Hvt SPIDER TOXIN CONFERRED INSECTICIDAL ACTIVITY AGAINST APHIS GOSSYPII AND MYZUS PERSICAE SULZER

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INTRODUCTION

Sucking insects are the most destructive pests of economic crop plants as they directly feed on plant nutrients through extracting food assimilates from phloem cells, deprive of plants from essential nutrients, vie with plant vigor and serve as plant disease vectors (Chakrabarti et al., 2009; Dhaliwal et al., 2010., Bharathi et al., 2011). No doubt, transgenic plant technology based on δ-endotoxins of Bacillus thuringiensis (Bt), a gram-positive, spore forming soil bacterium has transfigured global crop production through development of insect resistant transgenic plants (Gatehouse et al., 2011; Kos et al., 2009). Although, Bt transgenic technology is effectively contributing to control herbivorous insect species but commercialized δ-endotoxins are not effective against plant sucking insects (Malone et al., 2008; Chougule and Bonning, 2012). Hence, there is a solemn need of evaluation of other non-Bt insecticidal proteins against economically important plant sucking insects (Gatehouse, 2008).

The seminal advancements in plant biotechnology have driven the development of new strategies for plant protection and search for novel entomo-toxic molecules with substantial insecticidal activity is of challenging interest to develop insect resistant crop plants (Gatehouse et al., 2011). Plant viral vectors offer a useful tool in molecular biology for high throughput transient expression of proteins in plants (Donini et al., 2005; Lacomme and Chapman, 2005; Maimbo et al., 2007). Two cosmopolitan aphid species Aphis gossypii and Myzus persicae are major pests of wide range of economic crops like cotton, maize, tobacco, potato and other vegetables (Ahmad

ABSTRACT

Plant sucking insects being brutal competitors are active during all developmental phases of economic crop plants and cause massive yield and quality losses of agricultural produce. Potato Virus X (PVX) based transient expression was used to achieve high throughput of Hvt peptide toxin from H. versuta spider in tobacco plants were bioassayed in aim to test the insecticidal activity of Hvt again aphids of species Aphis gossypii and Myzus persicae. The insecticidal and antagonistic effects of Hvt were evaluated in A. gossypii and M. persicae in terms of mortality, population survival and nymph production by feeding adult aphids. The characteristic symptoms of Hvt toxicity were characterized and results strongly revealed significant insecticidal activity of Hvt in tested insect species at p<0.01%. In a positive control bioassay, Helicoverpa armigera and Spodoptera exigua fed on transiently expressed Hvt/PVX plants showed significant insecticidal and entomo-toxic effects of Hvt in terms of larval mortality and lower dry weight of survived larvae. The results demonstrated in this study are important to evaluate the substantial insecticidal activity of Hvt for the purpose of its potential application in developing insect resistant transgenic plants technology.

Keywords: Hvt, PVX, transient expression, Agrobacterium infiltration, RT-PCR,
commercially synthesized 117bp nucleotide DNA from *Hydronyche versuta* (funnel web spider) was cloned in pgR107 vector using MCS restriction sites sequence of Hvt was cloned in pgR107 vector using MCS. A PVX based transient expression system was established for swift, inexpensive and high throughput system as compared to constitutive transgene expression. 

**MATERIALS AND METHODS**

**Gene sequence and primers designing**

Hvt, a 37 amino acid long synthetic peptide toxin was deduced from published nucleotide sequence (Fletcher et al., 1997). The codon optimization was performed for its proper expression in plants (Mukhtar et al., 2004). Primers were designed using (NCBI, BLAST, DNASTAR) bioinformatics tools for further analysis. 

