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Full Length Article

Development and Evaluation of Synthetic *vip3A* Gene in Transgenic Cotton for Protection against Chewing Insect Pests

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Abstract

Insect pests are among the main factors limiting crop productivity worldwide, amongst which lepidopteran pests contribute a significant amount of damage. Genetically modified crops provide useful germplasm for the protection of various commercial agricultural commodities against insect pest infestation. The codon-optimized synthetic *vip3A* gene was designed, synthesized and cloned in a plant expression vector under CaMV 2X 35S promoter and CaMV terminator that subsequently transformed in *Gossypium hirsutum cv.* Coker-312 to estimate its efficacy against two lepidopteran insects, i.e., *Helicoverpa armigera* and *Spodoptera litura*. Twenty-eight putative T_0 transgenic plants were recovered from the agro-inoculated hypocotyls following callus formation, selection and embryogenesis. PCR amplification and Southern hybridization revealed the successful *vip3A* transgene integration into plant nuclear genome. Transgenic lines of various transformed events were subjected to detached leaf insect bioassays using first-instar larvae of *H. armigera* and *S. litura*. A complete insect mortality of *H. armigera* and *S. litura* was observed in 96 h after the release of caterpillars under laboratory conditions. Moreover, the observed LT_{50} for *H. armigera* (1.01 days) and *S. litura* (1.26 days) suggests the high expression of self-designed codon-optimized synthetic *vip3A* toxin in these cotton lines. The enhanced insecticidal expression of synthetic *vip3A* gene has shown great potential for its inclusion in the list of promising genes that can be used to develop insect resistant transgenic plants.

Keywords: *Agrobacterium* mediated transformation; Transgenic cotton; Insect bioassays; Insect resistance; Synthetic gene technology; *Vip3A* toxin

Introduction

Upland cotton (*Gossypium hirsutum* L.) formally known as American cotton (tetraploid) is among the widely grown species of cotton in the world (Ali and Ullah 2018). It has been estimated that the annual economic impact of cotton crop is around $600 billion worldwide (Shuli *et al.* 2018). *G. hirsutum* is well known for its fiber quality (about 40–60% use in textile industry) and various applications in Agro-based industries (15–25% use in cotton seed oil and 20–40% use in paper and pulp industry) *etc*. (Siddiqui *et al.* 2019). Cotton crop encounters various challenges including biotic stress like insect pest attack as well as a number of abiotic factors such as drought, salinity and temperature variation (Lobell and Asner 2003). Insects/pests pose prolonged and devastating effects on crop yield. The herbivorous insects of cotton crop are mainly bollworms *e.g.*, armyworm (*Spodoptera litura*), cotton bollworm (*Helicoverpa armigera*), spotted bollworm (*Earias insulana*) and pink bollworm (*Pectinophora gossypiella*) (Farooq *et al.* 2014).

To overcome the pest infestation, farmers use insecticidal chemicals (pesticides) that provide robust, reliable and effective pest eradication (Saeed *et al.* 2019). However, the use for such pesticides are discouraged, as their excessive usage poses many health hazards to all living organisms, potential development of resistance among insects and the prevalence of long-lasting toxic residues in the environment (Eede *et al.* 2004). As an alternative strategy, eco-friendly genetically modified (GM) crops expressing *Bacillus thuringiensis* insecticidal proteins have played a vital role in managing insect control with less insecticide usage thus providing farmers with higher profits. In 1990s, cotton varieties expressing parasporal crystalline δ-endotoxins (Cry1Ac and Cry2Ab) were launched as Bollgard-I and Bollgard-II in USA, Australia and various other counties (Adamczyk and Gore 2004). The area under GM crops cultivation has greatly increased from 90 million hectares in 2005, to 191.7 million hectares globally by the end of 2018 (ISAAA Brief 54, 2018). Moreover, various other *Bt*-toxins that have been stacked in GM crops have

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shown excellent control over pest infestation in agricultural fields (Naqvi *et al.* 2017). However, field evolved resistance among the various insect species (thought to be due to fluctuation of toxin level in the course of plant age and various parts; cross-resistance among *Bt*-toxins; mutational evolution in insects due to injudicious use of pesticides; and lesser or no use of refugia) have been reported against previously commercialized GM crops. This emphasizes the need of developing potentially new *Bt*-toxins that may help in delaying the development of resistance among the insect population and provide more realistic insecticidal spectrum to overcome the current scenario for pest management (Gunning *et al.* 2005; Caccia *et al.* 2010).

Vip (vegetative insecticidal protein), another class of toxins isolated from *B. thuringiensis* has gained popularity nowadays, that remains active throughout its course of synthesis (Lemes *et al.* 2017). It is considered to be more efficient compared to δ-endotoxins as it does not share any sequence homology and lacks cross-resistance, making it more suitable candidate for gene stacking as well. The Bt. Toxin Nomenclature Committee has categorized *Vip* proteins into four different families namely *Vip1*, *Vip2*, *Vip3* and *Vip4* (Chakroun *et al.* 2016). Vip1 and Vip2 constitutes a binary toxin expressed concomitantly with insecticidal spectrum over coleopteran and sap-sucking insect pests. *Vip3* proteins are single-chain toxins that exhibit a wide host range, including several major lepidopteran pests. Surprisingly, no target insects have been found for *Vip4* toxin, although *Vip4Aa1* has found to be phylogenetically similar to *Vip1* proteins (Palma *et al.* 2014; Chakroun *et al.* 2016). Among the *Vip3* proteins, *Vip3A* is most explored insecticidal toxin as it remains active throughout the vegetative growth stage of the plant (Gayen *et al.* 2012; Palma *et al.* 2014). The first *vip3* genes were cloned as *vip3Aa1* and *vip3Ab1*, isolated from *B. thuringiensis* strains AB424 and AB88, respectively (Estruch *et al.* 1996). Moreover, *Vip3A* toxins have been reported to be more effective against *Spodoptera* and *Helicoverpa* spp. that are quite resistant to δ-endotoxins and even lower dosage of *Vip3A* toxin have shown tremendous growth inhibition in various insect pest populations (Khan *et al.* 2019). Therefore, *Vip3A* toxins are considered to provide more effective control over insect populations that are resistant to the Cry toxins. For instance, genetically modified cotton made with synthetic *vip3A* gene fused to a chloroplast transit peptide, results in accumulation of *Vip3A* protein in the chloroplasts, causing 100% mortality in *S. frugiperda*, *S. exigua* and *H. zea* caterpillars (Wu *et al.* 2011). Later, this gene was stacked with other cry genes and two commercialized varieties, VipCot® (cotton) and Agrisure Viptra® (maize) were registered in the USA in late 2000s (ISAAA Brief 54, 2018).

