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ORIGINAL ARTICLE

Development of broad-spectrum and sustainable resistance in cotton against major insects through the combination of *Bt* and plant lectin genes

Salah ud Din[1,2] · Saira Azam[1] · Abdul Qayyum Rao[1] · Mohsin Shad[1] · Mukhtar Ahmed[1] · Ambreen Gul[1] · Ayesha Latif[1] · Muhammad Azam Ali · Tayyab Husnain[1] · Ahmad Ali Shahid[1]

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Abstract

Key message **Second generation *Bt* insecticidal toxin in comibination with *Allium sativum* leaf agglutinin gene has been successfully expressed in cotton to develop sustainable resistance against major chewing and sucking insects. The first evidence of using the Second-generation *Bt* gene in combination with *Allium sativum* plant lectin to develop sustainable resistance against chewing and sucking insects has been successfully addressed in the current study.**

Abstract Excessive use of *Bt* δ-endotoxins in the field is delimiting its insecticidal potential. Second-generation *Bt* Vip3Aa could be the possible alternative because it does not share midgut receptor sites with any known cry proteins. Insecticidal potential of plant lectins against whitefly remains to be evaluated. In this study, codon-optimized synthetic *Bt* Vip3Aa gene under CaMV35S promoter and *Allium sativum* leaf agglutinin gene under phloem-specific promoter were transformed in a local cotton variety. Initial screening of putative transgenic cotton plants was done through amplification, histochemical staining and immunostrip assay. The mRNA expression of Vip3Aa gene was increased to be ninefold in transgenic cotton line L_6P_3 than non-transgenic control while ASAL expression was found to be fivefold higher in transgenic line $L_{34}P_2$ as compared to non-transgenic control. The maximum Vip3Aa concentration was observed in transgenic line L_6P_3. Two copy numbers in homozygous form at chromosome number 9 and one copy number in hemizygous form at chromosome number 10 was observed in transgenic line L_6P_3 through fluorescent in situ hybridization. Significant variation was observed in transgenic cotton lines for morphological characteristics, whereas physiological parameters of plants and fiber characteristics (as assessed by scanning electron microscopic) remained comparable in transgenic and non-transgenic cotton lines. Leaf-detach bioassay showed that all the transgenic lines were significantly resistant to *Helicoverpa armigera* showing mortality rates between 78% and 100%. Similarly, up to 95% mortality of whiteflies was observed in transgenic cotton lines when compared with non-transgenic control lines.

Keywords Vip3Aa · ASAL · Insecticidal crystal proteins · Cotton pests

Introduction

Cotton is the foremost cash crop and an essential source of raw material to the textile industry of Pakistan; usually known as "white gold" due to its importance in the country's economy (Azam et al. 2013; Puspito et al. 2015). Pakistan is the fifth leading producer of cotton; the third major exporter and consumer and the largest exporter of cotton yarn all over the world (Mansoor et al. 2020). Cotton production for the financial year 2018/19 was recorded to be 9.861 million bales, a decrease of 17.5% over the last year's production of 11.946 million bales, still significantly behind the expected target of 14.4 million bales. The lower than

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✉ Abdul Qayyum Rao
qayyumabdul77@yahoo.com

[1] Plant Transformation Lab, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab Lahore, Lahore 53700, Pakistan

[2] Institute of Molecular Biology and Biotechnology (IMBB), Centre for Research in Molecular Medicine (CRiMM), The University of Lahore, 1 Km Defence Road, Lahore 54500, Pakistan

expected production is due to the considerable contraction of 12.1% in the cultivation area which was 2.3 million hectares as compared to 2.7 million hectares last year (Abbas et al. 2020). Being a developing country, Pakistan needs to grow more cotton to meet the export and domestic requirements (Farooq 2016).

The cotton industry of Pakistan is facing several challenges in the global market such as competition with synthetic fibers, poor fiber quality and low yield, mainly due to infestation of different insect pests (Bakhsh et al. 2005). The cotton crop is attacked by more than fifteen insect species which can be divided into two categories, i.e. sucking and chewing insects (Shah et al. 2017). Sucking insects damage the crop by sipping essential plant nutrients and result in wilting, yellowing, drying of the plant and reduced fiber quality (Chandrasekhar et al. 2014; Chaudhry et al. 2009). Second, these insects also act as carriers of different plant viruses such as Begomoviruses that are transmitted by whitefly (*B. tabaci*) (Ahmad et al. 2017). On the other hand, chewing insects attack directly the vegetative parts of the plants. It is estimated that almost half of the cotton yield is lost due to the infestation of sucking and chewing insect pests (Saini 2011).

Intensive use of chemical pesticides on crop plants to inhibit insect/pest attack is not only harmful to the plant itself; environment; human health but also creates economic pressure on the farmer's input. These synthetic pesticides adversely affect non-target beneficial insects directly or indirectly due to their non-selective properties, moreover, insects have also developed resistance to these synthetic insecticides (Ali et al. 2016; Wojciechowska et al. 2016). It has also been estimated that the use of insecticides in Pakistan increased from 5000 tons to 44,872 tons during the last thirty years (Khan et al. 2015).

The era of modern genetic engineering started in the late 1970s; since then, using sophisticated genetic transformation tools, scientists are now able to develop inbuilt resistance in crop plants against chewing or lepidopteran insects through the introduction of insecticidal crystal proteins (ICPs) derived from *Bacillus thuringiensis*, a gram-positive and spore-producing bacterium (Chandan et al. 2017; Javaid et al. 2016). Due to the continuous plantation and poor insect pest management in the field, over the years, many pest species have now developed resistance against these toxins that may minimize the overall performance of this technology. Therefore, to expand the insecticidal spectrum of pest control programmes and to combat resistance build-up, novel insecticidal proteins with greater toxicity are required (Javaid et al. 2018; Tabashnik et al. 2013).

The vegetative insecticidal protein 3A (Vip3A) from *Bacillus thuringiensis* (*Bt*), secreted during the vegetative growth stage (Estruch et al. 1996), could be the best possible candidate toxin due to the fact that it does not share sequence homology with any known *Bt* crystal proteins. Interaction of Vip3Aa with brush border membrane vesicle (BBMV) proteins of target insects suggests that it has ion channel properties (Lee et al. 2006) and binds with different midgut receptors (Chen et al. 2017). Despite being structurally different, both the crystal and vip protein families may exert their toxicity apparently through a similar mode of action (Liu et al., 2011). The Vip3 genes have been successfully transformed in different crops like cotton, maize and cowpea (Reisig et al. 2018; Burkness et al. 2010; Bett et al. 2017) and in some studies, pyramided with different crystal toxin genes to enhance broad range pest protection and delay resistance build-up (Chen et al. 2017).

Insecticidal crystal proteins from *Bacillus thuringiensis* have not been reported yet to control sucking insects (Vajhala et al. 2013). A lot of work has been done to explore possible substitutes of *Bt* insecticidal toxins to engineer resistance against sucking insect pests. In the last three decades, many plant-derived mannose-binding lectins have shown insecticidal activity against Hemiptera, Coleoptera and Lepidoptera (Macedo et al. 2015). The expression of lectin genes under the influence of phloem-specific promoters could be a possible alternative to chemical insecticides against major sucking pests (Javaid et al. 2016). Recently, a 25-kDa mannose-binding homodimeric leaf agglutinin lectin isolated from *Allium sativum* (ASAL) has been successfully transformed into different economically important crops and shown to have entomotoxic effects on major sap-sucking pests of cotton, mustard and rice (Vajhala et al. 2013; Yarasi et al. 2008).

In the current study, an effort was made to develop transgenic cotton plants through the transformation of codon-optimized synthetic *Bt* Vip3Aa gene driven by constitutive CaMV35S promoter and leaf agglutinin gene from *Allium sativum* (ASAL) under phloem-specific RTBV promoter to minimize the losses caused by the infestation of major sucking and chewing insect pests.

