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Full Length Article

Novel Approach using Digestive and Neurotoxins to Confer Resistance against *Helicoverpa armigera* and *Myzus persicae* in Tobacco

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Abstract

A new and improved strategy for the delaying of insect resistance by stacking two different genes with different mode of action was practiced. In the present study stacked gene construct having a spider toxin gene *i.e.*, ω-ACTX-Hv1a toxin (*Hvt*, a neurotoxin) and digestive toxin *Vip3A* derived from *Bacillus thuringiensis* was developed and transformed into tobacco (*Nicotiana tabacum* L. cv. Spade). The expressed transgenic tobacco expressing stacked genes showed the paralysis of *Helicoverpa armigera* Hübner larvae after 24 h and 100% mortality within 48 h. Loss of weight after 48 h was also observed for *H. armigera* larvae feeding on transgenic plants as compare to control plants where gain in weight was observed. The transgenic tobacco plants also showed reduced colony growth of aphid (*Myzus persicae* Sulzer) after 10 and 14 days as compared to control plants. Aphid showed less gain in weight on transgenic plants as compare to control plants. Commercial use of this construct in agronomical important crops will reduce the use of chemical pesticides as it is effective against both sucking and chewing insects. © 2019 Friends Science Publishers

Keywords: *Helicoverpa armigera; Hvt*; Insect Bioassay; Insect resistance; Aphid; *Vip3A*

Introduction

The world is facing a huge food crisis. Food requirements have increased with increasing population and reduced arable land (Shetty *et al*., 2018). There are numerous factors, which are reducing overall crop yields. Among them, insects are one of the major factors. Different approaches ranging from chemical pesticides to transgenic plants have been used to control insects. Most of the chemical insecticides currently in use are extremely toxic to non-target organisms and often cause severe adverse effects on human and animal health. Several studies showed that insecticides cause serious health problems such as cancer, birth defects and nerve damage (Rekha *et al.,* 2005). Moreover, these are a source of soil and water pollution. Due to their injudicious usage, many insects have developed resistance against these chemicals.

One approach to cope with this issue is to engineer plants to produce insect specific toxins through genetic transformation of genes encoding insect resistance from bacteria *B. thuringiensis* into a variety of useful plant cultivars (Shelton *et al*., 2002). Resistance to insect activity has been shown in crop plants by expressing toxins from *B. thuringiensis* (Mehlo *et al*., 2000), protease inhibitors, enzymes and plant lectins (Abdeen *et al.,* 2005). Many of the plant-based genes produce persistent effects rather than being toxic and most insect do not show any sensitivity to these factors. Mostly, these toxins are derived from *B. thuringiensis* (Schnepf *et al*., 1998). In order to increase the effective life of insect resistant crops, we need to deploy genes having different modes of action like different Bt genes or combination of Bt genes with insecticidal toxins from other organisms. Spider venoms are complex mixture of toxins but their use for biotechnological application has been very limited. A large number of toxin proteins were reviewed then cloned and expressed in crop plants for insect resistance. It was concluded that Bt toxins are very specific to particular insects but are less effective against other insects (Khan *et al*., 2006). Spider venoms have insecticidal mini proteins which can cause paralysis or death of insects (Windley *et al*., 2012). Spider toxins are very complex mixture of toxins which can be used for controlling insects. They demonstrated that the *ω-ACTX-Hv1a* toxin (*Hvt*) is a component of the venom of the Australian funnel web spider (*Hadronyche versuta* Rainbow, 1914). The peptide is toxic to a range of agriculturally important arthropods in the orders (Khan *et al*., 2006). Mechanism of action of these toxins is very wide and many organisms are susceptible to

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them, thus it makes them suitable for use against insect pests (Grishin, 1999). *Hvt* disturbs calcium ion channel and this activity is persistent even when it is expressed in plants. Mortality of *Spodoptera littoralis* Boisduval and *H. armigera* larvae within 2 days was observed in plants expressing *Hvt* gene. *Hvt* is an efficient toxin for controlling insects and should be studied and used in future insect control programs (Khan *et al*., 2006). Effect of *Hvt* toxin on four non-target insect species belonging to Coleoptera, Neuroptera and Hymenoptera demonstrated that this toxin had no adverse effect on these friendly insects. *Hvt* gene is toxic to lepidopteron insects when expressed in tobacco or in purified form (Ullah *et al*., 2015).