The main objective of this study was to assess the efficacy of synthetic *vip3A* gene for its insecticidal activity in transgenic cotton. For this purpose, the *vip3A* gene was codon-optimized, synthesized and transformed in *G. hirsutum cv.* Coker-312. Putative transgenic cotton lines were developed and screened against the *H. armigera* and *S. exigua* through laboratory bioassays. So far, no second or third generation *Bt*-crop has been commercialized in Pakistan, so it is the need of time to introduce new toxin genes to overcome insect pest infestation problem in Pakistan.

Materials and Methods

Designing of synthetic *vip3A* gene by codon bias optimization

The *vip3A* gene sequence (accession DQ539887) was retrieved from the National Center for Biotechnology Information nucleotide database. This gene sequence was codon optimized by using *Gossypium* spp. codon usage table (Kazusa, Japan) and Geneious 8.0 software (Genious, CA, USA) with the intentions to achieve enhanced expression of *Vip3A* toxin in transgenics. Next, this codon-optimized *vip3A* gene was commercially synthesized by ATUM (Newark, CA, USA) under commercial cloning vector pCR-Blunt (3.5 kbp) (Life Technologies, Carlsbad, CA) and the nucleotide sequence was deposited in GenBank database (Accession number MK761073).

Development of synthetic *vip3A* gene construct

The synthetic *vip3A* gene (~2.4 kbp) was amplified by PCR (using Phusion high-fidelity DNA polymerase, Thermo Scientific, MA, USA) from pCR-Blunt+*vip3A* using forward (GACCTGCAGATGAACAAAAACAACACTAAGC) and reverse (GACGGATCCTTTGATGCTCACGTCATAG) primers having *Pst*I and *Bam*HI restriction sites introduced at the 5` and 3` ends respectively. The amplified product was ligated at the corresponding sites between the double CaMV35S promoter and CaMV terminator in pJIT60 vector (~3.7 kbp) to acquire complete gene cassette. The resultant trans-conjugant plasmid was confirmed by restriction analysis and named as pMHK360.

Further, this synthetic *vip3A* gene cassette was cloned in the plant expression vector pCAMBIA2300 (~8.6 kbp). The gene cassette was amplified from pMHK360 by PCR using forward (GACGGTACCCCTACTCCAAAAATGTCA) and reverse (GAGGTCGACAGATCTCTCGAGGATATC) primers having *Kpn*I and *Sal*I restriction sites introduced at the 5` and 3` ends, respectively. The amplified PCR product (~3.9 kbp) was cloned at *Kpn*I and *Sal*I sites of pCAMBIA2300 and confirmed by restriction analysis. This plant expression vector was named as pMHDK-98 (Fig. 1–D).

Transformation of pMHDK-98 in *Agrobacterium tumefaciens* strain LBA4404

A. tumefaciens strain LBA4404 (Invitrogen, CA, USA) was

transformed with pMHDK-98 through electroporation method as described previously by Asad *et al.* (2003). Transformation setup comprised of 300 ng of pMHDK-98 mixed with 100 µL of *A. tumefaciens* LBA4404 electro-competent cells, transferred to chilled BTX electroporation cuvette (2 mm gap) and pulse was given using the following parameters: voltage 1.44 kV, resistance 200 Ω and capacitance 25 µF. After electroporation, 1 mL liquid Luria-Bertani (LB) medium was added into the cuvette, the cells were shifted to 15 mL tube and allowed to grow with the constant agitation at 28°C for 2 h, followed by spreading on the petri plates containing solidified LB medium supplemented with rifampicin (100 mg L^{-1}) and kanamycin (50 mg L^{-1}). These plates were incubated at 28°C under dark condition for 48 h for colony formation. The presence of pMHDK-98 in *Agrobacterium* cells was confirmed by colony and culture PCR.

Cotton transformation with *vip3A* gene for the production of transgenic plants

Cotton (*G. hirsutum* L. cv. Coker-312) was transformed with LBA4404 carrying pMHDK-98 following protocols as described by Wilkins *et al.* (2004). The hypocotyls sections (2–3 cm) of aseptically grown Coker-312 plants (8 to 10 days old) were used as a source of explants in transformation experiment. The hypocotyls were dissected with sterile sharp blade, agro-inoculated with *Agrobacterium* culture harboring pMHDK-98 for 15 – 20 min, blot dried on sterile filter paper and then co-cultivated on callus induction medium (CCi) (Table 1) with incubation at 26 ± 1°C in the dark for 2 days. These hypocotyls were then shifted to callus induction media (CCiCK) supplemented with kanamycin (50 mg L^{-1}) as plant tissue selection agent and cefotaxime (250 mg L^{-1}) as antibacterial agent to eliminate the bacterial growth after co-cultivation. Upon callus formation, calli were shifted to solidified embryo maturation selection medium (MSK) and subsequently cultured until embryo formation. Upon embryogenesis, mature embryoids were transferred to solidified embryo germination medium (EGK). The successfully germinated somatic embryos were shifted into jars having the same nutrition medium for shoots and roots development. All the developmental stages were maintained in a controlled growth chamber at 26 ± 1°C and 16/8 h light/dark cycle. The well-established T_0 plants were then transferred to pots in sterilized sand and later hardened in earthen pots under greenhouse conditions for the molecular confirmation of *vip3A* transgene and seeds setting.

Molecular analysis

Screening of putative transgenic plants by PCR*:* Genomic DNA was isolated from control (un-transformed and empty plasmid plants) as well as putative transgenic cotton plants following cetyl trimethyl ammonium bromide (CTAB) methods as described by Iqbal *et al.* (2016). The PCR amplification of the transgene with gene specific primers (*Vip3A*-FF/*Vip3A*-FR, VIP.HF/VIP.HR, given in Table 2) as well as antibiotic selection marker *i.e.*, *npt*II gene primers (Kana.HF/Kana.HR, given in Table 2) were carried out to confirm the transgenic status of cotton plants. The genomic DNA isolated from putative transgenic plants was used as a template, pMHDK-98 plasmid as positive control and DNA isolated from an untransformed plant was used as negative control. The empty plasmid transgenic cotton plants were also screened with *npt*II gene primers as a control of transformation experiment.

The PCR master mix included; 2.5 µL (10 ng) of template DNA, 2.5 µL *Taq* polymerase buffer (10X), 1.5 µL $MgCl_2$ (1.5 m*M*), 0.5 µL each of forward and reverse primers (13 pmol) each, 1 µL dNTPs (2.5 m*M*), and 0.2 µL (1 unit) of *Taq* DNA polymerase (Fermentas, CA, USA). The PCR reaction was profiled as; initial denaturation at 94°C for 5 min, 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min) and final extension of 10 min at 72°C.