Materials and methods

Construction of binary vector

Full-length nucleotide sequences of Vip3Aa (JQ946639.1) and *Allium sativum* leaf agglutinin ASAL (EU252577.1) were retrieved from GeneBank. The codons were optimized according to cotton (*Gossypium hirsutum*) to get high transgene expression by using Gensmart software. The Vip3Aa and ASAL genes were chemically synthesized by BioBasic Inc. under CaMV35S and phloem-specific RTBV promoter, respectively, in a single cassette (Vip3Aa + ASAL) with X*ho*I and H*ind*III restriction sites. The pUC57 cloning vector harboring gene cassette was transformed into

competent cells of *E. coli* (Top 10) through the heat-shock method (Sambrook et al. 2001). Recombinant plasmid DNA (600 ng) was digested with 10 units of FastDigest X*ho*I and H*ind*III restriction enzymes (Thermo Fisher Scientific cat # FD0694 & FD0504, respectively). Full-length gene cassette was ligated with pCAMBIA-1301 expression vector in 1:1 (vector to insert) ratio. The map of the binary vector (pCAMBIA-1301_Vip3Aa + ASAL) is shown in Fig. 1. The recombinant expression vector was then transformed into 100 µL freshly prepared chemically competent cells of *Agrobacterium tumefaciens* strain LBA4404 by using Electroporator (BioRad Gene Pulser Model 165–2105).

Agrobacterium-mediated cotton transformation

Shoot apex method of *Agrobacterium*-mediated cotton transformation was used to incorporate Vip3Aa + ASAL gene cassette into a locally developed cotton variety (CEMB 33) as described by (Rao et al. 2011). In this study, approximately 4500 cotton seedlings were co-cultivated with *Agrobacterium* cells ($OD_{600} = 1$) containing gene cassette and harvested at 3000 × g, 4° C for 10 min and resuspended in MS-zero (Murashige and Skoog 1962) broth (Thermo Fisher Scientific cat #50–254-673). After *Agrobacterium* treatment, the seedlings were blot dried and shifted on MS plates containing 250 µg/mL cefotaxime (Thermo Fisher Scientific cat # 64,485-93-4) to avoid bacterial contamination and kept in a growth room at 25 ± 2 °C and 16: 8 h light:dark cycle for the next 2–3 days. Totally, 600 rooted plantlets were then shifted to glass test tubes containing MS

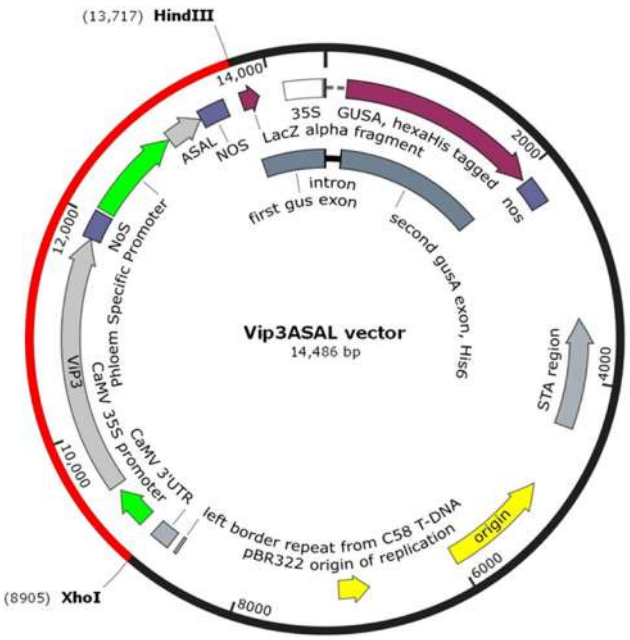


Fig. 1 Map of pCAMBIA1301_Vip3Aa + ASAL

medium supplemented with kinetin and B_5 vitamin complex (Thermo Fisher Scientific cat # 525-79-1 and 50-254-603, respectively) for efficient growth. After 4–6 weeks of regeneration on MS medium, fifty-three putative transgenic plants were able to survive and transferred to sigma pots containing sterilized loamy soil (Rashid et al. 2004). The initial screening of the plants was done with the expression of GUS reporter gene (Satyavathi et al. 2002) and Vip3Aa-specific immunostrips/dipsticks (Cat# STX 83,500/0050 Agdia) (Supplementary Figs. 1 and 2). The putative plants were kept covered with polythene bags to maintain proper humidity and were kept in a growth room at 28 ± 2° C with a photoperiod of 16 h light and 8 h dark. After 2 weeks of acclimatization in sunlight, the putative transgenic cotton plants were transferred to the field.

Molecular analyses of transgenic cotton plants

The genomic DNA from fresh cotton leaves was isolated following a combination of two protocols (Sukumar et al. 1997; Zhang et al. 2000) with some modifications. The putative transgenic cotton plants were confirmed through polymerase chain reaction using gene-specific detection primers (Supplementary Table 1). For the mRNA expression analysis of transgenic cotton plants, total RNA was extracted following a modified protocol as described by Jaakola et al. (2001) and Sarwar et al. (2019). The complementary DNA (cDNA) was synthesized through one-step reverse transcriptase RT-PCR with random hexamers using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622). The mRNA was quantified through quantitative Real-time qRT-PCR in an iQ5 cycler (BioRad) with a 96-well plate using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, K0221). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a comparative control to normalize the data. The C_t values obtained from different transgenic cotton plants were statistically analyzed using iQ5 software (BioRad) version 1.0. The Vip3Aa protein in transgenic cotton plants was quantified through ELISA using Agdia ELISA kit (Cat #83,500/480).

Agronomic traits of transgenic cotton plants

Several morphological parameters including (plant height; number of monopodial and sympodial branches per plant; number of bolls per plant; ginning out turn percentage) were measured in T_1 generation of transgenic and non-transgenic cotton plants (Jul–Nov 2017). Physiological parameters (net photosynthetic rate; transpiration rate; gaseous exchange rate were measured using an infrared gas analyzer (CIRAS-3 portable photosynthesis system, USA) in transgenic cotton lines of T_1 generation. The data were collected from 1000 to 1300 h (Aug–Sep 2017) on fully

expanded cotton leaves with the specific adjustments of molar flow rate of air (403.3 µmol m^{-2} s^{-1}), atmospheric pressure (99.9 kPa), water vapor pressure in chamber (6.0–8.9 mbar), photosynthetically active radiation (PAR) at leaf surface (1000–1711 µmol m^{-2} s^{-1}), temperature of leaf (28.4–32.4 °C), ambient temperature (22.4–27.9 °C) and ambient CO_2 concentration (352 µmol mol^{-1}). One-way analysis of variance (ANOVA) was performed to compare the significance level between transgenic and control cotton lines (Dunnett's test) at 95% ($P < 0.05$) confidence level using GraphPad Prism software version 5 for Windows (Prism 2007). The samples were sent to the Centre for Electron Microscopy, Zhejiang University, China to study the surface morphology of fiber samples from transgenic and non-transgenic cotton plants using a scanning electron microscope (Model SU8010 Hitachi Japan). Each fiber was excised into three parts, i.e. tip, middle and base. The screw pitch of fiber and the distance of fiber rotation in 360° were measured three times for each sample by SEM with an accelerating voltage of 20 kV and 10 µA current at 400X, 1000X and 4000X magnifications.

Insect bioassays

The transgenic cotton plants were subjected to the standard laboratory leaf-detach bioassays with American bollworm (*Helicoverpa armigera*) and clip bioassay with whitefly (*Bemisia tabaci*) to assess the efficacy of transgenes. The bioassay with American bollworm was performed in triplicates, i.e. three plants of T_2 generation from each of the transgenic and non-transgenic control cotton lines; one fresh leaf per plant was taken and placed on a wet sterile filter paper in 20-cm Petri dishes. Three pre-fasted 2nd instar larvae were placed in each of the plate and allowed to feed on the leaf. The plates were sealed with parafilm and kept at 25 ± 2 ºC, 70% relative humidity and 16 h:8 h (light to dark) cycle in a growth room. The percentage mortality rate was recorded on the third day of infestation (Siddiqui et al. 2019; Puspito et al. 2015).

The transgenic cotton plants in T_2 generation at (6–10 leaf stage) were grown in a glasshouse at 37 ± 2 °C, 14L/10D and ≈60% humidity. The plants were left un-infested for one week by carefully isolating them in a net cage. The whitefly culture was maintained on non-transgenic cotton plants in a separate greenhouse at the same conditions. Before the start of bioassay, the 0–24-h-old whitefly adults were captured using a manual aspirator and kept on ice to minimize environmental stress. Three biological replicates, from each transgenic and non-transgenic cotton lines, were used in the bioassay and to each replicate; three clip cages having four ice-inactivated whiteflies (2 M/2F) were released. The mortality data were recorded on the third day of infestation. One-way Analysis of Variance (ANOVA) was performed to compare the significance level of difference between transgenic and non-transgenic control lines fed whiteflies (Dunnett's test) at 95% ($P < 0.05$) confidence level (Ludgate 2013).

