Title: Transgenic maize inbred lines expressing high levels of *Bacillus thuringiensis* vegetative insecticidal protein (Vip3Aa86) offer effective control of maize stem borer (*Chilo partellus*).

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Abstract

The increasing incidence of field-evolved resistance in Lepidoptera (bollworms) insects towards Bt δ-endotoxins necessitates the need for an alternate strategy to prolong crop resistance. We have investigated the efficacy of *Bacillus thuringiensis* (Bt) derived toxin, the Vegetative Insecticidal protein Vip3Aa86 to manage maize stem borer in transgenic maize lines. Vip3A proteins do not share any midgut receptors or mechanism of action with any Cry insecticidal proteins and therefore are expected to possess toxicity even in the Bt resistant insects. The transgenic maize inbred lines generated through Agrobacterium mediated transformation expressing a codon optimized, synthetic *vip3Aa86* gene under the influence of the Poly ubiquitin promoter. The T_0 progenitor plants were screened initially through GFP reporter gene expression and transgene insertion by specific amplifications that identified four *vip3Aa86* transgenic maize lines. Highest *vip3Aa86* transcript abundance was observed in the V1 transgenic line while lowest was observed in the VA8 transgenic maize line when subjected to relative mRNA expression analysis. The concentration of Vip3Aa86 protein in T_1 transgenic maize lines ranged from 0.94 to 2.24 μg g^{-1} leaf fresh weight. The percentage mortality of *Chilo partellus* was 76.6%, 56.7%, 40% and 53.3% respectively when fed on V1, V10, V12 and VA8 transgenic maize lines of T_1 plants, for a period of 72 h in comparison to a control, non-transgenic maize sample. The study concluded that *vip3Aa86* insecticidal gene holds great potential against maize stem borer and can be used in gene-pyramiding with Bt crops to prolong the crop resistance.

Key words: Maize stem borer, Insecticidal Cry toxin, insect resistant maize.

Introduction

Maize is a leading cereal with global production of more than 1 billion tonnes and covers an area of approximately 200 million hectares (Erenstein et al. 2022). United States of America and China produces approximately 54.5% of the world's maize production followed by Brazil, Argentina, Ukraine, Indonesia, India and, Mexico which contribute 77.4% in total production (Erenstein et al. 2022). However, according to Pakistan Bureau of Statistics, maize is grown on approx. 1.418 million hectares throughout the country and its production stood at 8.465 million tonnes in the year 2020–21 (Pakistan Economic Survey 2020–21).

During 2001–2003, nearly 18% of the maize crop was lost globally (Oerke 2006) where insects and molds were the major contributors (Dowd and Johnson 2016). The severity of the damage caused by insects depends on type of seed, agronomic and environmental conditions, and storage practices (Arabjafari and Jalai 2007; Nabeel et al. 2018; Adeyinka et al. 2018). *Chilo partellus* (maize stem borer) is a major insect that can decrease about 10–30% of maize yield. The overall loss may increase under conditions favourable to the insect and early sowing. It is estimated that average production can be increased by at least 14% if such predation by insects is eliminated (Falak 2003).

A soil bacterium, *Bacillus thuringiensis*, forms parasporal crystal proteins belonging to several structural groups

(Crickmore et al. 2021) during sporulation that contained δ-endotoxins (Maria Cristina Gonzalez-Vazquez et al. 2021). Proteins belonging to the Cry family are the main agents used to produce plant resistance against corn borer and are effectively used in numerous other crops (Viktorov 2015). However, over time, some insect pests have evolved resistance against Bt toxins (Tabashnik et al. 2013). Even though Cry proteins have been extensively efficacious in insect control, some important pests were found to be highly tolerant to Cry proteins, such as lepidopterans (Chakroun et al. 2016). The development of resistance could be due to selection pressure applied on the crop pests, both temporally and spatially, by single Bt toxin genes (Sheikh et al. 2017). Until the present, 17 insect species have been reported to have evolved resistance to Bt crops (Sheikh et al. 2017), necessitating the search for an alternative toxin for prolonged resistance to insects.