Vegetative insecticidal proteins (*Vip3A*) are unique proteins with insecticidal activity and transgenic plants having *Vip3A* genes have shown resistance to lepidopteran pests. *Vip3A* protein, produced during the vegetative growth stage of *B. thuringiensis*, is a new family of proteins that can be used for controlling insects (Lee *et al.,* 2003). *Vip3A* gene has a slightly different mode of action as compared to Cry proteins as its protein domain is different. Among those different genes we selected *Hvt* and *Vip3A* genes on the basis of their different modes of action and there is immense potential of these genes for controlling different insects.

Pyramiding three *Bt* genes is a useful strategy for improving the durability of Bt cotton in China (Liu *et al*., 2016a). It has also been found that the insect-resistance in plants transformed with four *Bt* genes was decreased due to homozygous suppression (Guo *et al.,* 2001). Thus, use of genes with a different mode of actions is useful. Gene stacking is an efficient method for widening the number of insect species controlled by transgenic plants, but should be used in conjunction with other Bt resistance management strategies (Manyangarirwa *et al.,* 2006). Fusion protein of AaIT/GNA is used for controlling chewing and sucking pests (Liu *et al*., 2016b). Fusion of two insecticidal proteins enhances the insecticidal activity (Javaid *et al*., 2018; Khan *et al.*, 2018). Evolution of insect resistance can be delayed to transgenic crops when it is combined with the refugee strategy (Tabashnik and Yves, 2017). Biosafety studies for synthetic *ω-ACTX-Hv1a* gene showed that the transgenic *Hvt* plants have no adverse effect on the environment (Naeem *et al*., 2015). Further they showed that there was no significant effect on plant morphology and physiology. The objective of the current study was to use genes from different origins to develop insect resistant crops with broaden spectrum and target both sucking and chewing insects.

Materials and Methods

Stacked Gene Construct

Experimental work was done at National Institute for Biotechnology and Genetic Engineering (NIBGE) in 2015. *Vip3A* gene was obtained from ICGEB (International Centre for Genetic Engineering and Biotechnology) New Delhi, India, *Vip3A* gene was cloned in pN6 vector under 35S promoter and OCS terminator (Fig. 1a). The *ω-ACTX-Hv1a* toxin (*Hvt*) gene sequence was optimized by codon optimization from published amino acid sequence for expression in plants (Mukhtar *et al.,* 2004) and was synthesized from Medigenomics, Germany. *Vip3A* gene caste was cloned in pSAKII clone (Khan *et al*., 2006) shown in (Fig. 1b) to make the stacked gene construct pJSVD (Fig. 1c). The vector pJSVD was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. In order to clone *Vip3A* gene under 35S promoter and OCS terminator in pSAKII (Khan *et al.,* 2006) the 2400 bp PCR product of *Vip3A* gene was cloned in T/A cloning vector. The PCR product from T/A cloning vector and vector pN6 (35S x2 the promoter and OCS terminator) were digested with *Cla*I and *BamH*I, and then ligated. The resultant vector was named as pJSVB (Fig. 1a). Vectors pJSVB and pSAKII (Fig. 1b) were digested with *Not*I and ligated to get stacked gene construct named as pJSVD (Fig. 1c).