Detection of transgenes

The transgene location and copy number in the cotton genome were determined through Fluorescence in situ hybridization (FISH) in T_2 generation using Vip3Aa-specific probe. The fluorescent signals were detected by fluorescent microscope (Olympus Model BX6l) on a blue filter for 4′,6-diamidino-2-phenylindole (DAPI) stain and red filter for propidium iodide (PI) stain. The pictures of fluorescence signals were taken by a charged-coupled device (CCD) camera attached with a microscope and analyzed/enlarged using Adobe Photoshop 7.0.

Results

Molecular analyses of transgenic cotton plants (T_0 progeny)

The successful integration of transgenes (Vip3Aa + ASAL) in putative transgenic cotton plants was confirmed through amplification using genomic DNA extracted from fresh cotton leaves. Recombinant expression vector containing gene cassette was used as positive control and genomic DNA isolated from non-transgenic cotton plant was used as negative control. The amplification of 587 bp fragment with ASAL-specific primers and 682 bp fragment with Vip3Aa-specific primers confirmed the incorporation of gene cassette in seven transgenic cotton plants as shown in Fig. 2a, b. The overall transformation efficiency remained 1.17%. Further, the Vip3Aa protein expressed in transgenic cotton plants was quantified through ELISA with the help of a standard curve of known Vip3Aa concentrations (Supplementary Fig. 3). The maximum protein concentration (4.26 µg/mL) was observed in plant L_6P_3 when compared with non-transgenic control as depicted in Fig. 3.

Molecular analyses of transgenic cotton plants (T_1 progeny)

Five transgenic cotton plants that showed maximum Vip3Aa concentration in T_0 generation having better morphological/physiological parameters and yield performance in field conditions (data not shown) were further selected for the advancement of T_1 generation consisting of five replicates per transgenic line. Total of eleven plants were found positive from the progeny of five transgenic lines/events (Fig. 4a, b).

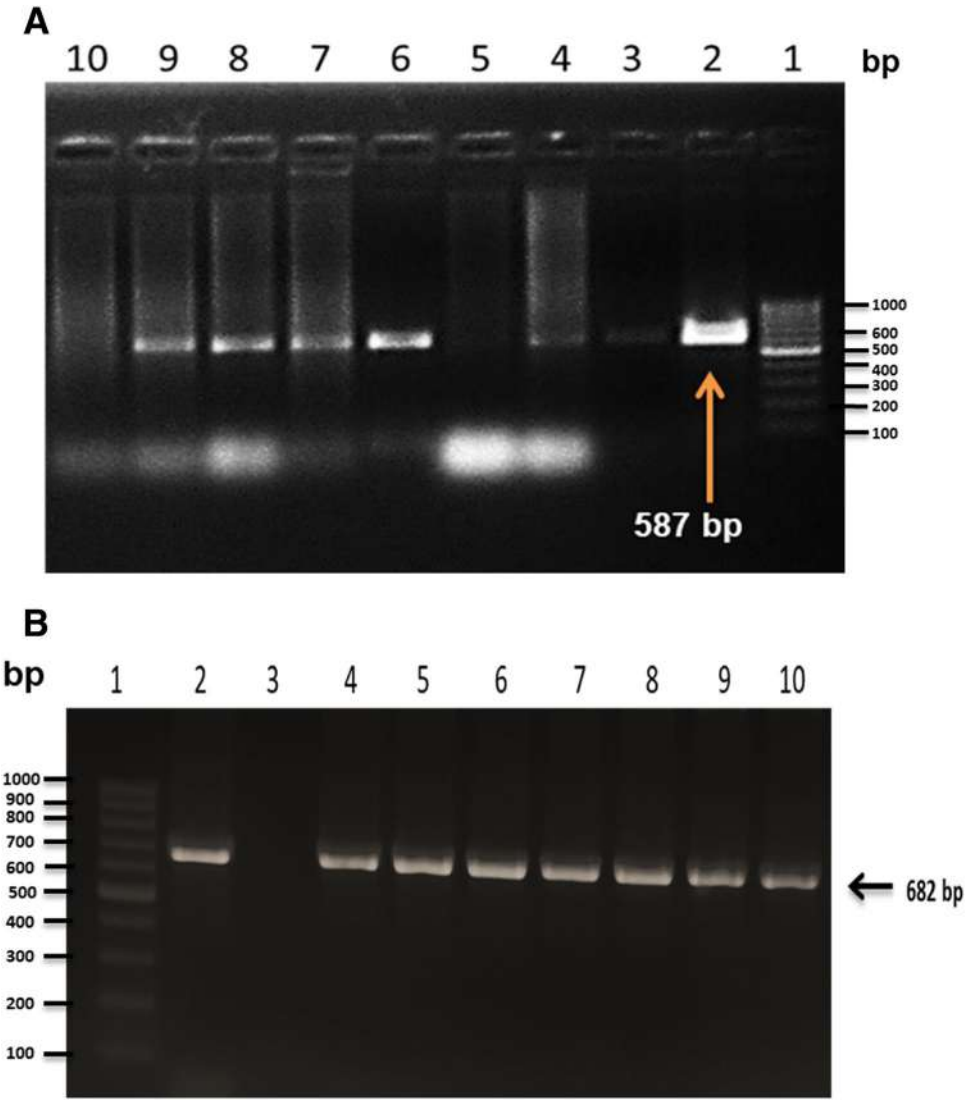


Fig. 2 **a** Confirmation of putative transgenic cotton plants through ASAL primers. Lane 1: 100 bp ladder; Lane 2: positive control (recombinant expression vector containing gene cassette); Lane 3–9: Genomic DNA isolated from transgenic cotton plants. Lane 10: negative control (DNA isolated from non-transgenic cotton plant). **b** Confirmation of putative transgenic cotton plants through Vip3Aa primers. Lane 1: 100 bp ladder; lane 2: positive control (recombinant expression vector containing gene cassette); lane 3: negative control (DNA isolated from non-transgenic cotton plant); lane 4–10: genomic DNA extracted from transgenic cotton plants

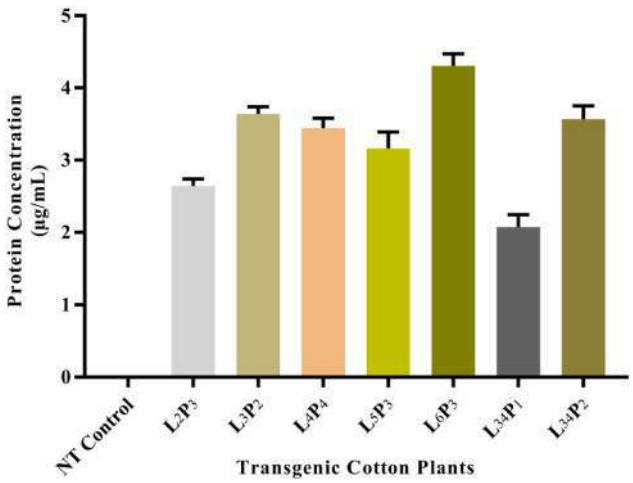


Fig. 3 Quantification of Vip3Aa protein through ELISA

The quantitative RT-PCR analysis of Vip3Aa and ASAL mRNA expression revealed relative fold change in all the transgenic cotton lines. The maximum mRNA expression level of Vip3Aa gene (8.7-fold) was observed in transgenic cotton line L_6P_3 (Fig. 5a) while the ASAL gene (fivefold) was observed in the line L_4P_4 (Fig. 5b) as compared to non-transgenic control cotton plants.

The transgenic cotton line L_6P_3 showed maximum Vip3Aa concentration in T_1 transgenic cotton lines as compared to non-transgenic cotton plants (Fig. 6).