Amongst such insecticidal proteins, are a group of proteins produced during the vegetative growth stage of Bt and which have no sequence homology with Cry proteins (Xu et al. 2018). The most important of these vegetative proteins are the Vip3 family, which are secreted proteins of around 88.5 kDa. One of its members, Vip3Aa, was found to be highly toxic towards lepidopterans and other chewing insects (Raybould and Vlachos 2011). Once the midgut proteases activate pro-toxins, these toxins pass through peritrophic membrane and reach apical membrane; where they bind to specific proteins of epithelial cells and finally, cause pore formation (Chakroun et al. 2016). Histopathological analysis of the insects that were infected with Vip3A, have revealed widespread destruction of the insect midgut (Chen et al. 2018). Moreover, the damage includes the lumen with leaked cellular material, and enflamed, degenerated epithelial cells (Abdelkefi-Mesrati et al. 2011; Ben Hamadou-Charfi et al. 2013; Boukedi et al. 2015; Sellami et al. 2015; Yu et al. 1997; Zhang et al. 2012). The entomotoxic effect of Vip3A protein against Chilo spp has been reported previously in few crop species (Yu et al. 1997; Wang et al. 2018; Riaz et al., 2020). Riaz et al. (2020) has reported significantly high mortality of sugarcane stem borer (*Chilo infuscatellus*) in transgenic sugarcane lines. Moreover, the synergistic effects of Vip and crystalline proteins has been reported to enhance mortality in Chilo spp, for instance, Vip3Aa29/Cyt2Aa3 exhibited synergistic behavior against *Chilo suppressalis*, *Spodoptera exigua* (Yu et al. 1997); while Vip3Aa/Cry9a showed synergistic behavior against *Chilo suppressalis* (Wang et al. 2018; Burkness et al. 2010) have evaluated the efficacy of *B. thuringenesis* derived Vip3A against *Helicoverpa armigera* on a transgenic maize event and documented that the maize hybrids expressing Vip3A provide effective control against *H. armigera* when compared them with non-Bt hybrids. A similar finding was reported from Canada documenting successful management of *Striacosta albicosta* in Vip3A transgenic maize lines (Farhan et al., 2018).

The current study focuses on the induction of resistance in local maize varieties against chewing insect pests, particularly *Chilo partellus* (maize stem borer) through Agrobacterium mediated transformation with *vip3Aa86* gene. This is the first study evaluating the insecticidal activity of *vip3Aa86* against *C. partellus* in maize.

Materials and Methods

Recombinant Vip3A protein expression in Prokaryotic host

The *vip3Aa86* gene sequence was retrieved from GenBank (Accession No. HM536938.2) and codon-optimized (~ 2370 bp) as per maize usage frequency. For expression of Vip3Aa86 recombinant protein in a prokaryotic expression host, the gene was cloned in pET30(a) vector by using the *KpnI* restriction enzyme. The resulting construct, pETUVip3A was introduced into *E. coli* strain NEB SHuffle T7 cells and induced with 1mM IPTG for recombinant Vip3A protein expression. The recombinant protein was purified by using Protino Ni-TED2000 packed column and was blotted onto nitrocellulose membrane. The recombinant vip3Aa86 protein was detected with anti-His-HRP conjugated antibody (BioRad). Westar SUN (cynagen) was used as substrate and the membrane was developed by using C-DiGit Blot Scanner (Li-Cor Biosciences) and visualized using Image studio.

Construction of plant expression vector

For construction of plant expression vector for efficient expression in maize plants, the TMVΩ translational enhancer sequence (GTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACA) was retrieved from the NCBI database. The *vip3Aa86* gene cassette comprised of the poly ubiquitin promoter, TMVΩ enhancer, *vip3Aa86* gene (2370 bp), GFP exon, and NOS terminator [SacI-PolyUbi-enhancer-KpnIVip3A- KpnI-GFP-BamHI-Nos-HindIII] (Fig. 1). The *vip3Aa86* gene cassette was synthesized from Bio Basic Inc. and was cloned in pCAMBIA1301 vector with *SacI* and *HindIII* restriction sites.