Plant Genetic Transformation

Stable transformation of vector pJSVD via *A. tumefaciens* cv. 4404 in tobacco (*Nicotiana tabacum* L. cv. Spade) was carried out following the protocol of (Grimsley *et al*., 1987). Selection of the transformed cells was done on Kanamycin 50 mg/L, Tetracycline 10 mg/L and Streptomycin 50 mg/L. The transgenic insertion was confirmed by RT-PCR using nptII forward (5' ATTCGGCTATGACTGG 3') and reverse (5' AGGCGATGCTGCGAATC 3') primers. This *A. tumefaciens* culture was used to inoculate the leaf disks of tobacco (*Nicotiana tabacum* L. cv. Spade). Selection was done on MS salt media containing Kanamycin 50 mg /L and cefotaxime 500 mg/L. Shooting was induced on media containing 1-naphthaleneacetic acid (NAA) 0.1 mg/L and 6-benzylaminopurine (BAP) 1 mg/L. Shooting was induced after 37 days. Then these plants were shifted to new MS media to induce roots. After the development of roots plants were transferred to soil. RNA was isolated from five putative transgenic calli and one non-transformed control. The cDNA was synthesized from RNA of each callus and PCR was carried out for these cDNA using nptII forward (5' ATTCGGCTATGACTGG 3') and reverse (5' AGGCGATGCTGCGAATC 3') primer to confirm the transgene insertion.

Insect Bioassays

To check the efficiency of transformed genes against *H. armigera*, insect bioassays was performed. Insect bioassays were carried out by placing detached leaves from transgenic and control plants in petri plates. Underneath the leaves, blotting paper was placed to keep the leaves moist. All experiments were performed at room temperature. *H. armigera* 2nd instar larvae were placed on detached leaves from both transgenic and control tobacco plants. Along with

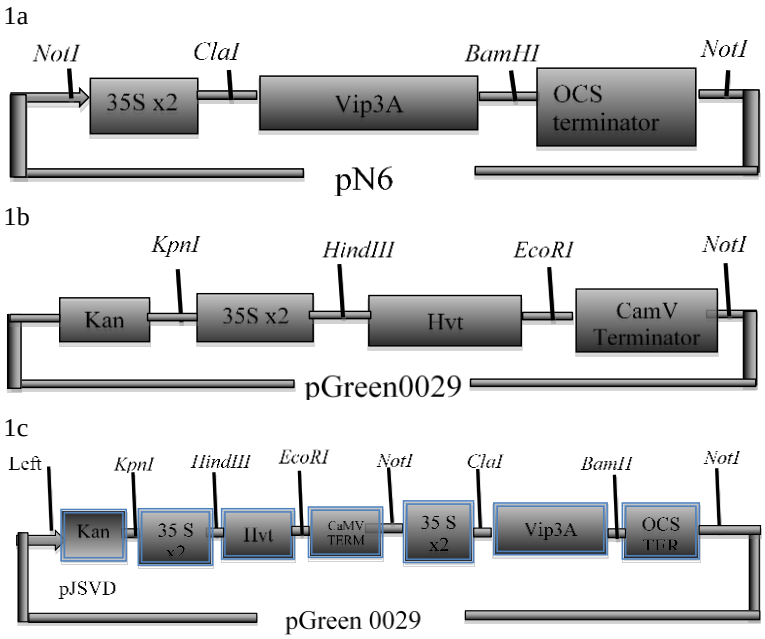

Fig. 1: a) Schematic diagram of clone pJSVB showing Vip3A gene clone under 35S promoter and OCS terminator in vector pN6. **b)** Schematic diagram of clone pSAKII (Khan et al., 2005). **c)** Schematic diagram of clone pJSVD showing stacked gene construct of *Hvt* and *Vip3A* clone in plant expressing vector pGreen 0029 having Kanamycin resistant gene for transgenic plant selection

insect bioassays larvae weight was also measured at 0 and 48 h. Aphid (*Myzus persicae* Sulzer) colony growth was recorded by placing 1 day old nymphs on 10 transgenic and 10 control plant leaves and these aphids were contained on leaf by placing clip cages. Aphid colony size was recorded after 10 and 14 days of inoculation. Aphid *M. persicae* weight on transgenic and control plants was recorded on 1st and 5th days of inoculation.

Real Time PCR

To measure the expression of pJSVD construct in transgenic tobacco plants, real time PCR analysis was performed. Real time PCR primers were designed using primer3 software. Real time PCR analysis was performed by using forward primer (5′TGGACGAGTACGGGATCAAC3′) reverse primer (5′TAGAATCCCTCGTCCTCCCTG3′). Standard curve was used to quantify the cDNA. Standard curve was obtained by series dilution of cDNA. Biorad CFX 96 software was used for the data analysis. The reaction was performed in Biorad CFX real time PCR detection system, by using program 95°C for 3 min followed by 40 cycles, in which each cycle consisted of 95°C for 35 sec, 55°C for 35 sec, 72°C for 35 sec. This was followed by melt curve analysis from 50°C. Each sample was repeated for 3 times.