Southern hybridization for the estimation of transgene copy number

Southern blotting was done to confirm the integration of *vip3A* transgene in T_0 transgenic cotton lines. Ten microgram of genomic DNA was digested with *Hin*dIII restriction enzyme and size fractionated on 1% Tris-acetate-EDTA (TAE) agarose gel by electrophoresis and transferred onto nylon Hybond $–N^+$ membrane (Roche, Penzber, Germany) by standard blotting procedures (Sambrook *et al.* 1989). The digoxigenin-labeled probe was synthesized by PCR amplification of an internal fragment of *vip3A* gene (889 bp amplicon). Hybridization and all other procedures were followed according to manufacturer's instructions using the DIG High Prime DNA Labeling and Detection Starter Kit-I (Sigma Aldrich, St. Louis, MO).

Reverse transcriptase PCR for the detection of *vip3A* transcript synthesis

To determine the synthesis of *vip3A* gene transcript at the mRNA level in cotton lines, the total RNA from *vip3A* transgenic plants along with controls (*i.e.*, untransformed Coker-312 and empty plasmid transgenic line *i.e.*, pCAMBIA2300) was extracted using TRIzol reagent (Invitrogen, Carlsbad, C.A.) by following the manufacturer's instructions. Prior to cDNA synthesis, the purified total RNA was treated with DNase-I (Invitrogen) to remove the genomic DNA residues that may hinder downstream applications. Then, cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania, Italy) by following the manufacturer's instructions. Finally, the synthesized cDNA was used as a template in a PCR reaction to confirm *vip3A*

and *GhUBC1* (an endogenous reference) gene transcripts synthesis (using HK-qV$_1$-F + HK-qV$_1$-R and HK-UBC1-F + HK-UBC1-R primers sets; given in Table 1) at post-transcription level.

qPCR to quantify the relative abundance of *vip3A* transcripts in cotton plants

To estimate the relative abundance of synthetic *vip3A* transcripts in T_0 transgenic cotton lines, quantitative PCR (qPCR) was performed using *GhUBC1* (ubiquitin conjugating enzyme-E2) as a reference gene to normalize the Ct values (primers efficiencies are given in Supplementary Table 2). A 10 μL volume of reaction mixture was used in each well of a 384-well microtiter plate (USA Scientific, Ocala, FL). qPCR was performed with all positive and negative controls in triplicate format using an Applied Biosystems QuantStudio^{TM} 6 Flex System (Thermo Fisher Scientific, Waltham, MA). The specificity of the amplification product was assessed using a melt curve analysis, performed from 60 to 95°C at the end of every run, with an increment of 0.5°C every 10 sec. Quantification results were analyzed using the $2^{\Delta\Delta CT}$ method. Gene expression was measured in both transgenic cotton lines (*vip3A* and pCAMBIA2300) and untransformed control (*i.e.* Coker-312) to evaluate the relative transcripts abundance level of *vip3A* transgene.

Insect Bioassays

To check the efficacy of *vip3A* transgene against *Helicoverpa armigera* and *Spodoptera litura*, detached leaf insect bioassays were carried out with transgenic as well as control cotton plants in three replicates under laboratory conditions. A total 15 first-instar larvae (5 larvae in each replicate) were placed on each transgenic line as well as the leaves of control plants (untransformed Coker-312 and empty plasmid transgenic plant). The experiment was setup in petri plates having leaves placed on moist filter paper. The plates were kept at 25 ± 1°C and 50–60% relative humidity. Mortality data was recorded after 24, 48, 72, 96 and 120 h post release of caterpillars. Larvae lacking any movement on gentle teasing with camel hair brush were considered to be dead.

Statistical Analysis

The plant transformation efficacy was calculated in percentage, while the insect survival data (in percentage) were corrected for control mortality using Abbott's formula as described by Abbott (1925). Dunnett's tests were conducted with JMP (SAS, Cary, NC, USA). Data compilation (mean, standard deviation and standard error) and graphs were made using Microsoft Excel®. LT_{50} (time to 50% lethality) for *vip3A* transgenic cotton were calculated using the Solver add-in of Microsoft Excel to fit a curve of the form $Y = A + (1 - A)/(1 + \exp(B - G \ln(X))$ to the data, where X is the time (in days) to kill a particular insect population in detached leaf insect bioassay, Y is the fraction of larvae killed (%), A is the fraction of larvae killed on control plants, and B and G are parameters that are varied for optimal fit of the curve to the data points.

Results

Designing of novel synthetic *vip3A* gene by computational tool

The previously reported *vip3A* gene sequence (GenBank ID DQ539887) was retrieved from NCBI nucleotide database and codon-optimized for *Gossypium* spp. using a specialized computational tool (Geneious 8.0). The newly designed *vip3A* gene showed 99% query coverage and 76% nucleotide identity in BLAST comparisons to the original sequence. Moreover, both DNA sequences were translated (Translate tool-ExPasy) and aligned by using Clustal Omega (EMBL-EBI) to ensure similarity of newly designed *Vip3A* protein (Supplementary Fig. 1 & 2). The hypothesis was made that newly predicted *Vip3A* protein might be a potential candidate for better insect pest resistance in transgenic cotton. With the satisfactory *in silico* analysis, this codon-optimized *vip3A* gene was commercially synthesized by ATUM (Newark, California, USA) under commercial cloning vector *i.e.*, pCR-Blunt (3.5 kbp) and annotated (as Accession No. MK761073) in NCBI GenBank database.

Development of *vip3a* gene construct

Vip3A plant expression cassette was constructed by the PCR amplification of pCR-Blunt+*vip3A* plasmid. Fig. 1A, illustrates the amplification of corrected sized PCR fragment (*i.e.*, ~2.4 kb) of *vip3A* gene that was subsequently ligated at the *Pst*I and *Bam*HI restriction sites in pJIT60 vector. The successfully cloned trans-conjugant plasmid was named as pJTV360 and confirmed by enzymatic digestion of pMHK360 with the same restriction enzymes (Fig. 1C; lane 2) and by PCR (Fig. 1C; lane 1).

Later, the *vip3A* plant expression cassette was sub-cloned in plant transformation vector by the PCR amplification of pMHK360 recombinant plasmid. Fig. 1B illustrates the amplification of corrected sized PCR fragment (*i.e.*, ~3.9 kb) of *vip3A* plant expression cassette that was subsequently ligated at the *Kpn*I and *Cla*I restriction sites in plant transformation vector *i.e.*, pCAMBIA2300. The successfully cloned trans-conjugant plasmid was named as pMHDK-98 and confirmed by enzymatic digestion of pMHDK-98 with the same restriction enzymes (Fig. 1C; lane 5) and by PCR (Fig. 1C; lane 4).