Ligated products were propagated in *E. coli*. Top 10 cells and positive clones were confirmed through restriction digestion. The pCAMBIA-UVip3A was moved into *A. tumefaciens* strain LBA4404 through the freeze-thaw method (Weigel and Glazebrook, 2006). Potential transformants were screened through amplification of a ~ 872 bp gene fragment with specific primers. The PCR reaction mixture contained 100 ng of plasmid DNA, 1X Taq Buffer with Ammonium Sulphate, 10 pM forward (ACA CCT TCA GCA ACC CAA AC) and reverse primer (GTG GAT GTA GCC GGT GTT CT), 1.25 mM $MgCl_2$, 1mM dNTPs, 03 units of DNA Taq DNA polymerase (Thermo scientific) and nuclease free water. The PCR was performed in Veriti® Thermal cycler (Applied Biosystems) by using the profile; initial denaturation at 95 °C for 3 min, 35 cycles with denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 40 s followed by the final extension of 10 min at 72 °C.

Agrobacterium mediated transformation and regeneration of transgenic maize plants

Immature Seeds from maize inbred line (CEMB02) were kindly provided by Dr. Abdul Munim Farooq, CEMB Lahore. The seeds were separated from the cob and were sterilized before isolation of the embryos. The intact embryos were injured via minor incision on their coleoptile and co-cultivated with over-night grown cells of *Agrobacterium tumefaciens* (strain LB-4404) harbouring pCAMBIA-UVip3A construct, resuspended in 5 ml MS medium (Murashige and Skoog, 1962) supplemented with 100 mM acetosyringone (Thermo scientific). Post-co cultivation, the embryos were allowed to germinate in the dark at 25 °C for5–6 days. Later, the embryos were placed onto N6 medium (Phyto Technologies) and placed at 18 ± 2 °C under 16/8 h photoperiod.

Screening of transformed maize plants

GFP fluorescence detection

To detect the presence and/or absence of transgenes in early developmental stages of the transformed maize, the first leaf of germinating seedlings other than cotyledonary leaves were subjected to initial screening through GFP fluorescence. The cross sections of each leaf were mounted onto glass slides and images were taken using a Leica Confocal Microscope (Leica) equipped with a GFP filter attached to a digital camera. The seedlings with positive GFP fluorescence were further were hardened in soil pots and grown to maturity in a glasshouse under contained conditions. Untransformed maize embryos, used as control, were also grown in parallel.

Transgene insertion by PCR assay

Total genomic DNA was isolated from leaves of GFP positive transformed maize plants (T_0 & T_1 plants) by the CTAB method (Porebski et al. 1997). The transgene insertion was detected by *vip3Aa86* specific PCR amplification. The PCR reaction mix, primers and cycling profile was same as described in the previous section. The T_0 positive maize plantlets (T_0) were brought to maturity, selfed, harvested and seeds were sown in the next growing season for transgenic maize lines as T_1 plants that were tested for transgene presence through PCR.

Southern Blotting

To reveal the genomic integration of the *vip3Aa86* cassette in the genome of T_1 maize plants, Southern blot analysis was performed. Briefly, 20 µg of genomic DNA was digested with *SacI* and *HindIII* and the restricted products were resolved on 0.8% agarose gel. The blotting was performed using the Hybond N membrane (Amershan Bioscences) and the DNA was hybridized with Biotin labeled probe [*Vip3Aa86* cassette: SacI-PolyUbi-enhancer-Vip3AGFP-Nos-HindIII] that was synthesized by using the Biotin DecaLabel DNA Labelling Kit (Thermo Scientific). The hybridization was carried out at 42 °C in a hybridization chamber. The hybridization signals were detected by using the Biotin Chromogenic Detection Kit (Thermo Scientific) in a chromogenic reaction of substrate (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)) hydrolysis.

Relative Quantification of *vip3Aa86* transcript in transgenic maize lines

To measure the relative abundance of *vip3Aa86* transcript in transgenic maize lines of T_1 plants, a real time PCR assay was performed. For the T_1, ten plants per transgenic line were grown in soil and tested through PCR for transgene presence. Only three positive maize plants per transgenic line were selected for sampling as biological replicates. Three biological replicates (three *vip3Aa86* positive plants) were taken from each transgenic maize line. Total RNA was isolated from transgenic maize plants using TriZol reagent (Invitrogen) and complementary (c)DNAs were prepared with the First strand cDNA synthesis kit (Thermo Scientific). For ~ 151 bp Vip3A transcript amplification, specific primers were designed using online available Primer3 (version 4) software (https:// bioin fo. ut. ee/ prime r3-0. 4.0/). 10 µM of forward primer 5′ -AACAACACCAAGCTGTCCAC-3′