Agronomic traits of transgenic cotton plants

Different morphological characters of the transgenic cotton plants were critically observed for evaluation of the best transgenic cotton line as the yield must not be compromised in the best insect-resistant cotton line. Although all transgenic cotton lines (except $L_{34}P_2$) were found to have higher

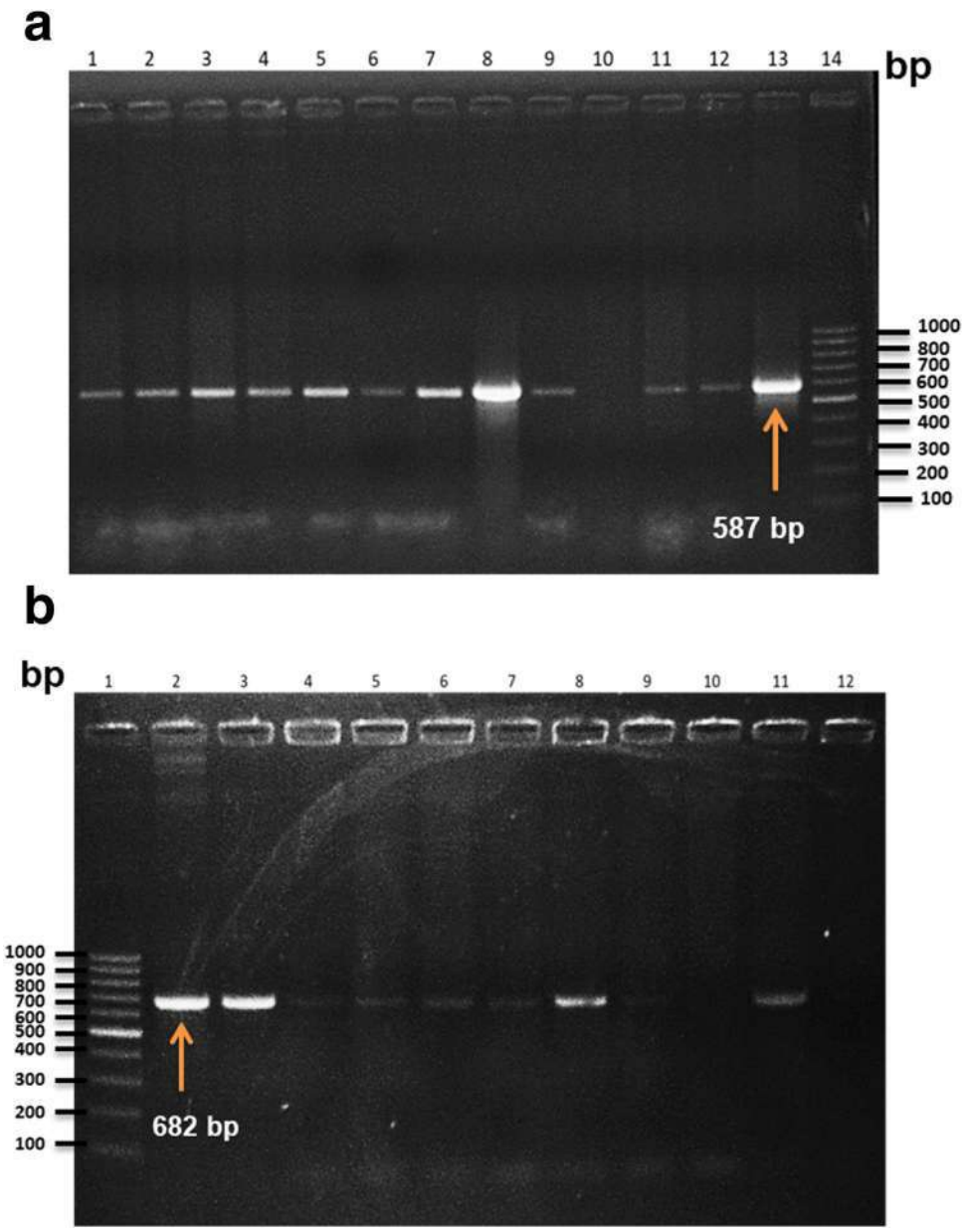


Fig. 4 **a** Confirmation of transgenic cotton plants through PCR in T_1 progeny (ASAL primers). Lane 1–9, 11–12: genomic DNA isolated from transgenic plants of T_1 progeny; lane 10: negative control (DNA extracted from non-transgenic cotton plant); lane 13: positive control (recombinant vector containing gene cassette); lane 14: 100 bp DNA ladder. **b** Confirmation of transgenic cotton plants through PCR in T_1 progeny (Vip3Aa primers). Lane 1: 100 bp DNA ladder; lane 2: positive control (recombinant vector containing gene cassette); lanes 3–11: Genomic DNA extracted from transgenic plants of T_1 progeny; lane 12: negative control (DNA extracted from non-transgenic cotton plant)

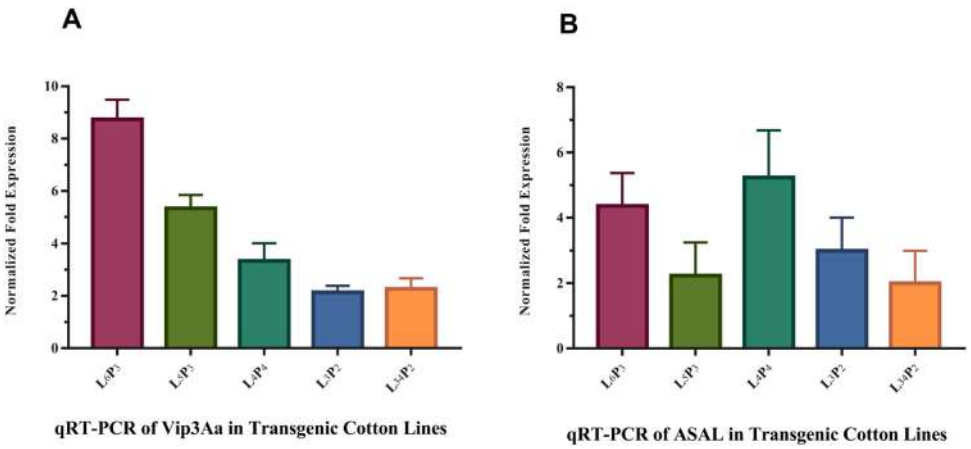


Fig. 5 **a**, **b** mRNA expression of Vip3Aa and ASAL genes through qRT-PCR

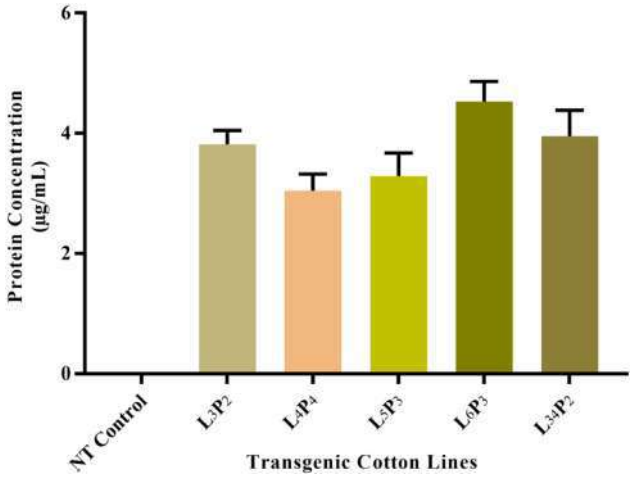


Fig. 6 Quantification of Vip3Aa protein through ELISA in T_1 transgenic lines

yields as compared to non-transgenic control cotton plants, the maximum cotton yield was observed in transgenic line L_6P_3 (Fig. 7a). A significant increase in plant height was observed in transgenic cotton lines L_6P_3 and L_3P_2 as compared to non-transgenic cotton line (Fig. 7b). Maximum number of bolls per plant (108) was recorded in transgenic cotton line L_6P_3 which is almost double as compared to non-transgenic control (58). Similarly, the transgenic line L_3P_2 also showed a significant increase in the number of bolls, whereas non-significant increase was seen in the rest of the transgenic cotton lines (Fig. 7c). The number of monopodial branches per plant ranged from 4 to 8 in transgenic cotton lines as compared to 7 in non-transgenic control. Likewise, the number of sympodial branches increased up to 16 in transgenic cotton lines as compared to 12 in non-transgenic control. Two-way ANOVA was applied to this grouped data which showed that the number of branches per plant found to be significantly variable among each other. On the other hand, interaction between transgenic cotton lines and number of branches per plant did not show any significant difference from control (Fig. 7d). The ginning out-turn (GOT) percentage (weight of the lint divided by the weight of seeds multiplied by 100) in transgenic cotton line L_6P_3 was significantly higher than non-transgenic control cotton plants, while a non-significant increase was seen in the rest of the transgenic cotton lines (Fig. 7e).