**Cloning of PVX based expression construct**

A PVX based binary vector pgR107 (Gene Bank Acc#AY297842.1) was used to express the Hvt protein in *Nicotiana tabacum* L. plants. The 117nucleotide gene sequence of Hvt was cloned in pgR107 vector using MCS restriction sites *Cla*I and *Sal*I. For the amplification of Hvt by PCR, a reaction mixture of 25μl containing 1μg DNA template, 5μl 10X Taq polymerase buffer, 5μl 2mM dNTPs, 1.5mM MgCl2, 0.5μM of each primer and 1 unit of Taq DNA polymerase enzyme (Fermentas, Life Sciences) in thermal cycler. The PCR profile 94°C 5min, 94°C 3min, 48°C 30sec, 72°C 45sec, 72°C 10min followed 4°C 10min using gene specific primers 5’ATCGATATGTACACACCTGCATACCC and 3’GTCGACTTAATCGCATACTTATCGGAT from commercially synthesized 117bp nucleotide DNA from Medigenomix, Germany (Khan et al., 2006). DNA products were resolved on 1.7% agarose gel stained with ethidium bromide (0.5μg/ ml) in 0.5X TAE buffer (20mM Tris acetate and 0.5mM EDTA [pH 8.0]) co-electrophoresis a 100bp DNA ladder. The DNA/RNA were analyzed in UV transilluminator (Eagle Eye, Strategene). The DNA from agarose gel was isolated and purified by Wizard SV Gel and PCR Clean-Up System kit (Promega). The 500ng Hvt DNA from PCR amplification and circular cloning vector pgR107 were digested separately to expose sticky ends (10.0μl volume) using 1.5μl of 10X Tango Buffer (Final conc. 1X Tango), 5 units of *Cla*I (0.5μl), 0.5μl of RNAsae 2.5μl sterile ddH2O (final volume of reaction 15μl) and incubated for 3-4H. Following 2nd enzyme *Sal*I (0.5μl), 0.5μl of 10X Tango Buffer (Final conc. 2X Tango), sterile ddH2O of 4.0μl was added (final volume 20μl of reaction) and incubated at 37°C for 3-4H. The restricted vector and DNA products were purified from proteins by phenol-chloroform (24:1) Treatment. To ligate both PCR product and the vector, and 500ng of restricted DNA, 3μl restricted pgR107 vector, 6μl 5X ligation buffer and 5 units (0.5μl) of T4 DNA Ligase enzyme (Fermentas, Life Sciences) were added to obtain a final volume of 30μl and incubated at 16°C overnight. The Recombinant PVX vector was confirmed by respective restriction endonucleases *Cla*I and *Sal*I (Fermentas, Life Sciences) after miniprep plasmid isolation using (GeneJET™ Plasmid Miniprep Kit, Thermoscientific). The positive clones of Hvt/PVX were confirmed by sequencing from (Macrogen, Korea).

**Transformation in Escherichia coli cells**

The recombinant PVX vector was transformed into *E. coli* Top10 cells by heat shock method at 42° for 30sec and cultured in sterilized solid Luria Bertani (LB) agar medium (1% Tryptone, 0.5% yeast extract and 1% NaCl) supplemented with Kanamycin (25μg/ml) and incubated at 37°C 220-rpm overnight.

**Agrobacterium transformation and infiltration**

For transient expression of Hvt in tobacco, recombinant PVX was co-transformed with pSoup binary vector into *Agrobacterium tumefaciens* strain (GV3101) by electroporation at 1.44kV (2.5kv) using (ECM 600, BTX, U.S.A) apparatus. Cultures of *Agrobacterium* cells were grown at 28°C for 48H and 125-rpm with Kanamycin(25μg/ml), Rifamycin(25μg/ml) and Tetracycline (12.5μg/ml) antibiotics in LB liquid medium and inoculum was prepared by pelleting and suspending agrobacterium cells in 30ml of 10mM MgCl2, 30μl of 100μM acetosyringone added and incubated at 4°C overnight. The tobacco plants of 4-6 weeks old were agrobacterium-infiltrated with 1ml surgical syringe (B.D Syringe) following a method illustrated by (Leckie and Stewart 2011). 

**Reverse transcriptase (RT) PCR of tobacco plants**

In order to confirm the expression of Hvt in tobacco, total RNA was extracted from tobacco plants by TRIAGENT (Invitrogen). The 1μg quantity of RNA quantified on Spectrometer (Smart spec, BioRad) was used to synthesize first strand complementary DNA (cDNA) by the RevertAid® H Minus first strand cDNA synthesis kit (Fermentas, Life
replications for each treatment. The results revealed that
83% scored mortality in released on each symptomatic leaf in petriplates with five bioassay. The 5 numbers of neonate larvae of bollworm species therefor, PVX based expression of Hvt was Hvt is known for strong insecticidal activity in herbivorous armigera