Analysis of Data

Insect Bioassay was performed as complete randomized block (CRD) and data was arranged accordingly. The Analysis of variance was calculated using Statistix® 8.1 software. The means were compared by Student T test. The mean value of 4 larvae weight on each transgenic line, aphid colony size and weight of 10 aphids on transgenic plants and as well as control tobacco leaves was measured and presented in graphically.

Results

Transformation of pJSVD (Stacked Gene Construct) in Tobacco

The genetic transformation of *N. tabacum* was carried out with vector pJSVD (*Hvt+Vip3A* genes) under control of 35S promoter to express the toxins in all the tissues. Approximately 150 explants (leaf discs) of tobacco were transformed with *A. tumefaciens* having pJSVD vector. Out of 150 explants, 31 explants survived on selection media and regenerated plants with an average transformation efficiency of 20%. Rest of the explants bleached out indicating that these were not transformed. Kanamycin resistant plants were recovered and successfully established in soil. Different stages of tissue culture are shown in Fig. 2.

Molecular Analysis of Putative Transgenic Plants

Reverse transcriptase-PCR analysis: A large number of putative transgenic green calli were formed. Only 5 calli representing each experiment from individual transformation events were randomly selected and analyzed by RT-PCR. The results showed that all 5 putative transgenic calli were positive for the transgenes. No

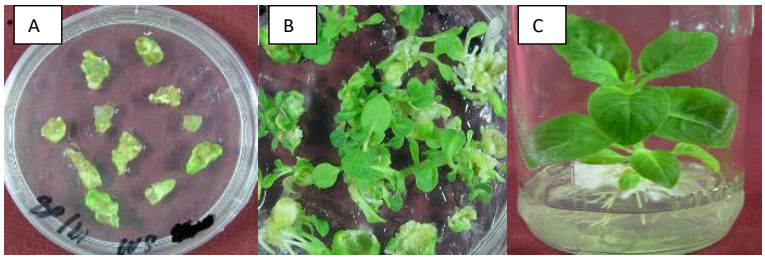

Fig. 2: Sequential stages involved in transformation of tobacco plants through *Agrobacterium* mediated transformation. From Left to Right **A)** Transformation of leaf discs with *Agrobacterium* mediated transformation and bleaching of non-transformed leaf discs; **B)** Regeneration from leaf disc on selection media; **C)** *In vitro* kanamycin resistant plant with roots

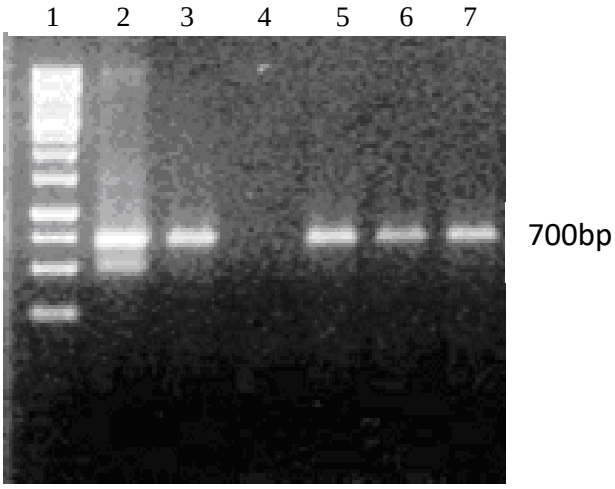

Fig. 3: RT-PCR confirmation of transgenic tobacco plants. (On 1% agarose gel) Lane1 1kb DNA ladder, lane 4 cDNA of non-transformed plant, Lane 2, 3,5,6,7 using cDNA from transformed tobacco plants