Fiber analysis of transgenic cotton plants

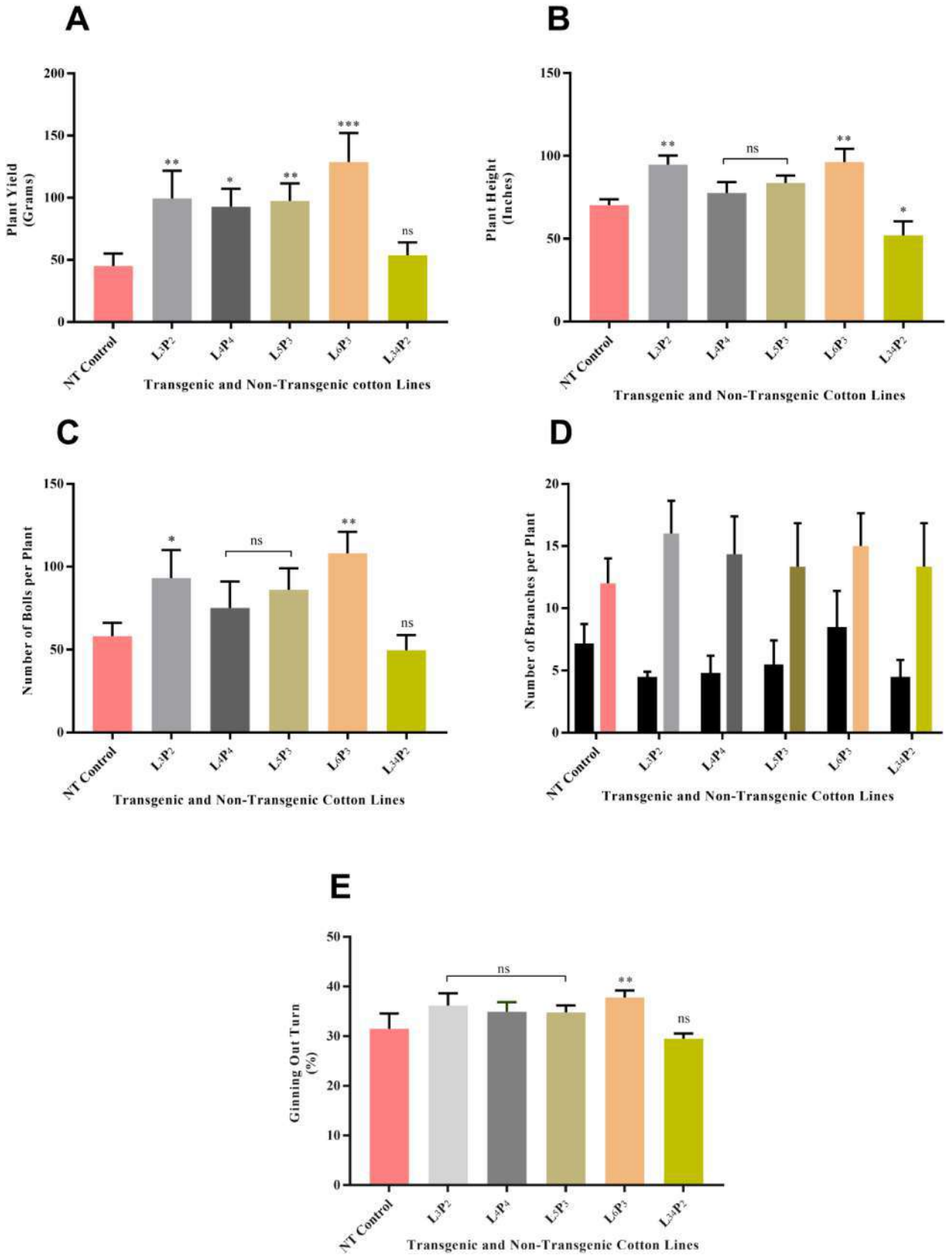
The scanning electron microscopic (SEM) analysis of fiber samples from both, the transgenic and non-transgenic control cotton plants showed flat, twisted ribbon-like structure and natural folds running parallel along the length of cotton fibre. No visible difference in fiber morphology (fineness, smoothness, numbers of twists and presence of ribbon-like structures) was seen among transgenic and non-transgenic control cotton plants which depicts that there is no positive or negative correlation exist between transgene expression and cotton fibre quality (Fig. 8a–f).

Physiological characteristics of transgenic plants

Net photosynthetic activity in fully expanded cotton leaves of transgenic and non-transgenic cotton lines was measured through a portable infrared gas analyzer (IRGA). Statistical analysis showed that only the transgenic cotton line L_6P_3 was found to have significantly higher photosynthetic rate (5.93 mmol CO_2 m^{-2} s^{-1}) as compared to non-transgenic control cotton plants, no significant difference in photosynthetic rate was observed among other transgenic cotton lines. A significant increase in transpiration rate (4 mmol H_2Om^{-2} s^{-1}) was seen in transgenic cotton line L_4P_4 as compared to control. The statistical data also showed that a significant increase in the gaseous exchange rate was measured in transgenic cotton line $L_{34}P_2$ (182 mmol CO_2 mol^{-1}) when compared with non-transgenic control (Fig. 9a–c).

Insect bioassay with cotton bollworm (*Helicoverpa armigera*)

Transgenic cotton lines expressing Vip3Aa + ASAL insecticidal proteins were evaluated for their toxicity against cotton bollworm larvae in T_2 generation by standard laboratory leaf detach bioassay. The results showed that the transgenic cotton plants were highly resistant to *H. armigera* as compared to non-transgenic control. Almost whole leaves of non-transgenic control cotton plants were found to be damaged significantly or even consumed, while the transgenic cotton leaves were barely fed. Similarly, the larvae feeding on non-transgenic control cotton leaves were significantly grown up and developed into the next instar with a maximum increase in weight. Besides the minor damage to the leaves of transgenic cotton plants, a significant delay in the larval growth with ultimate death was observed. All the larvae that survived after the third day of bioassay were extremely weak and immotile as compared to the larvae fed on non-transgenic cotton plants as shown in Fig. 10I a–f. In terms of mortality, transgenic cotton lines L_6P_3B, L_6P_3C, and $L_{34}P_2$ showed 89%; 100% and 89% mortality, respectively, while L_3P_2 and L_5P_3 showed 78% mortality as compared to non-transgenic control cotton line (Fig. 10II).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Transgenic and Non-Transgenic Cotton Lines

◂**Fig. 7** Comparison of morphological characteristics between transgenic and non-transgenic lines (**a**) plant yield; (**b**) plant height; (**c**) number of bolls per plants; (**d**) number of branches per plant; (**e**) ginning out-turn. Each bar in graphs represents the mean of three plants ($n=3$). Asterisk (*) shows significant variation at 95% confidence interval ($P \leq 0.05$); **significant at 99% confidence interval ($P \leq 0.01$); ***significant at 99.9% confidence interval ($P \leq 0.001$); *ns* = non-significant ($P > 0.05$)

Insect bioassay with whitefly (*Bemisia tabaci*)

After testing the transgenic cotton plants against cotton bollworm, the same plants were also exposed to whitefly feeding in specialized clip cages as shown in Fig. 11I (A). After 24 h of feeding on transgenic cotton plants, the bodies of insects started stretching abnormally, Fig. 11I (B), while the insects on non-transgenic control cotton plants remained alive and active as depicted in Fig. 11 (C). The mortality data were collected after three days of infestation. Transgenic cotton lines L_3P_2, L_5P_3, L_6P_3B and L_6P_3C showed 95%; 89%, 89% and 72% mortality, respectively (Fig. 11).

Determination of transgene copy number and location

One transgenic cotton plant (L_6P_3C) from transgenic cotton line L_6P_3 in T_2 generation was subjected to determination of the copy number and transgene location through fluorescence in situ hybridization. The transgenic cotton plant showed homozygosity, i.e. one copy number at chromosome number 9 at both chromatids and one copy number at chromosome number 10, whereas no signal was observed in non-transgenic cotton plant as shown in Fig. 12a, b.