RESULTS (S.D) and Standard Error (S.E) in Microsoft Office Excel were graphically plotted using Means, Standard Deviation significance as test and Student’s t-test were applied for estimation of Variance (ANOVA) using MSTAT-C software and Fisher’s F-analyzed by computing means and variances Analysis of petriplate wrapped with cling film. The statistical data was of treatment plants placed on moist filter paper, caged in bollworm species were introduced on each symptomatic leaf and 8-10 days old (plants. The 25 adult aphids of 12-14 days old (aphids were collected from host plants and bioassayed. The tobacco plants of 10-12 day post-infiltration (dpi) after arrival of mild PVX symptoms on systemic leave were used. Bioassays were designed in Complete Randomized Block Design (CRD) with ten replications. Hvt was tested on neonate larvae of bollworms as positive control. Data was scored for mortality and dry weights gain of surviving larvae. The surviving larvae were collected in 1.5ml eppendorf tubes, lyophilized using dry hot oven at 50°C for 48H and weighed on digital weigh balance (Sturtious®) of 0.001mg accuracy. The data were scored for mortality, number of nymphs produced by adult aphids that survived on Hvt/PVX plants were compared to PVX empty vector and healthy as control plants. The 25 adult aphids of 12-14 days old (M. persicae) and 8-10 days old (A. gossypii), while 5 neonate larvae of bollworm species were introduced on each symptomatic leaf of treatment plants placed on moist filter paper, caged in petriplate wrapped with cling film. The statistical data was analyzed by computing means and variances Analysis of Variance (ANOVA) using MSTAT-C software and Fisher’s F-test and Student’s t-test were applied for estimation of significance as p-value against p<0.01% and p<0.05% probability. The bioassay pictures were captured by using Sony Cybershot® 16mpx with 10X Zoom and data scored were graphically plotted using Means, Standard Deviation (S.D) and Standard Error (S.E) in Microsoft Office Excel 2010.

RESULTS

Insecticidal activity of Hvt in bollworms, S. exigua and H. armigera

Hvt is known for strong insecticidal activity in herbivorous bollworm species therefor, PVX based expression of Hvt was tested in S. exigua and H. armigera as positive control bioassay. The 5 numbers of neonate larvae of S. exigua were released on each symptomatic leaf in petriplates with five replications for each treatment. The results revealed 83% scored mortality in S. exigua larvae after feeding Hvt/PVX plants for 5 days feeding duration (Fig. 2). The results of positive control bioassay also revealed that Hvt exerted strong insecticidal effects in bollworm that caused significant lethality with obvious symptoms of Hvt toxicity in larvae of tested insect species. The results of positive control bioassay confirmed that PVX based transient insecticidal protein expression system is potentially working and ready to bioassay other insect species.

Insecticidal activity of Hvt in cotton aphid, A. gossypii

The 25 adult aphids were released on each treatment plants of Hvt/PVX, PVX and Healthy as control plants with 15 replications for 5 days feeding duration. The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 3). The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 3). The mean number of survivors of adult aphids of A. gossypii on (Hvt/PVX 4.3, PVX 21.3, healthy 21.4 ±1.513 S.E) and mean number of nymphs produced that survived on (Hvt/PVX 38.30, PVX 57.1, healthy 61.66 ±0.281 S.E) were found significant at p<0.01% (Table 1). The insecticidal effects of Hvt on adult aphid, A. gossypii in terms of adult aphid mortality and nymphs produced that survived on Hvt/PVX were compared to control plants and plotted for 5 days feeding duration as illustrated in (Fig. 4).

Insecticidal activity of Hvt in potato peach aphid, M. persicae

The 25 adult aphids were released on ACA Lectin/PVX plants, PVX and Healthy as control plants with 15 replications bioassayed for 5 days feeding duration. The Hvt exerted significant insecticidal effects on aphids and characteristic symptoms of Hvt appeared like melanization of cuticle, disturbed locomotion, disoriented coordination with continuous antennal and cerci movements, resting body posture with reduced fecundity of adult aphids fed on Hvt/PVX plants compared to control plants (Fig. 5). Hvt spider toxins exerted severe entomotoxic effect on adult aphid population and reproduction that significantly declined as less number of adult aphids were survived to reproduce normally. Secondly, less number of nymph were scored because newborn aphid babies could not survive soon after started feeding on Hvt/PVX plants. The mean number survivors of M. Persicae were found significant on (Hvt/PVX 2.400 PVX 21.9, healthy 21.4)±1.704 S.E and mean numbers of nymphs produced that survived on (Hvt/PVX 23.80, PVX 59.30, healthy 62.1 ±3.834 S.E) after 5 days feeding duration at p<0.01% (Table 1). The insecticidal effects of Hvt on adult aphids M. persicae in terms of population survival and nymphs produced on Hvt/PVX compared to control plants as illustrated in (Fig. 6).
Table 1

ANOVA of Hvt for adult aphid mortality and nymph production and survival.