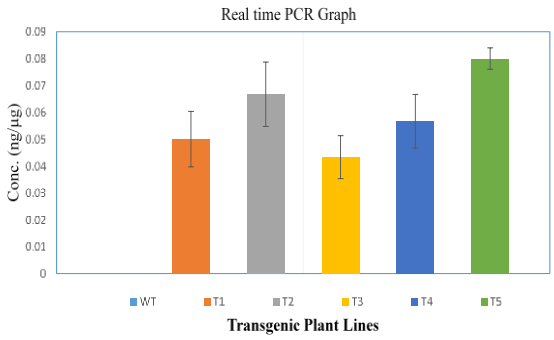

Fig. 4: Graphical representation of real time PCR analysis of transgenic and wild type control plants. Data represent means ± S.D. of at least 3 repeats for each line. Differences in mean values for each transgenic line were not statistically significant. Where T represents transgenic and WT represents wild type control

amplification from the non-transformed control authenticates results (Fig. 3).

Real-time PCR: Real time PCR analysis showed the expression of transgene in all transgenic tobacco plant lines. Transgenic line 5 showed maximum expression (0.08 ng/μg), followed by line 2 and 4. Line 3 showed comparatively less expression (.047 ng/μg) and wild type control showed no expression (Fig. 4).

Insect bioassay: Insect bioassay was performed by detaching leaves from transgenic and non-transgenic plants shown in Fig. 5. *H. armigera* larvae were placed on detached leaves from both transformed and non-transformed tobacco plants. The Larvae feeding on transgenic plants showed the effect of toxin within 24 h. These Larvae initially stopped feeding, reduced movement leading to paralysis and finally larvae died within two days. The larvae feeding on controlled leaves showed no such effects and their feeding was normal and finally eating whole of the leaf. Larval weight measured at 0 and 48 h showed a significant weight loss for larvae feeding on transgenic plants. On the other hand, there was gain in weight for larvae feeding on non-transgenic leaves (Fig. 6). Larvae feeding on control plants showed a significant increase in growth. One day old nymphs of aphid (*M. persicae*) were caged in clip cages on 10 transgenic and 10 controlled tobacco plants five Aphid colony growth was recorded after 10 and 14 days. Transgenic plants displayed reduced aphid growth compared to control plants (Fig. 7). Aphid weight was measured after 5 days of inoculation. This clearly showed that aphid weight feeding on transgenic plant was much reduced as compare to aphid feeding on control plants (Fig. 8).

Discussion

The present study resulted in the development of stacked gene construct of *Hvt* and *Vip3A* genes under CaMV35S promoter for expression in plants. Here, we have transcriptionally fused the *Vip3A* gene with *Hvt* gene, to have multiple genes from different sources. Results have demonstrated that combination of *Vip3A* and synthetic ω-ACTX resulted in 100% mortality in 2nd instar larvae of *H. armigera* within 48 h after exposure to the detached leaves from transgenic plants. It also showed reduced growth of aphid after 10 and 14 days. Measurement of aphid weight clearly showed that aphid feeding on transgenic tobacco plants showed less gain in weight as

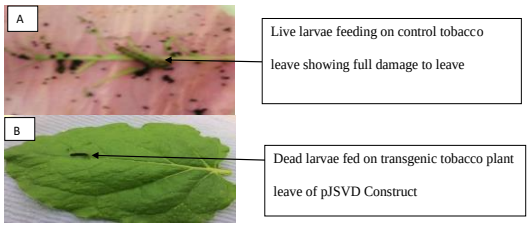

Fig. 5: Insect bio assay of tobacco plnats having stacked gene construct of *Hvt* and *Vip3A*: A) Control plants after after 48 h. Larvae caused full damage to leaf B) Tobbaco transgenic plants with no damage, larvae died with in 48 h