and Reverse 5′ -AGCTGCTGGTTCTTCAGGAT-3′ primers were used in a reaction mixture composed of 5 µl of Maxima SYBR Green/ROX qPCR master mix (2X) (Thermo Fisher), 1 µg cDNA and nuclease free water to a final volume of 10 µl. The reaction was performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems) with the following cycling profile: initial denaturation (95 °C for 5 min) was followed by 40 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 30 s). For data normalization, beta actin was used as a reference gene, and was simultaneously amplified from the sample.
The relative gene expression among transgenic maize lines was evaluated by using the comparative CT method, also known as the 2^(-DDCt) method (Livak and Schmittgen 2001). The data were subjected to statistical analysis though One Way ANOVA, using the Microsoft Excel and Graph-Pad-Prism Software, Dunnet's test was applied as a post-test.

Enzyme-linked Immunosorbent assay (ELISA)

To measure the concentration of Vip3Aa86 protein in transgenic maize lines (T_1), ELISA was performed using Vip3A ELISA kit (Agdia). A standard curve was generated with a pure standard of Vip3A present in the kit. Fresh, 0.1 g of young leaf samples were taken from selected maize plants and were homogenized in 1ml of 1X PBST. The ELISA was performed as per the kit manual. The absorbance values of Vip3Aa86 in test samples were then plotted on the standard curve for protein quantification.

Insect-Plant Bioassays

In-vitro detached leaf bioassays of maize stem borer (*Chilo partellus*) were conducted on leaf sample of T_1 progeny plants of maize. First instars of *C. partellus* were obtained from the Insectary facility, CEMB Lahore and fed on soft leaf sections obtained from transgenic maize lines. As control, samples were collected from non-transgenic maize plants and stem borer 1st instar larvae were allowed to feed. Three biological replicates of each transgenic maize line (three leaf sections from 3 independent plants per transgenic line) were tested for insect resistance through insect feeding assays while for each replicate, ten first instars of stem borer (30 insect larvae per transgenic line) were fed on leaf segments. Data of live and dead insects were recorded every 24 h for 72 h. All the data were subjected to independent sample t-test for statistical analysis.

RESULTS

Recombinant Vip3Aa86 protein expression in Prokaryotic host

The ~ 2375 bp *vip3Aa86* gene was successfully cloned in the pET30(a) vector for expression in *E. coli* and recombinant Vip3Aa86 protein was purified via the His-tag on a metal affinity column and eluted samples were analysed by Coomassie stained SDS-PAGE prior to the Western Blot. The SDS-PAGE revealed the presence of a ~ 88 kDa protein corresponding to Vip3Aa86 (Fig. 2a), as confirmed by western blot (Fig. 2b).

Generation of transgenic maize lines

Approximately 800 immature seeds were collected from maize inbred lines and transformed with pCAMBIAUVip3A via *Agrobacterium* mediated transformation. Only 224 transformed embryos regenerated into small plantlets and post hardening in glass house, only 65 survived to mature plants. Example transformants are illustrated in Fig. 3. Preliminary screening of the 65 transformants was performed through GFP reporter gene expression in maize seedlings. It was found that five seedlings (V1, V4, V10, V12 and VA8) transformed with pCAMBIA-UVip3A showed fluorescence, when visualized under the fluorescent microscope. However, no fluorescence was observed in non-transgenic maize seedlings (Fig. 4a). Pre-screened GFP positive maize 05 plantlets were further confirmed through PCR analysis using gene specific primers. An amplicon of ~ 1 kb was observed in five T_0 maize plants; V1, V4, V10, V12 and VA8 while no amplification was observed in non-transformed maize plant (Fig. 4b). The seeds from positive maize plants were used for T_0 progeny (T_1) in the following season (Fig. 3e and f). The T_1 plants of maize were verified for transgene presence through *vip3A* specific amplifications by PCR. The segregating population of T_0 progeny (T_1 plants) revealed that more than 3 plants in each of the four transgenic maize lines (V1, VA8, V10 & V12) tested positive for *vip3Aa86* amplifications (segregation data not shown) while no plant was detected positive for V4 maize plants in T_1 during initial screening. The Southern blotting assay confirmed *vip3Aa86* transgene integration in T_1 maize plants. Genomic DNA isolated from transgenic lines of maize namely; V1, V10, V12 and VA8, when digested with *KpnI* and probed with the *vip3Aa86* gene revealed strong and detectable hybridization signals on nitrocellulose membrane (Fig. 4c). Conversely, no band was observed in non-transformed maize plants.