Generation of *vip3A* transgenic cotton plants

To explore the insecticidal potential of synthetic *vip3A* gene in transgenic cotton plants, *Agrobacterium*-mediated plant

Table 1: Composition of different medium used in cotton transformation

Label	Description of Medium	Constituents	Sub-culturing timeline
CCi	Cotton callus induction medium	4.3 g L^{-1} MS basal salts, 100 mg L^{-1} Myo-inositol, 3% Glucose, 3.7 m*M* $MgCl_2.6H_2O$, 0.1 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} Kinetin, pH adjusted to 5.7 to 5.8 using 1 *M* KOH or HCl, 0.25% Phytagel	One round of 2 days
CCiCK	Cotton callus induction selection medium	4.3 g L^{-1} MS basal salts, 100 mg L^{-1} Myo-inositol, 3% Glucose, 3.7 m*M* $MgCl_2.6H_2O$, 0.1 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.1 mg L^{-1} Kinetin, pH adjusted to 5.7 to 5.8, 0.25% Phytagel, 50 mg L^{-1} Kanamycin and 500 mg L^{-1} Cefotaxime	Three rounds with 4 weeks interval
MSK	Embryo maturation selection medium	4.3 g L^{-1} MS basal salts, 100 mg L^{-1} Myo-inositol, 3% Glucose, 3.7 m*M* $MgCl_2.6H_2O$, 1.9 g L^{-1} KNO_3, pH adjusted to 5.7 to 5.8, 0.25% Phytagel and 25 mg L^{-1} Kanamycin	Two rounds of with 4-6 weeks interval
EGK	Embryo germination selection medium	Macro salts (m*M*): KNO_3 (5), NH4NO3 (3), $MgSO_4.7H_2O$ (2), $CaCl_2$ (1.2) and KH_2PO_4 (0.2); micro salts (m*M*): H_3BO_3 (30), $MnSO_4$ (30), $ZnSO_4.7H_2O$ (9), KI (1.5), $NaMoO_4.2H_2O$ (0.9), $CuSO_4.2H_2O$ (0.03), $CoCl_2.6H_2O$ (0.03) and Fe-EDTA (15); vitamins: 4 µM each of Nicotinic acid, Pyridoxine HCl and Thiamine HCl, 3% Glucose, 0.25% Phytagel, pH adjusted to 5.7 to 5.8and Kanamycin 25 mg L^{-1}	One round for 2 weeks in Petri-plate, followed by second round for 4-8 week in glass jars

Table 2: PCR primers used in this study

Primer name	Purpose of amplification	Primer Sequence	Amplicon Size
Vip3A-FF	Forward primer to amplify full length *vip3A* gene	GACCTGCAGATGAACAAAAACAACACTAAGC	~2.4 kb
Vip3A-RF	Reverse primer to amplify full length *vip3A* gene	GACGGATCCTTTGATGCTCACGTCATAG	
VIP.HF	Forward primer to amplify an internal fragment of *vip3A* gene	GGGCGTGGTATATTGAAACAAAATC	267 bp
VIP.HR	Reverse primer to amplify an internal fragment of *vip3A* gene	GGATCCTTTGATGCTCACGTCATAG	
Kana.HF	Forward primer to amplify an internal fragment of *nptII* gene	TCAGAAGAACTCGTCAAGAAGGCG	329 bp
Kana.HR	Reverse primer to amplify an internal fragment of *nptII* gene	ATGATCTGGACGAAGAGCATCAGG	
HK-VdigP-F	Forward primer for the synthesis of digoxigenin-labeled probe for *vip3A* gene	CTTACGCTGACAACCTGCCG	889 bp
HK-VdigP-R	Reverse primer for the synthesis of digoxigenin-labeled probe for *vip3A* gene	GACGGGGGCATTTCTCAATTCATTGG	
HK-qV_1-F	Forward primer to amplify *vip3A* gene in qPCR	CTTACGCTGACAACCTGCCG	89 bp
HK-qV_1-R	Reverse primer to amplify *vip3A* gene in qPCR	TGGCATCTTCGTCACTTCCCTAAC	
HK-UBC1-F	Forward primer to amplify *GhUBC1* gene in qPCR	TGGCATTATATTGTCATTGTTACTATCC	130 bp
HK- UBC1-R	Reverse primer to amplify *GhUBC1* gene in qPCR	ACCATGTTATCTTATTCTAAGACAAGCTC	

Table 3: Regeneration and transformation efficiency of cotton (*G. hirsutum L.*)

No. of Batch	No. of explants used	No. of explants producing calli	Callus induction efficiency (%)	No. of calli shifted to regeneration medium	No. of embryogenic events	No. of regenerated plants	Regeneration efficiency (%)	No. of regenerated plants producing roots	Transform-ation efficiency (%)
1	40	28	70	28	1	9	32.14	6	22.5
2	40	27	67.5	27	2	15	56	13	37.5
3	40	29	72.5	29	1	8	27.5	7	20
4	40	32	80	32	1	2	0.07	2	5
Total	160	116	72.5	116	5	34	28.93	28	17.50

transformation of *G. hirsutum* L. cv. Coker-312 was carried out with the plasmid pMHDK-98 through agro-inoculation. The experiment was setup in four batches having forty explants each as hypocotyl sections. Fig. 2 illustrates the steps involved in cotton transformation with pMHDK-98. The mean callus induction, embryo regeneration and transformation efficiency in all the batches were 72.5, 29 and 17.5%, respectively (Table 3). Any successfully regenerated embryo from the embryogenic calli was considered as an independent event. A total 5 independent events (having 2 to 7 plants in each event) were recovered in this study. These transgenic plants were successfully recovered and shifted to pots containing sterilized sand.

Molecular analysis

Screening of putative transgenic plants by PCR: Presence of *vip3A* gene along with plant selection marker (*nptII* gene) in plant genome was confirmed through PCR (Fig. 3). Amplification of correct sized fragment of ~2.4 kbp (full gene amplification, Fig. 3A) and 267 bp (internal fragment amplification, Fig. 3B) indicates the integration of *vip3A* gene in the putative T_0 transgenic cotton lines of 5 events (Fig. 3A, B; lanes 2 to 6). Similarly, amplification of correct sized fragment of ~329 bp (internal fragment of *npt*II gene, Fig. 3C), validates the integration of kanamycin selection marker in the previously screened putative T_0 transgenic cotton lines of 5 events as well (Fig. 3C; lane 2 to 6). No amplification was observed in the un-transformed control and negative controls (lacking any DNA template; Fig. 3A, B, C; lane 1 & 7).