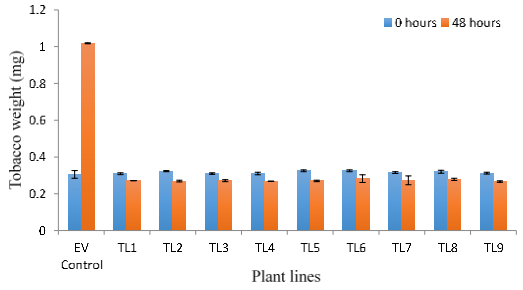

Fig. 6: Comparison larvae mean weight at 0 and 48 h. stage feeding on transgenic and non-transgenic tobacco leaves. Larvae feeding on non-transgenic leave tobacco shows gain in weight while weight-loss was detected in larvae feeding on transgenic tobacco leaves. EV stands for empty vector control and TL stands for transgenic line

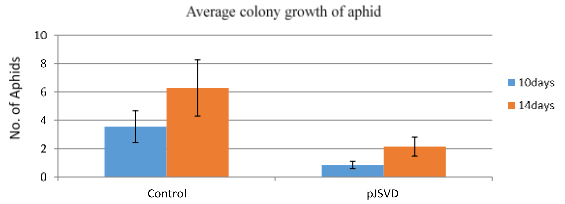

Fig. 7: Graph showing aphid *M. persicae* average colony growth on 10 control and 10 transgenic tobacco plants with standard deviation after 10 and 14 days

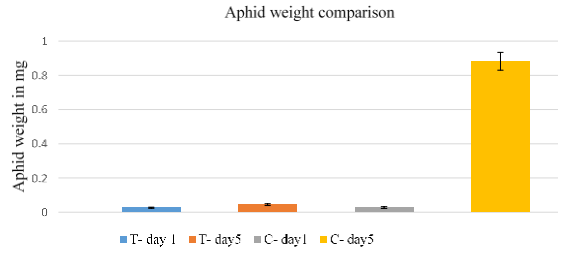

Fig. 8: Graphical comparison of *M. persicae* weight gain on 5th day, feeding on transgenic and non-transgenic tobacco leaves. Mean Aphid weight from 10 Aphid feeding on transgenic and non-transgenic tobacco leave is shown with standard deviation. Whereas T represents transgenic and C represents control

compare to control tobacco plants after 5 days of inoculation. Aphid health feeding on transgenic plants was very much compromised as compare to aphids feeding on control plants. This study clearly suggests that if we clone synthetic *ω-ACTX-Hv1a* under phloem specific promoter and *Vip3A* under constitutive promoter then it has great potential to control both sucking and chewing insects. Shah *et al*. (2011) showed that *ω-ACTX-Hv1a* gene cloned under phloem specific promoter can result in 93–100% mortality in *H. armigera* larvae. Transgenic plants expressing *ω-ACTX-Hv1a* (*Hvt*), either alone or in combination with onion leaf lectin under phloem specific promoter, are resistant to *Phenacoccus solenopsis* (cotton mealybug) and *Bemisia tabacaci* (Javaid *et al*., 2016). Further they showed that expression of both proteins under different phloem-specific promoters resulted in close to 100% mortality.

To cope with the issue of controlling both sucking and chewing insects, it is extremely important that we discover broader spectrum solution. And utilise those genes which have less commercial use and are highly effective. Due to the extensive exposure of pests to *Cry* proteins and low exposure to vegetative insecticidal proteins (Vips), no field evolved resistance has been reported in Vips (Chakroun *et al*., 2016). To date there is no proven evidence of development of resistance to *Hvt* as well.

Results revealed that the combination of two genes has immense potential for controlling major lepidopteran as well as sucking pests as *Vip3A* effecting the gut epithelial cells (digestive toxin) and *Hvt* is calcium channel antagonist (neurotoxin). Since the two genes have different modes of action, therefore, it is expected that development of resistance in the insect population will also be minimized or delayed. As aphids are one of the major carriers of plant viruses, controlling aphid will reduce the spread of viral diseases. These constructs can be used in agronomic crops like wheat and cotton to reduce the crop caused by insect damage.

Conclusion

Stacked gene construct of *Hvt* and *Vip3A* is effective for controlling larvae of *H. armigera* within 48 h and reducing the growth of aphid *M. persicae* due to the presence of two different toxins with different modes of actions, it is most likely that development of resistance in insects can be delayed. The stacked genes used in this study showed immense potential for future applications in the insect control programs and must be explored further for developing transgenic insect resistant crops of economic importance.

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