Discussion

Yield improvement of genetically modified *Bt* crops has been attributed to its specific features of suppressing pests, decreased treatments with conventional insecticides and conserving natural enemies. However, limitations arise due to narrow insect spectrum along with continuous use of *Bt* crops in field without buffer zone and lead to the development of resistance in a number of insect species (Tabashnik and Carrière 2019). Characterization of next-generation toxins to broaden the insect spectrum could be the ultimate solution to this emerging problem (Bravo et al. 2017). The vegetative insecticidal protein 3Aa (Vip3Aa) from *Bacillus thuringiensis* shares no sequence homology with already characterized cry proteins and displays a wider insecticidal spectrum against a broad range of chewing insects (Estruch et al. 1996; Song et al. 2016). Similarly, the carbohydrate-binding plant lectins infer toxicity to agronomically important sap-sucking insects (Ahmed et al. 2017; Chandrasekhar et al. 2014; Rani et al. 2017; Vajhala et al. 2013). In the current study, codon-optimized and chemically synthesized Vip3Aa and ASAL genes under a constitutive promoter (CaMV35S) and phloem-specific promoter from Rice tungro bacilliform virus (RTBV), respectively, were introduced in cotton using *Agrobacterium* transformation system.

In this study, a transformation efficiency of 1.17% was attained using the shot-apex-cut method comparable with previous reports (Bajwa et al. 2015; Rao et al. 2011; Gul et al. 2020). The recalcitrant behavior of cotton, cell density of *Agrobacterium* cells, transgene size and more importantly the selection pressure of antibiotic used are hindering factors in low transformation efficiency (Bazargani et al. 2010). In some studies, higher transformation efficiencies in cotton were reported (Majeed et al. 2000; Wamiq et al. 2016) because of different efficiency calculation formulas used. The maximum concentration of Vip3Aa protein (4.26 µg/mL) was observed in plant L_6P_3 when compared with negative control. The concentration of *Bt* toxins in commercial *Bt* cultivars (cotton, maize, and potato) generally ranges from 1 to 11 µg/g of fresh leaf weight, which is considered to be sufficient for "high dose/refuge" strategy requirement (Cohen et al. 2000).

The mRNA expression of Vip3Aa gene varied from 2- to 8.7-fold; similarly, in case of ASAL gene, 2- to 5-fold increase was observed in transgenic cotton lines. Similar outcomes are also reported by Wu et al. (2011) while evaluating synthetic Vip3A gene and reported varied expression in different transgenic cotton lines. The transgene expression is directly related to different factors like promoter, insertion point of genes, copy number and location on host chromosome (Rao et al. 2011). Owing to fast and easy approach and similarity of results obtained when compared with southern blot (Yasmeen et al. 2016) the copy number and location of transgene were determined through fluorescent in situ hybridization (FISH) (Tsuchiya and Taga 2001). One transgenic cotton plant from line L_6P_3 was subjected to FISH analysis in T_2 generation using Vip3Aa-specific probe following the same procedure as explained by Ali et al. (2016) and showed homozygosity (signal on each chromatid) on chromosome number 9 and one copy number at chromosome number 10. The results are in accordance with Ali et al. (2016) who evaluated two cotton varieties (CRSP-I and CRSP-II) for determining copy numbers of Cry1Ac and Cry2A using the same approach and obtained single copy number in transgenic cotton line having higher expression of transgene.

Different agronomic characteristics, scanning electron microscopic analysis of fibre and physiological parameters (photosynthetic rate; transpiration rate; gaseous exchange rate) of transgenic cotton lines in T_1 progeny were studied in comparison to negative control plants. The transgenic cotton lines showed significant variation in studied morphological

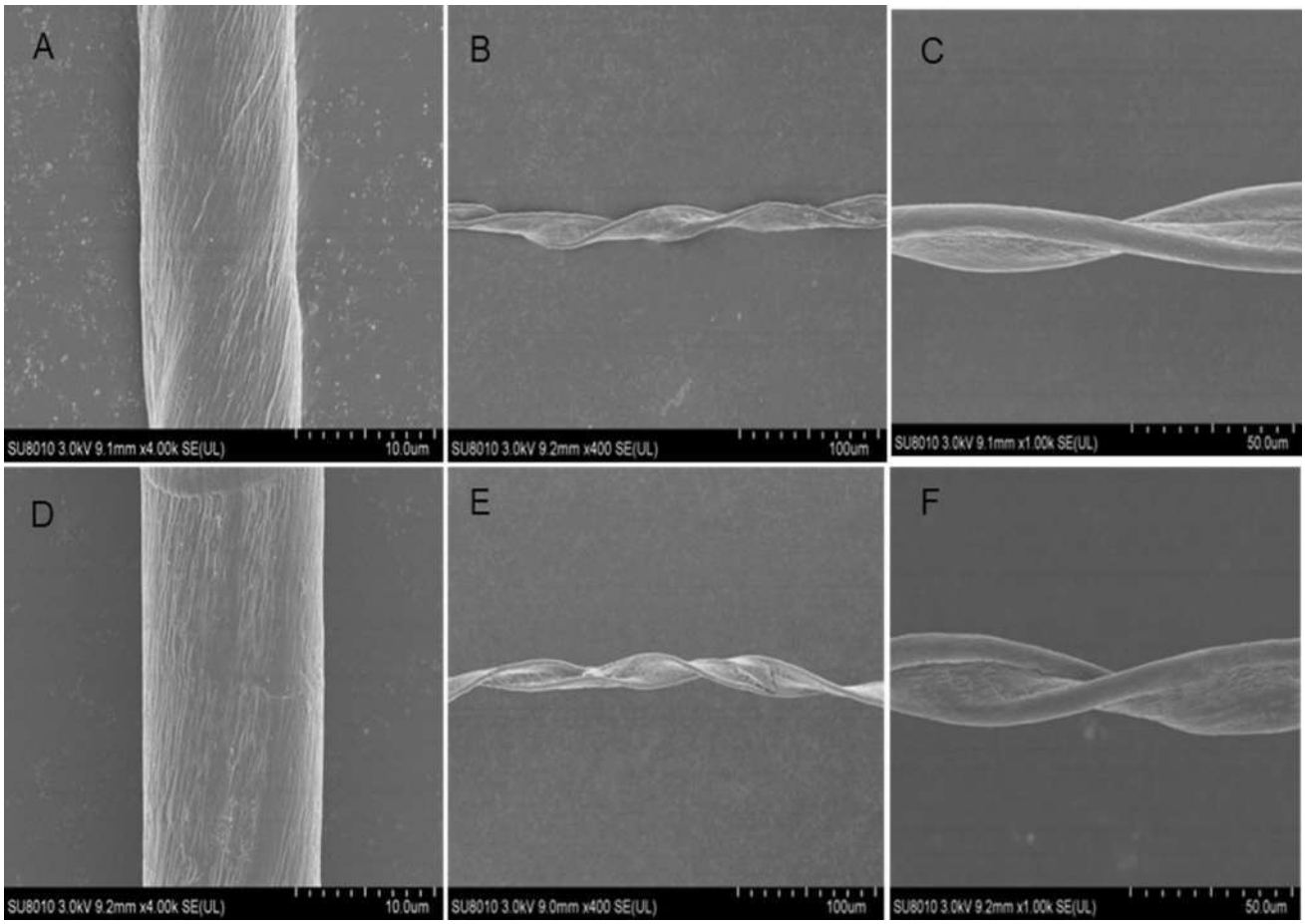

Fig. 8 Scanning electron microscopic analysis of fiber surface. A, B, C SEM Images of cotton fiber from non-transgenic cotton plant at 400X, 1000X and 4000X magnifications, respectively (D, E, F) SEM Images of cotton fiber from transgenic cotton plant at 400X, 1000X and 4000X magnifications, respectively