Transgenic maize lines with high Vip3Aa86 expression showed increased mortality of maize stem borer

The transcript level of *vip3Aa86* was found to be variable among transgenic maize lines of T_1 when revealed through real-time PCR assay by the Livak method. Three maize plants that were found positive for transgene in segregating population at T_1 per transgenic line were picked to investigate mRNA expression as three biological replicates. The relative mRNA expression of *vip3Aa86* was based on the average expression values obtained from three biological replicates as shown in Fig. 5. The relative mRNA expression of *vip3Aa86* was found to be highest in the V1 maize line while lowest expression was recorded in the VA8 transgenic maize line (Fig. 5).

Enzyme linked immunosorbent assay (ELISA) was employed for quantification of Vip3A protein (derived from Vip3A standard curve; supplementary Fig. 1) in *vip3Aa86* transgenic maize lines (biological replicates included three *vip3Aa86* positive plants per transgenic line). It was revealed that transgenic maize line V1 had highest protein content of 2.241 ± 0.120 µg g^{-1} of fresh leaf weight. While lowest protein content of 0.94 ± 0.207 µg g^{-1} was found in V12 transgenic maize line, 1.34 ± 0.091 µg g^{-1} and 1.20 ± 0.173 µg g^{-1} of Vip3A protein content was observed in the transgenic lines V10 and VA8, respectively. However, no significant Vip3A protein content was detected in non-transgenic maize (Fig. 6).

The *in-vitro* detached leaf bioassay of *Chilo partellus* fed on the *vip3Aa86* transgenic maize lines (V1, VA8, V10 and V12) of T_1 showed varying mortality percentages. Three *vip3Aa86* positive plants were selected as three biological replicates per transgenic line. After 72 h, transgenic line V1 showed the highest mortality of maize stem borer at 76.6 ± 0.404%, whereas V12 showed lowest (40 ± 1.155%) insect mortality and V10 and VA8 transgenic maize lines produced 56.7 ± 0.981% and 53.3 ± 0.981% mortality of maize stem borer, respectively. However, only 6.6 ± 0.173% mortality was observed in insects fed on the non-transgenic maize line (Fig. 7f). Furthermore, the percentage mortality was found to be statistically significant at $p < 0.05$. Figure 7 shows a graph of percentage mortality of *C. partellus* in different transgenic lines of maize, after 72 h of exposure. Conclusively, the highest insect mortality observed in transgenic maize line V1 in comparison with non-transgenic control, indicates that the insect mortality was directly correlated with *vip3Aa86* gene expression and high concentration of Vip3Aa86 recombinant protein in the transgenic line.

DISCUSSION

The rapid change in environmental systems and ever-evolving pests, the Bt crops have been reported to lose resistance to their target pests (Giron-Calva et al. 2020; Taylor, 2017) and the problem appears to be particular in Asia (Gianessi 2013). Insecticidal proteins such as ribosome-inactivating proteins (RIPs) composed of toxic N-glycosidases (Zhu et al. 2018), recently discovered homologous family of proteins from *Adiantum spp.* (Liu et al. 2019) and vegetative insecticidal proteins such as Vip3A have shown high insecticidal activity towards lepidopterans and other pests (Jiang et al. 2020; Raybould and Vlachos 2011). Vip3A has been found to be highly effective against the Asian rice borer *Chilo suppressalis* (Xu et al. 2018). Escudero et al. screened five Vip3A proteins from *B. thuringenesis* and all were found to be effective against eight different lepidopterans (Escudero et al. 2014). Efficacy of Vip3A proteins was also tested against *Helicoverpa zea*, in transgenic maize by Burkness et al. (2010).

One of the major pests of maize crops in Pakistan are maize stem borers. The attack becomes severe if the crop is sown earlier. Moreover, the climate change positively increasing the infestation of insects (Arabjafari and Jalai 2007). It is estimated that if the threat from borer insects is reduced, the overall maize production may be increased by at least 14% (Falak 2003).