For the control in plant transformation experiments, few plants were developed with empty plasmid *i.e.* pCAMBIA2300. The amplification of correct sized fragment of ~329 bp from *npt*II gene from these cotton lines

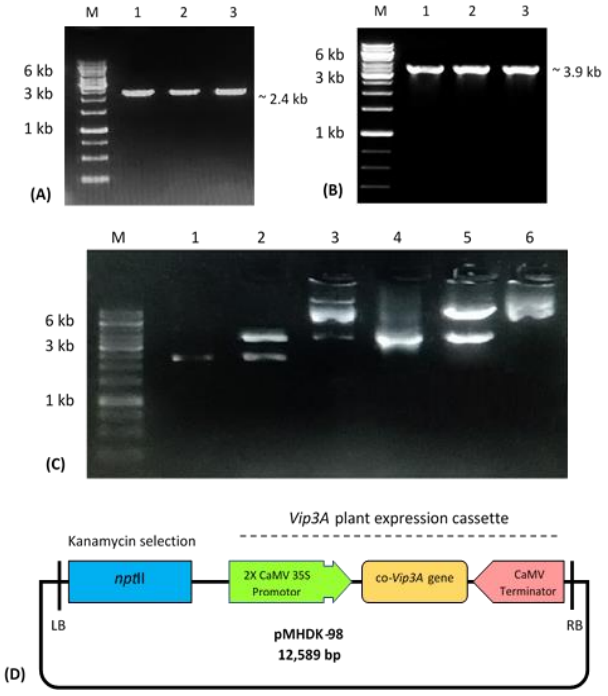

Fig. 1: Confirmation of plasmids with cloned *vip3A* gene
(**A**) PCR amplified *vip3A* gene (~2.4 kb), (**B**) PCR amplified *vip3A* plant expression cassette (~3.9 kb), (**C**) Agarose gel electrophoresis of enzymatically digested clone (pMHK360 & pMHDK-98) to confirm the cloning of synthetic *vip3A* gene in plant expression vector *i.e.*, pJIT60 and plant transformation vector *i.e.*, pCAMBIA2300 vector, (**D**) Diagrammatical representation of plant expression vector containing synthetic *vip3A* gene, under double CaMV 35S promoter and CaMV terminator (pMHDK-98)

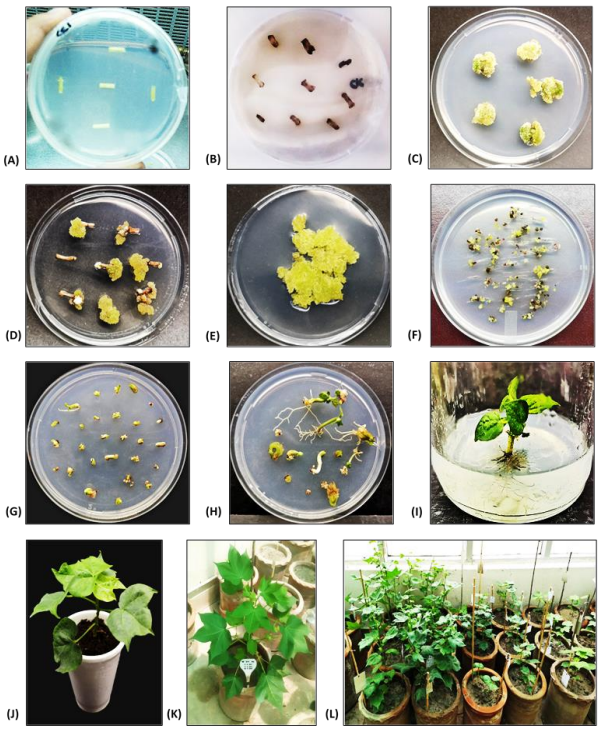

Fig. 2: Development of the synthetic *vip3A* transgenic cotton through *Agrobacterium*-mediated transformation
(**A**) agro-inoculated hypocotyl sections (**B**) un-transformed hypocotyl sections (callus selection medium) (**C**) un-transformed hypocotyl sections (on callus induction medium) (**D**) agro-inoculated hypocotyl sections (on callus induction medium) (E) transgenic callus (on callus induction medium) (**F**) transgenic embryogenic calli with immature, mature and germinating embryos (**G**) transgenic embryos (on germination medium) (**H**) transgenic embryoid (embryo maturation medium) (**I**) transgenic plant with established rooting system (on embryo maturation medium) (**J**) putative transgenic plant in small plastic pot (**K**) hardening of transgenic cotton plant in earthen pot (**L**) transgenic plants in containment glass house

also confirmed and validated the development of true transgenic cotton lines (Fig. 3D; lane 4 to 7).

Southern hybridization for the estimation of transgene integration events

Southern hybridization of the putative *vip3A* T_0 transgenic cotton plants have shown one or more integration events of *vip3A* transgene in cotton plant genome. Fig. 3E clearly showed the integration of 2 copies of transgene in HK-Vip-E.61 (Fig. 3Ei; lane 3) and HK-Vip-E.177 (Fig. 3Eii; lane 4), whereas HK-Vip-E.16, HK-Vip-E.71 (Fig. 3Ei; lane 2 & 4) and HK-Vip-E.85 (Fig. 3Eii; lane 3) showed one copy of *vip3A* transgene. Moreover, no hybridization was observed in the both controls, *i.e.*, untransformed control plant (Fig. 3Ei & Eii; lane 1) and empty plasmid control plant (*i.e.*, pCAMBIA2300; Fig. 3Eii; lane 2).

Reverse transcriptase PCR to detect *vip3a* transcripts at post-transcription level

The reverse transcriptase PCR was carried out after isolating total RNA from the young leaves of the transformed cotton lines (HK-Vip-E.16-P4, HK-Vip-E.61-P3, HK-Vip-E.71-P2, HK-Vip-E.85-P4, HK-Vip-E.177-P1) and control lines (untransformed and pCAMBIA2300). The isolated RNA was size fractionated on 1% agarose gel (Fig. 4a). Further, the isolated total RNA was treated with DNase-I to remove the downstream traces of genomic DNA (Fig. 4b). DNase-I treated total RNA was then used to synthesize cDNA and subjected to PCR (formally known as RT-PCR) for the amplification of small fragment of *vip3A* gene (~89 bp) and an endogenous reference gene *i.e.*, *GhUBC1* (130 bp) to confirm the synthesis of mRNA transcripts at post-transcriptional level (Fig. 4C, D).