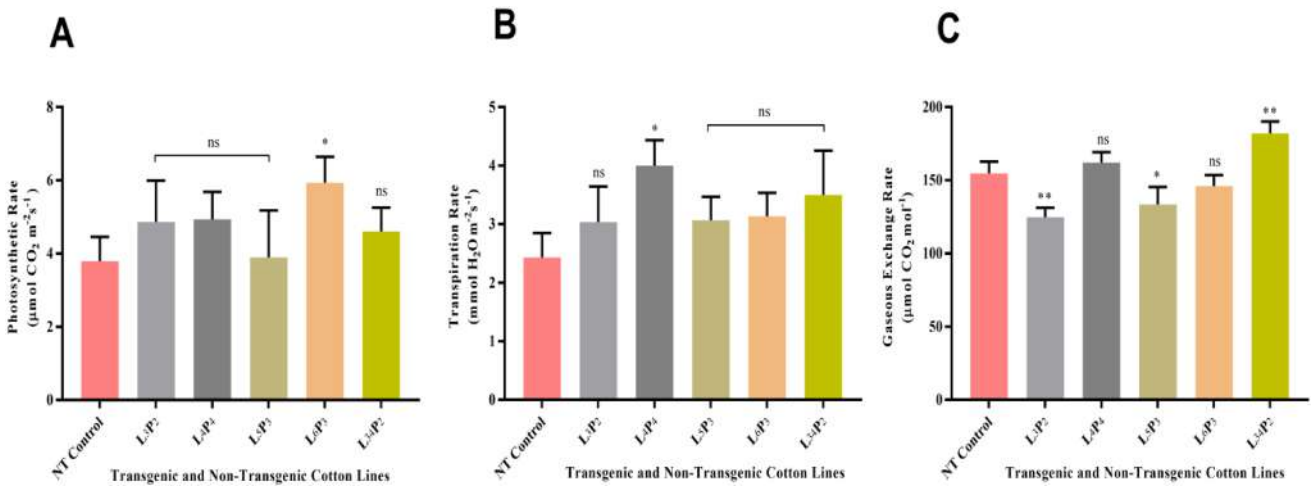

Fig. 9 Comparison of physiological parameters between transgenic and non-transgenic cotton lines: (**a**) net photosynthetic rate; (**b**) transpiration rate; (**c**) gaseous exchange rate. Each bar in graphs represents the mean of three plants ($n=3$). Asterisk (*) shows significant variation at 95% confidence interval ($P \leq 0.05$); **significant at 99% confidence interval ($P \leq 0.01$); ***significant at 99.9% confidence interval ($P \leq 0.001$); *ns* non-significant ($P > 0.05$)

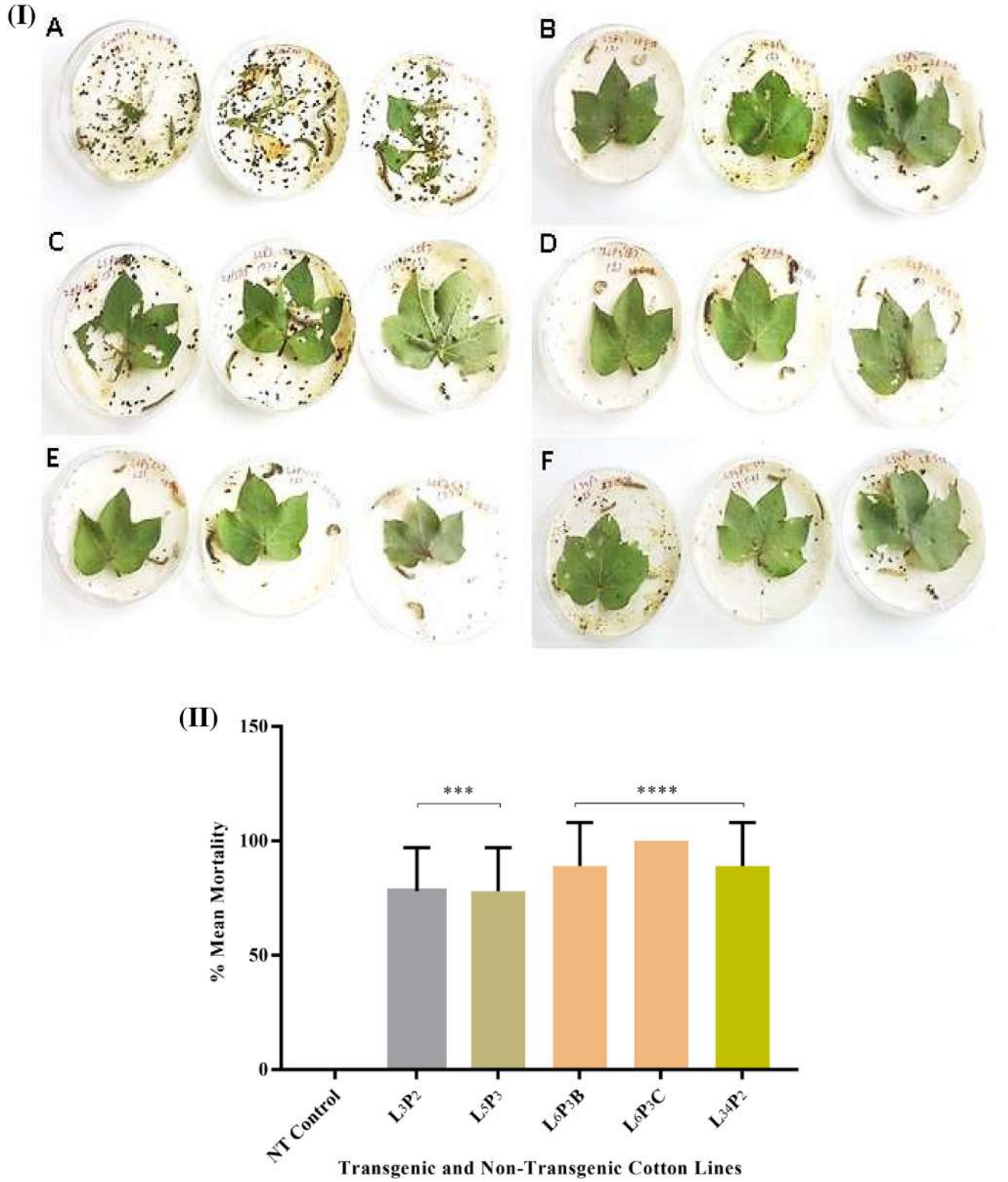

Fig. 10 I Leaf-detach bioassay of transgenic cotton lines with cotton bollworm (*Helicoverpa armigera*). (**a**) Leaf damage in non-transgenic control cotton plants, (**b**–**f**) leaf damage in transgenic cotton plants. II Mean percent mortality of Cotton Bollworm (*Helicoverpa armigera*) larvae Each bar in graph represents mean of three plants ($n = 3$); ****significant at 99.99% confidence interval ($P \leq 0.0001$); ***significant at 99.9% confidence interval ($P \leq 0.001$).