We used codon optimized synthetic *vip3Aa86* transgene in this study to enhance insect resistance in local maize inbred lines. The Vip3Aa86 protein was found to be effective against *Chilo partellus* by causing 76.6% insect mortality (Fig. 5). To our knowledge, this is the first study documenting development of *vip3Aa86* transgenic maize lines with protection against *C. partellus*. A comparable study was reported by Burkness et al. (2010) where they evaluated the efficacy of a Vip3A transgenic maize line (MIR162 event by Syngenta) against *Helicoverpa zea*, *Ostrinia nubilalis* (Hubner) and *Spodoptera frugiperda* in field trial study. They reported high mortality of insect pests.

For enhanced *vip3Aa86* expression, we used TMVΩ enhancer that was introduced upstream of the transgene. This enhancer was used because of its ability to enhance translation of plant proteins (Gallie 2002). This 5' leader sequence has a poly CAA region, which acts as a binding site for heat shock protein (HSP 101).We express the

vip3Aa86 transgene under the influence of maize polyubiquitin-1 promotor. This promoter is widely used for driving high levels of gene expression in monocots including maize (Streatfield et al. 2004; Bhatti et al. 2020). We used Agrobacterium mediated transformation in maize and found the transformation efficiency to be 1.78%. In a similar study, Du et al. (2019) used immature embryos of maize for transformation and were able to achieve the transformation efficiency of approximately 5.3%. Additionally, Frame et al. (2006) also used MS salts for transformation in inbred lines and were able to achieve a transformation frequency of 6.8%. The possible reasons for fluctuation in transformation efficiency may depend on factors including co-cultivation time, media formulations, and concentration of acetosyringone, genotype difference and temperature conditions.

In this study, we found five transformed maize lines positive for the *vip3Aa86* transgene at T_0 while only four T_0 progenitors produce T_1 with transgene. It is well understood that the heterozygous transgenic plants definitely produce non-transgenic offsprings (Raldugina et al. 2021) which supports our data of V4 transgenic line that was positive at T_0 while did not produce a T_1 plant with the transgene. The possible explanation for this under-representation may be the epigenetic gene silencing (Zhang et al. 2005), position effect, homozygosity and, ploidy level (Yin and Malepszy 2003). In the present study, we used the $2^{\Delta\Delta}$Ct method (Livak and Schmittgen 2001) to calculate relative gene expression in developed maize transgenic lines and found variable transcript levels among the generated maize lines (Fig. 7). A similar method has also been used previously for effective data analysis of SYBR Green q-RT PCR (Ingham et al. 2001; Li et al. 2004).

The mortality percentage of *C. partellus* documented in this study coincided with relative gene expression of *vip3Aa86* and amount of Vip3Aa86 protein in each transgenic line. We found 0.94–2.241 μg g^{-1} of Vip3Aa86 protein concentrations in developed transgenic maize lines (Fig. 7). Levels of production have been correlated with mortality in other studies, for example in Asian stem borers (He et al. 2014)) and in yellow stem borer (YSB) *Scirpophaga incertulas*, rice leaf folder (RLF) *Cnaphalocrocis medinalis* and oriental army worm (OAW) *Mythimna separata* (Chakraborty et al. 2016). In a related study, Chakraborty et al. (2016) have documented generation of cry2AX1 transgenic rice lines with protein expression 0.68–1.34 μg g^{-1} leaf fresh weight and found 80–92% levels of resistance against rice pests at the vegetative and reproductive stages. It has been reported that stability in expression level of proteins, also plays a vital role in creating resistance in transgenic plants (Wu et al. 2011).

In conclusion, Vip3A proteins can be potential alternatives to Cry proteins for conferring insect resistance in maize and may also be used in gene pyramiding. Integrated methods of controlling plant pathogens using insecticidal genes along with conventional strategies could provide a sustainable solution against insect resistance (Nong et al. 2015; Xu et al. 2018). Moreover, gene pyramiding involving recombination with existing Bt transgenics that could serve as breeding material in the production of insect resistant maize varieties (Vajhala et al. 2013). The developed maize transgenic lines are expected to perform better in the field and appear promising for commercial cultivation. Furthermore, gene stacking and transformation of fusion proteins in maize, could be a considerable research area for future studies to combat rapidly evolving pests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability statement

This manuscript has no associated data set.