***Vip3A* transcript abundance profiling by quantitative PCR**

With the confirmation of transcript synthesis by RT-PCR, qPCR was performed. Fig. 5 illustrated the variable expression of *vip3A* transgene among the tested transgenic lines. The analysis of these transgenic lines suggested that HK-Vip-E.85-P4 showed the maximum relative gene expression *i.e.*, 5.8 times more compared to untransformed control plant, whereas the relative gene expression for HK-Vip-E.16-P4, HK-Vip-E.61-P3, HK-Vip-E.71-P2 and HK-Vip-E.177-P1 was 5.2, 4.7, 4.9, 5.4 times greater than

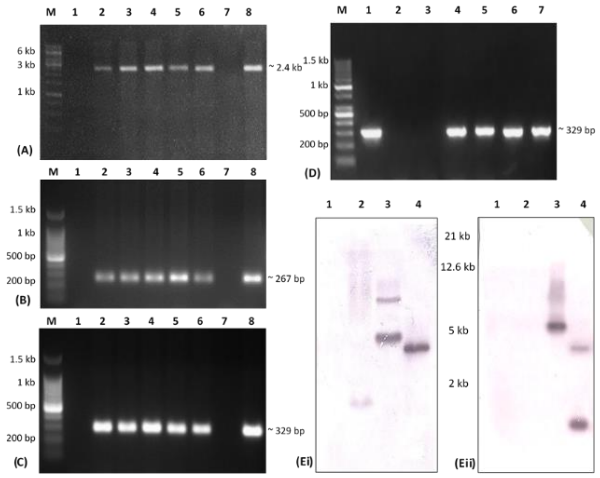

Fig. 3: Confirmation of *vip3A* transgenic cotton plants

(**A**) PCR amplification of ~2.4 kbp *vip3A* gene using full length primers from T_0 transgenic *vip3A* cotton plants, (**B**) PCR amplification of 267 bp of internal fragment of *vip3A* gene from T_0 transgenic *vip3A* cotton plants, (**C**) PCR amplification of 329 bp of internal fragment of *nptII* gene (selection marker) from T_0 transgenic *vip3A* cotton plants, (**D**) PCR amplification of 329 bp of internal fragment of *nptII* gene (selection marker) from empty plasmid (*i.e.*, pCMBIA2300) T_0 transgenic cotton plants, (E i,ii) Southern blot of *vip3A* transgenic cotton lines to estimate copy number of the transgene integration events in cotton genome

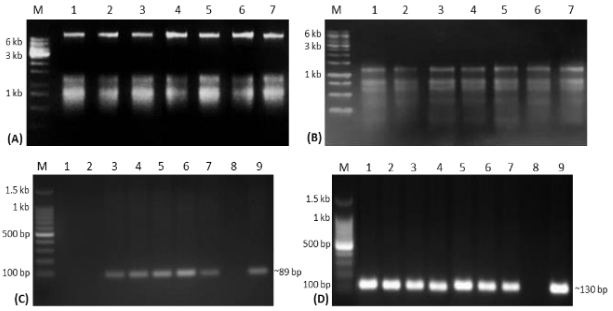

Fig. 4: RT-PCR for the detection of transcripts synthesis at post-transcription level

(**A**) Total RNA before DNase-I treatment, (**B**) Total RNA after DNase-I treatment; M= 1 kb DNA ladder, lane 1= untransformed cotton plant (control *i.e.*, Coker-312), 2= empty plasmid transgenic line (control, *i.e.*, pCAMBIA2300), lanes 3 to 7= transgenic cotton lines (HK-Vip-E.16-P4, HK-Vip-E.61-P3, HK-Vip-E.71-P2, HK-Vip-E.85-P4, HK-Vip-E.177-P1, respectively). (**C**) Amplification of *vip3A* gene (89 bp) from transgenic cotton lines, (**D**) Amplification of *GhUCB1* gene (130 bp) from transgenic cotton lines; M= 100 bp DNA ladder, lane 1= untransformed cotton plant (Coker-312), lane 2= control transgenic line (pCAMBIA2300), lanes 3 to 7= *vip3A* transgenic cotton lines, lane 7= negative control (PCR mixture lacking any template), lane 8= positive control (PCR mixture containing genomic DNA of positive *vip3A* transgenic plant)

untransformed control plant respectively. No expression of *vip3A* transgene was observed in both controls (i.e. untransformed and pCAMBIA2300).

Detached leaf insect bioassays

Toxicity of *vip3A* transgene in transgenic cotton lines was analyzed by conducting the detached leaf bioassays. A total 6 plants of four events (*i.e.*, HK-Vip-E.16, HK-Vip-E.61, HK-Vip-E.71 & HK-Vip-E.85) and 2 plants of event HK-Vip-E.177 were analyzed using *H. armigera* and *S. litura*

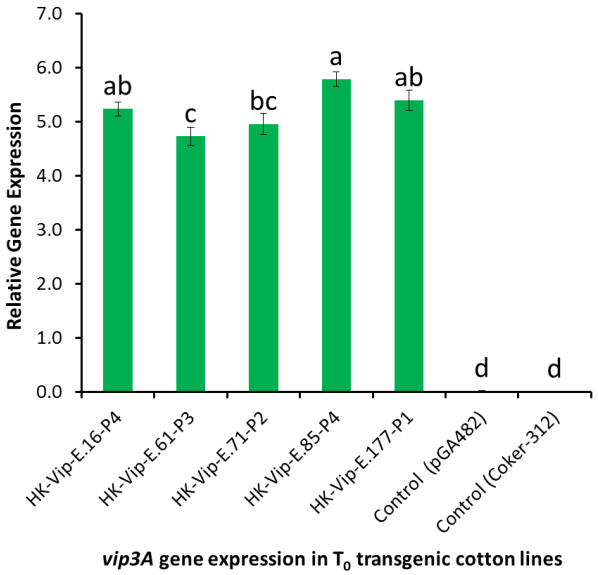

Fig. 5: Quantitative PCR analysis at post-transcriptional level in *vip3A* transgenic cotton lines

Mean ± S.E. N = 3, different letters indicate $P < 0.05$, ANOVA followed by Tukey's HSD T-test

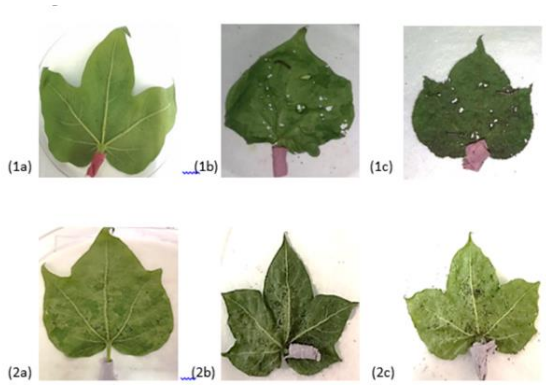

Fig. 6: Insect bioassays with leaves from *vip3A* transgenic cotton and different control plants

(**1**) screening for *H. armigera* (**2**) screening for *S. litura*; (**a**) T_0 *vip3A* transgenic plant (**b**) Control (empty plasmid-pCAMBIA2300 transgenic plant) (**c**) Control (untransformed Coker-312 plant)

larvae. For the control, leaves from untransformed cotton plants (Coker-312) and plants having empty plasmid (pCAMBIA2300) were also included in insect bioassay. Each plant was tested in three replicates (Fig. 6).