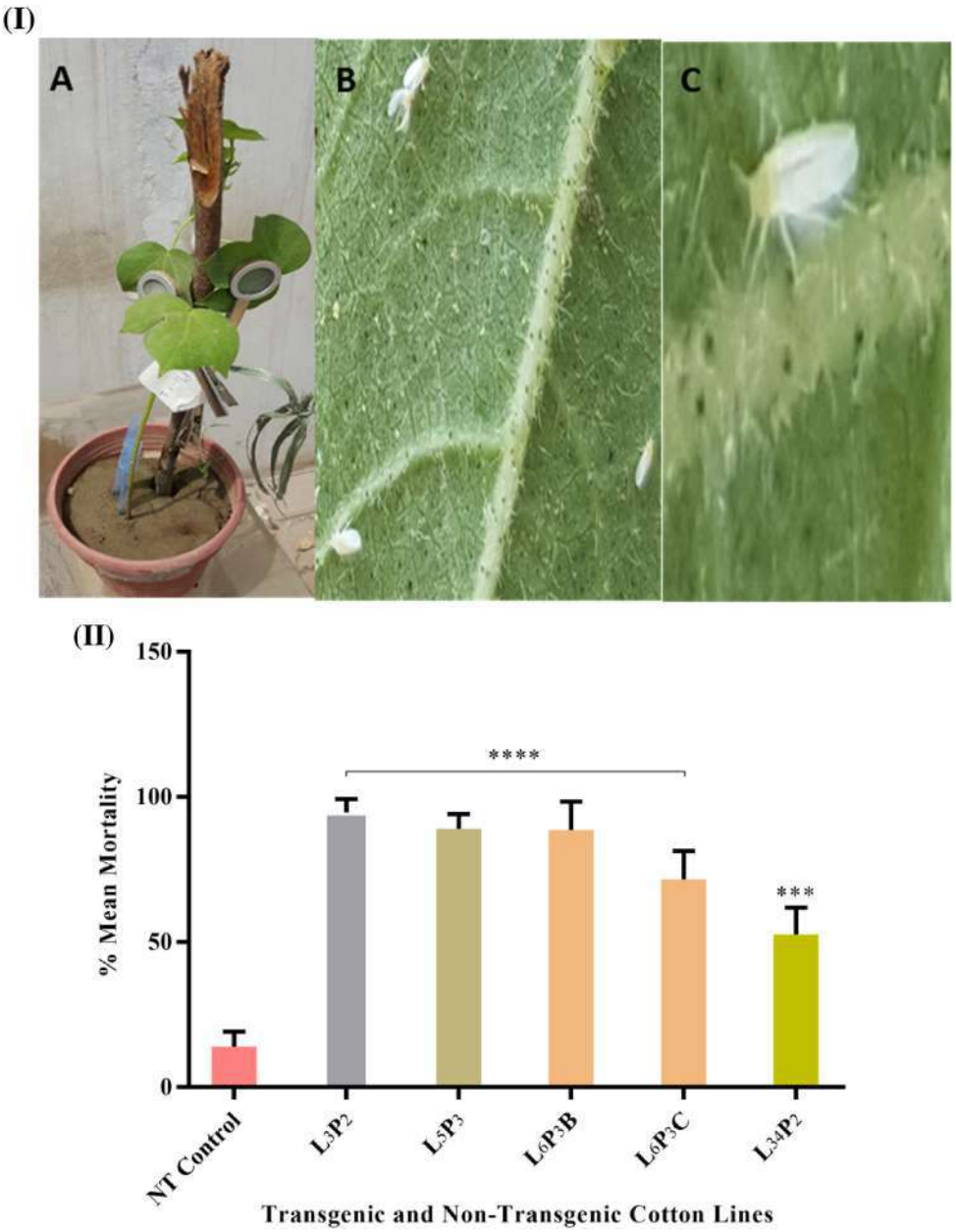

Fig. 11 I (**a**–**c**) Clip cage bioassay of transgenic cotton lines with whitefly (*Bemisia tabaci*). **a** Transgenic plant with clip cages. **b** Dead whiteflies on transgenic leaf. **c** Whitefly feeding on non-transgenic leaf. II Mean percent mortality of whitefly (*Bemisia tabaci*). Each bar in graph represents mean of three plants ($n = 3$); ****significant at 99.99% confidence interval ($P \leq 0.0001$); ***significant at 99.9% confidence interval ($P \leq 0.001$)

characteristics as previously published by Raybould and Vlachos (2011); Romeis et al. (2008) who claimed that statistically significant differences were observed between transgenic cotton plants expressing Vip3Aa in comparison to non-transgenic controls. The scanning electron microscopic (SEM) images of transgenic and non-transgenic cotton plants showed flat, twisted ribbon-like structure and natural folds running parallel along the length of the cotton fiber. There was no visible difference in fiber morphology (fineness, smoothness, number of twists and presence of ribbon-like structures) between transgenic and non-transgenic control cotton plants which depicts no positive or negative correlation between expression of insecticidal genes (Vip3Aa + ASAL) and cotton fiber quality.

In the present study, 78% to 100% mortality of *H. armigera* confirmed significant resistance development in transgenic cotton plants expressing Vip3Aa toxin. Besides the minor damage to the leaves of transgenic cotton line L_5P_3, all other lines remained undamaged. All the larvae that survived after the third day of bioassay were extremely weak and a significant delay in the larval growth was observed as compared to the larvae fed on non-transgenic cotton plants (de Oliveira et al. 2016). Wu et al. 2011 also reported that Vip3A expressing cotton plants showed 72.5% mortality against cotton bollworm

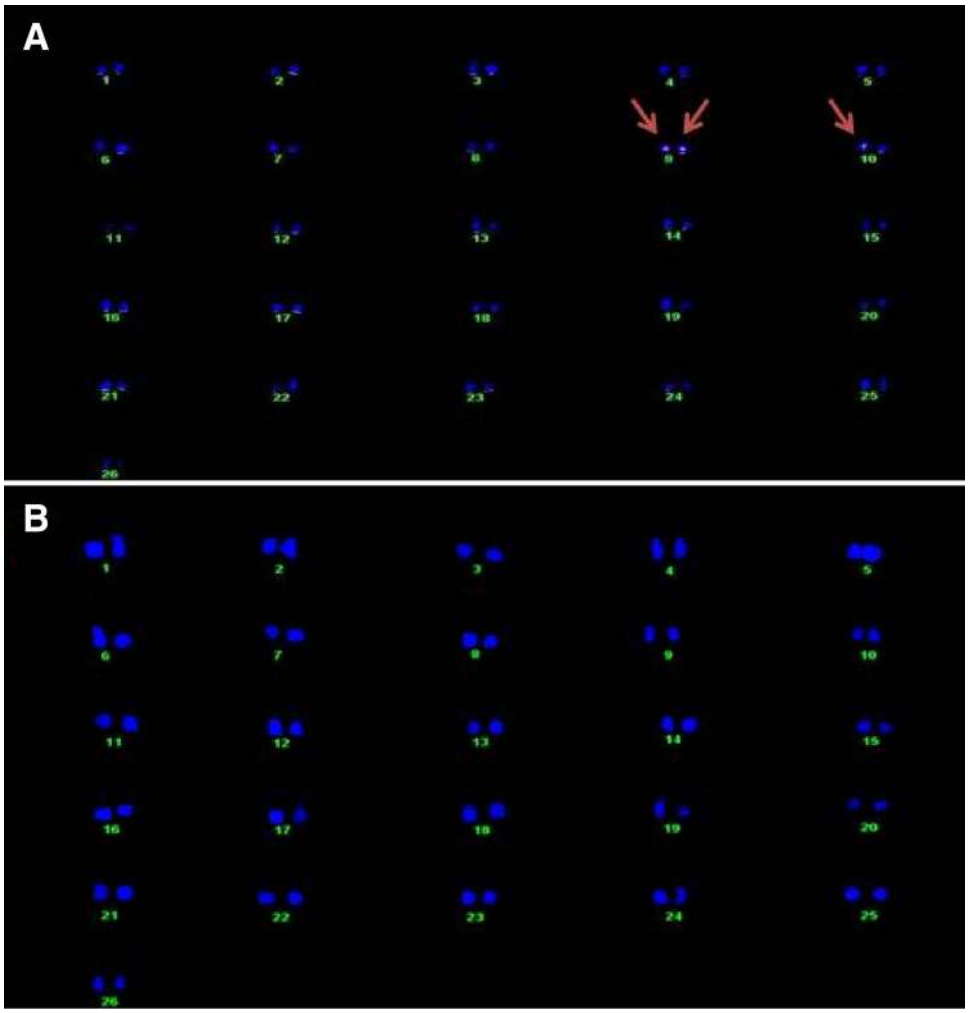

Fig. 12 Determination of copy number and transgene location through FISH. **a** Karyogram of transgenic cotton plant showing one copy number at chromosome number 9 in homozygous form and one copy number at chromosome number 10 determines the gene integration and location on genome. **b** Karyogram of non-transgenic control cotton plant showing no fluorescent signal

and 100% mortality against fall armyworm and beet armyworm. The transgenic cotton lines showed significant (72%–95%) mortality of whitefly in three days clip bioassay in controlled environment grown transgenic cotton plants as compared to non-transgenic controls where the mortality was calculated to be 10%. Moreover, notable physical changes like the abnormal stretching of the body were evident in dead larvae likely due to the effect of lectin on the digestive tract of targeted insect and proved the efficacy of transgenes in host plants (Javaid et al. 2016).

Conclusion

The current research work was proposed to utilize and evaluate the insecticidal spectrum of *Bt* Vip3Aa and garlic lectin ASAL genes in the cotton plant to enrich the insecticidal potential of the transgenic cotton plant against major chewing and sucking insects. Insect bioassays using viruliferous whiteflies and 2nd instar larvae of cotton bollworms confirmed the efficacy of transgenes. Significant mortality of sucking and chewing insects on transgenic cotton plants tells the success story of this study. The study is a part of a future strategy to overcome resistance build-up in insects due to extensive use of *Bt* toxins against specific receptors. Using next-generation *Bt* toxins having different target receptors will be helpful in delaying resistance and making longer and stable insect control which will impact on national economy and farmers.

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Author contribution statement AQR conceived and designed the research. SD performed cotton transformation, SA, AG and MA did molecular analysis. MS and AL have done insect bioassays. AAS

supervised insect assay experiments while field analyses were done by MAA. Manuscript was prepared by SD and TH. All the authors read and approved the manuscript.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information file.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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