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Author Contribution

Muhammad Umar Bhatti: Investigation. **Bushra Tabassum:** Supervision, Writing - review & editing, Data curation. **Colin Berry:** Advisor at School of Biosciences, Cardiff University during IRSIP fellowship. **Anwar Khan:** Writing - original draft. **Uzma Qaisar:** Validation. **Ejaz Ali:** Investigation, Bioassay. **Rida Khalid:**

Investigation, Bioassay. **Abdul Munim Farooq:** investigation. **Muhammad Tariq:** Validation. **Hasan Ayaz:** data curation

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Figure Legends

Figure 1: Graphical representation of *vip3Aa86* binary construct used to transform maize inbred lines. The *vip3Aa86* gene cassette [Ubiquitin promoter-Vip3A-GFP reporter gene-NOS poly A] was cloned in *SacI* and *HindIII* restriction enzymes.

Figure 2: Recombinant Vip3Aa86 protein expression and Western blot analysis. (a) SDS-PAGE showing purified Vip3Aa86 protein of ~88kDa: Lane M: Protein Ladder (BioRad), Lane 1-4: Protein elutions from purification column. (b) Western Blot representing Vip3A recombinant protein of ~88kDa: Lane M: Protein Ladder (BLueye Pre-stained), Lane 1: Blank Control, Lane 2: Vip3Aa86 protein purified fraction.

Figure 3: Schematic representation for generation of *vip3Aa86* transgenic maize inbred lines by *Agrobacterium* mediated transformation. (a) Co-cultivated immature maize embryos germinating on MS medium. (b) Transformed maize plantlets growing in test tubes. (c, d) Plants grown to maturity in greenhouse, (e) Selfing of T_0 plants to maintain inbred character in T_1, (f) Seed cobs obtained from T_0 plants for generation of T_1 maize progeny.

Figure 4: Screening of transformed maize plants in T_0. (a) Preliminary screening of transformed maize seedlings through GFP fluorescence detection by microscopic images. (b) Amplification of *vip3Aa86* gene fragment through PCR assay in putative transgenic maize plants to reveal ~872bp transgene insertion. +ve refers to DNA sample obtained from plasmid, pCAMBIA-Vip3A. While the control 'NT' refers to DNA sample obtained from non-transgenic maize plant. (c) Southern Blot analysis of PCR positive maize plants to reveal genetic integration of *vip3Aa86* cassette. The membrane bound genomic DNA of maize plants were probed with DIG labeled *vip3A86* fragment and detected through chromogenic reaction.

Figure 5: Relative mRNA expression of *vip3Aa86* transgene in transgenic maize lines of T_0 progeny (T_1). Graphical representation of mRNA expression level of *vip3Aa86* gene in transgenic lines of maize revealed through Livak method. The results were normalized with beta actin and compared with each other for relative expression levels. The data indicated that transgene's transcript level was variable among transgenic lines ($p<0.05$; $n=3$).

Figure 6: Relative quantification of Vip3Aa86 protein in transgenic maize lines. The concentration of Vip3Aa86 protein in each transgenic maize line was revealed through estimation from a standard curve (Supplementary figure 1).

Figure 7: Percentage mortality of maize stem borer post *in-vitro* insect feeding assay on transgenic maize lines. (a,b,c,d) Stem sections from V1, V10, V12, VA8 transgenic lines of maize, respectively, showing dead 1st instar larvae of *C. partellus* after 72 hours exposure. Black arrows point towards dead larvae. **(e)** Stem section from non-transgenic line of maize showing alive 1st instar larvae after 72 hours. Black arrows point towards living larvae. (**f**) Graphical representation of percentage mortality of maize stem borer fed on transgenic maize lines in comparison to control, non-transgenic maize.

Supplementary figure 1: Standard curve of Vip3Aa86 protein generated from standards present in the kit.