Surprisingly, all the tested population of *H. armigera* showed 100% mean mortality within 72–96 h of exposure to the *vip3A* transgenic lines (Table 4; Fig. 7A). Four out of the five lines showed 100% mortality within 72 h while line HK-vip-E-16 showed 100% mortality after 96 h (Supplementary Table 1). Similarly, the transgenic lines conferred 99% mean mortality in *S. litura* larvae in different lines (Table 4). Three transgenic lines showed 100% mortality *i.e.*, HK-vip-E-16 and HK-vip-E-177 after 72 h of

Table 4: Insecticidal trend of *vip3A* transgenic cotton against *Helicoverpa armigera* and *Spodoptera litura* larvae in detached leaf insect bioassays

Insect species	N[a]	n[b]	Mortality % (Mean ± S.E.) at 120 h	LT_{50}[c]	Insect Mortality (%) in 5 days Range		Toxicity status of transgenic lines
					Min	Max	
H. armigera	390	5	100 ± 0.0	1.01	33	100	Highly resistant
S. litura	390	5	99 ± 0.5	1.26	13	100	Highly resistant

[a] N = Total No. of caterpillars used in bioassay experiment,
[b] n = Number of caterpillars tested in each dish, three-fold replication,
[c] LT_{50} = Time in days until 50% of caterpillars are dead
Toxicity status of plant lines: > 80% =highly resistant, 50% to 80% = resistant, 30% to 50% = moderately resistant, < 30% = susceptible

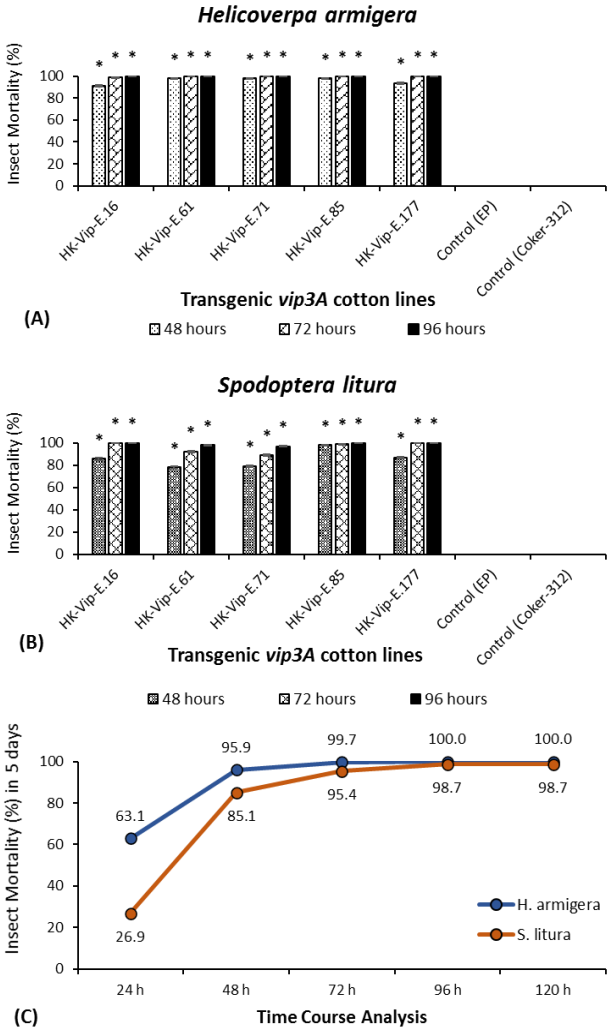

Fig. 7: Insect mortality status in *vip3A* transgenic cotton plants in detached leaf bioassays
(**A**) *Helicoverpa armigera* response to *vip3A* transgenic cotton, (**B**) *Spodoptera litura* response to *vip3A* transgenic cotton, (**C**) Accumulative insecticidal trend of transgenic *vip3A* cotton lines at each 24 hours.
Each bar corresponds to mean insect mortality (%) ± S.E. (N = 3), **P* < 0.05, Dunnett's test relative insect mortality in control plant

exposure while HK-vip-E-85 after 96 h of exposure, respectively. The remaining two lines *i.e*., HK-vip-E-71 and HK-vip-E-61 showed 97 and 98% *S. litura* larval mortality after 96 h of exposure (Supplementary Table S1). These two transgenic lines showed considerable consumption of leaf material by the larvae compared to other transgenic lines (Fig. 7B). However, the caterpillars released on both of the controls remained alive and continued to feed on the leaf material (Fig. 7A, B). The Fig. 7C illustrates the time course analysis at each 24 h progression of assay, along with the insecticidal pattern of each transgenic event against *S. litura* and *H. armigera.*

Moreover, the mortality trend was also assessed in terms of LT_{50} (time in days until 50% lethality) in detached leaf bioassay (Supplementary Table 1). The LT_{50} in T_0 *vip3A* transgenic cotton was determined for *H. armigera* (1.01 ± 0.1 days) and *S. litura* (1.26 ± 0.3 days). The data of insect bioassays was compiled accumulatively as a transgenic event. These results indicate conference of high resistance of transgenic *Vip3A* cotton against *H. armigera* and *S. litura* larvae (Table 4).

Discussion

In the progressing world population, it is difficult to maintain the food supply and demand ratio for sustainable food security. Losses due to biotic and abiotic stresses results in the depriving of food to the one third population in the world (Pimentel and Peshin 2014). Provision of food to all mankind is one of the biggest challenges being faced by the human beings on earth. Herbivorous insects are considered to be responsible for destroying one fifth of the total crop production annually in the world (Sharma and Prabhakar 2014). Insect pests are capable of evolving themselves and are adaptive to the new situations to overcome the effect of toxic substances or bypass natural or artificial plant resistance to maintain their integrity and existence on earth (Nwilene *et al.* 2008). These characteristics make them more problematic to meet the challenges of food security to the mankind. With the increasing threat of insect pests to the agricultural goods, there is a need of time to imply more advanced strategies to combat these pests (Waterfield and Zilberman 2012). Thus, exploitation of new potential toxin proteins with different mode of actions is one of the best strategies to improve resistance against various lepidopteran chewing insects (Burkness *et al.* 2010).

