Aa protein against major chewing insects. The efficacy of transgenes was evaluated through insect bioassays using 2nd instar larvae of cotton bollworm. Significant insect mortality on transgenic cotton plants determined the success of this study. The development of insect-resistant transgenic plants against chewing insects will have a significant impact on the national economy and farmers.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06972-z.

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Author contributions All authors equally contributed and gave their final approval to the submitted manuscript.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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