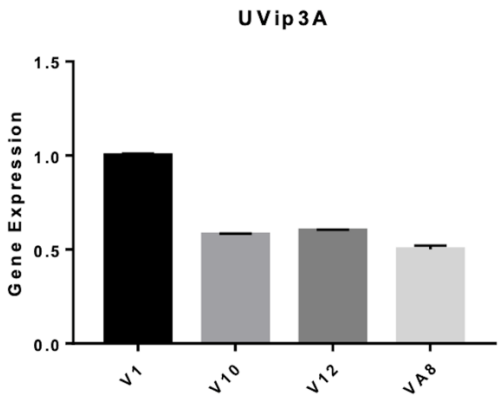


Figure 5

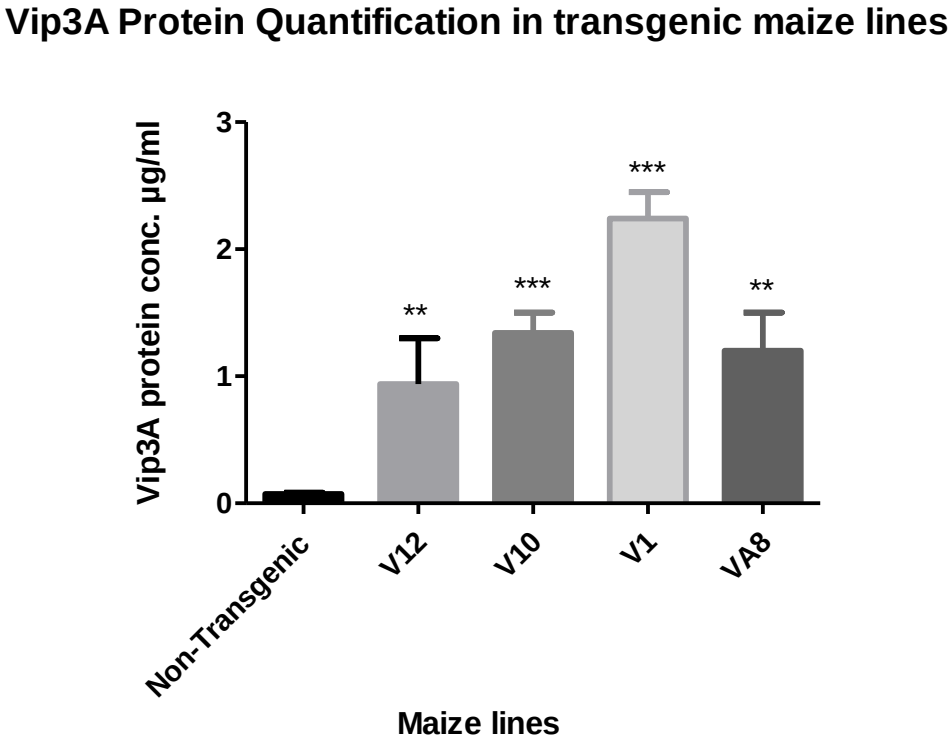
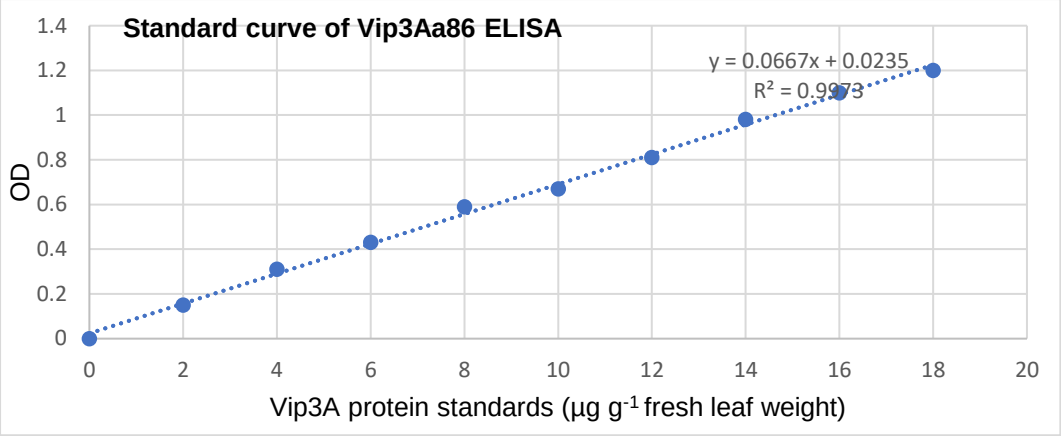


Figure 6



Supplementary figure 1: Standard curve of Vip3Aa86 protein generated from standards present in the kit.