Spodoptera and Helicoverpa spp. are among the most devastating and damaging lepidopteran insects that result in

significant yield reduction in agricultural crops across the world (Adamczyk *et al.* 2001). Ever since the introduction of *Bt* crops in the 1990s, *Bt* toxins have shown great success in pest control in the world (Wu *et al.* 2003; Kurtz *et al.* 2007). Unfortunately, a few reports have shown the development of resistance against Cry1Ac and Cry2Ab toxin in *H. armigera* (Gunning *et al.* 2005).

To delay the development of resistance among insect pests to these *Bt*-insecticidal proteins, gene stacking/pyramiding and refugia is being commonly practiced in the field. A number of reports suggests that gene pyramiding of *cry1Ac* and *cry2Ab*, conferred delayed resistance and provided better insecticidal spectrum (Tabashnik *et al.* 2013; Carrière *et al.* 2015). Still, concerns are reserved regarding the evolution mechanism among the insect pest populations that may enable them to develop resistance later in course of time due to the phenomenon of cross-resistance in both cry-toxins as they share the same binding site in midgut epithelial membrane of the lepidopteran insects (Kurtz *et al.* 2007; Jin *et al.* 2013). This scenario motivated the scientists to develop better crops which may provide long lasting pest management to the farmers.

Vip (vegetative insecticidal proteins) is another class of proteins from *Bacillus cereus* and *B. thuringiensis* that are compatible with Cry proteins, as both toxin does not share structural homology (Estruch *et al.* 1996; Lee *et al.* 2003; Burkness *et al.* 2010). Gouffon *et al.* (2011) reported that though both Cry and *Vip3* proteins are needed to be bound to membrane receptors in midgut to get activated and exert their toxic action, still both proteins show different levels of stability and processing (Gouffon *et al.* 2011). Furthermore, both proteins bind to different receptors in the BBMV of the susceptible insects. These differences in the mode of action are believed to ensure the absence of cross-resistance to *Vip3A* proteins. Moreover, several members of the *Vip3* family have high activity against lepidopteran pests (Chakroun *et al.* 2016; Khan *et al.* 2019).

Since the beginning of this millennium, *vip3A* gene has been characterized as a broad-spectrum insect controlling *Bt*-gene around the globe (Palma *et al.* 2014). *Spodoptera* and *Helicoverpa* spp. have been reported to be the most vulnerable insects to *Vip3A* protein, though still there are a number of findings that suggests the inability of naturally isolated *vip3A* gene from the *Bacillus thuringiensis* to provide a desirable insecticidal expression level in transgenic plants that may effectively control insect pest attack (Lee *et al.* 2003; Gayen *et al.* 2012; Lemes *et al.* 2017). Nowadays, synthetic gene technology has proven to provide better and enhanced expression of codon-optimized genes compared to the naturally occurring genes (Devine *et al.* 2018). Moreover, the codon optimization of insecticidal gene may help in acquiring desired level of expression in transgenic plants (Liu *et al.* 2004; Chen *et al.* 2018; Khan *et al.* 2019).

The codon-optimized *vip3A* gene used in study was modified preferably for the cotton crop and commercially synthesized with the intention of cotton transformation. Transformation of cotton is a time taking process and needs abundant resources. Fortunately, we managed to develop 28 putative T_0 transgenic cotton lines through *Agrobacterium* mediated transformation. The successfully developed T_0 transgenic lines were confirmed with gene specific PCR as well as with kanamycin selection marker gene. Moreover, Southern hybridization also confirmed the integration of one to two copies of *vip3A* transgene in the cotton genome. Furthermore, the expression profiling by qPCR also suggests that synthetic *vip3A* gene is expressing at higher rate (4.7 to 5.8 times greater) compared to the *GhUBC1* gene expression in control plant.

To assess the insecticidal property of *Vip3A* toxin protein, the developed T_0 transgenic lines were screened through detached leaf insect bioassays using first-instar larvae of *H. armigera* and *S. litura*. It was observed that all the transgenic lines exhibited 100% insect mortality for *H. armigera*, while 99% insect mortality for *S. litura* in 96 h of feeding in detached leaf insect bioassays. On the other hand, no insect mortality was observed in untransformed and empty-plasmid transgenic line. Empty-plasmid (*i.e.*, pCAMBIA2300) transgenic lines in this study were included to trace any endogenous insecticidal activity. Luckily, no insect resistance was found in these lines suggesting that the insecticidal activity found in our *vip3A* transgenic lines was solely conferred by *Vip3A* protein that is being expressed by our codon-optimized *vip3A* transgene. Furthermore, no cannibalistic effect was observed in *vip3A* transgenic cotton lines as well as in controls. Moreover, our results confirmed the quick toxin effect of *Vip3A* protein as LT_{50} for *H. armigera* was 1.01 ± 0.1 days and *S. litura* 1.26 ± 0.3 days, respectively. This suggests that this codon-optimized *vip3A* gene has enormous potential to control resilient insect pests.

This study infers that the codon-optimized synthetic *Vip3A* toxin when expressed in cotton provides a broad-spectrum insecticidal action towards the lepidopteran pests. Till to date, no commercial crop or cultivar expressing *vip3A* toxin has been launched in Pakistan. So, these findings may help in developing cotton cultivars for better insect control and tackling resistant insect management issues. In future, we will test our T_0 and T_1 transgenic cotton lines against *Pectinophora gossypiella* as well. The promising insect resistant transgenic cotton lines developed during the course of this study can be used as a germplasm source for *vip3A* gene to develop commercial cultivars of cotton and may provide a breakthrough in agriculture sector of Pakistan.

Conclusion

The expression of synthetic *vip3A* gene can efficiently tackle the insect pests in agricultural fields. Various reports illustrated the cross-resistance and adaptability of insect

populations to first generation *Bt*-crops, emphasizing the need of exploration new *Bt*-toxins with broad-spectrum action. The present study provides all the evidences that codon-optimized synthetic *vip3A* gene possesses a versatile insecticidal potential to control resilient insect pests. Moreover, it is convincing to use this *Bt*-toxin in cotton and other agronomical crops to reduce insect related crop losses in Pakistan.

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Author Contributions

All authors equally contribution to this work.

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