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Mean</th>
<th>SOV</th>
<th>df</th>
<th>MS</th>
<th>p-value</th>
<th>S.D</th>
<th>S.E</th>
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<td><em>S. littoralis</em></td>
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<tr>
<td>(Larval mortality)</td>
<td>Hvt/PVX</td>
<td>1.0</td>
<td>Rep.</td>
<td>9</td>
<td>0.578NS</td>
<td>0.1131</td>
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</tr>
<tr>
<td>PVX</td>
<td>4.7</td>
<td>Treat.</td>
<td>2</td>
<td>43.30**</td>
<td>0.0000</td>
<td>1.831</td>
<td>0.334</td>
</tr>
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<td>Healthy</td>
<td>4.5</td>
<td>Error</td>
<td>18</td>
<td>0.300</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Dry Weight)</td>
<td>Hvt/PVX</td>
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<td>Rep.</td>
<td>14</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PVX</td>
<td>0.806</td>
<td>Treat.</td>
<td>2</td>
<td>1.836**</td>
<td>0.000</td>
<td>0.037</td>
<td>0.06</td>
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<td>Healthy</td>
<td>0.786</td>
<td>Error</td>
<td>28</td>
<td>0.015</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td><em>H. virescens</em></td>
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<tr>
<td>(Larval mortality)</td>
<td>PVX</td>
<td>4.7</td>
<td>Treat.</td>
<td>2</td>
<td>54.90**</td>
<td>0.0000</td>
<td>2.013</td>
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<td>Error</td>
<td>18</td>
<td>0.233</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(Dry weight)</td>
<td>Hvt/PVX</td>
<td>0.143</td>
<td>Rep.</td>
<td>14</td>
<td>0.0458S</td>
<td>0.2543</td>
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<td>PVX</td>
<td>0.806</td>
<td>Treat.</td>
<td>2</td>
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<td>0.0000</td>
<td>0.37</td>
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<td>Healthy</td>
<td>0.872</td>
<td>Error</td>
<td>28</td>
<td>0.032</td>
<td>-</td>
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<td><em>A. gossypii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(Adult)</td>
<td>Hvt/PVX</td>
<td>4.30</td>
<td>Rep.</td>
<td>9</td>
<td>1.407</td>
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<td>PVX</td>
<td>21.3</td>
<td>Treat.</td>
<td>2</td>
<td>969.033**</td>
<td>0.0000</td>
<td>8.285</td>
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<td>Healthy</td>
<td>21.0</td>
<td>Error</td>
<td>18</td>
<td>2.219</td>
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<td>23.8</td>
<td>Rep.</td>
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<td>59.3</td>
<td>Treat.</td>
<td>2</td>
<td>4558.30</td>
<td>0.0000</td>
<td>18.533</td>
<td>3.384</td>
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<tr>
<td>Healthy</td>
<td>62.1</td>
<td>Error</td>
<td>18</td>
<td>29.781</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>M. persicae</em></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Adult)</td>
<td>Hvt/PVX</td>
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<td>Rep.</td>
<td>9</td>
<td>2.300</td>
<td>0.3622</td>
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<tr>
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<td>21.9</td>
<td>Treat.</td>
<td>2</td>
<td>1235.83</td>
<td>0.0000</td>
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<td>1.704</td>
</tr>
<tr>
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<td>21.4</td>
<td>Error</td>
<td>18</td>
<td>1.944</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>(Nymphs)</td>
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<td>38.3</td>
<td>Rep.</td>
<td>9</td>
<td>89.852</td>
<td>-</td>
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<tr>
<td>PVX</td>
<td>57.1</td>
<td>Treat.</td>
<td>2</td>
<td>1527.63**</td>
<td>0.0088</td>
<td>1.513</td>
<td>0.281</td>
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<tr>
<td>Healthy</td>
<td>61.6</td>
<td>Error</td>
<td>18</td>
<td>245.152</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>

SOV=Source of variation, df=degree of freedom, MS= Mean squares, Treat= Treatments, Rep= Replications, Err= Error, p-value= estimated at corresponding degree of freedom, df= degree of freedom, S.D=Standard deviation, S.E=Standard Error, NS= non significance, ** significant at p≤0.01%

**DISCUSSION**

PVX vector is a promising and attractive tool for screening and evaluation of gene assays accompanied with rapid high replication, strong gene expression, robust results analysis and inexpensive methodology of easy manipulation, inoculation and good range of solanaceae host plants (Lawrence and Novak, 2001). Previously, PVX has been evaluated for high throughput expression, screening and evaluation of candidate insecticidal proteins against chewing insects (Lawrence and Koundal, 2002; Lawrence and Novak, 2001; Lawrence and Novak, 2006). Besides, PVX was demonstrated as simple cheap, robust and time saving screening tool for insecticidal protein expression and evaluation of candidate proteins under atestation against sucking insect pest species. Moreover, the statistical results and commonality in phenotypes are evident of oral insecticidal toxicity of the Hvt in aphids and bollworms (Khan et al., 2006; Shah et al., 2011, Javade et al., 2016). The paralysis, convulsions, disoriented/irregular movement and resting appendages are the common characteristics of the venom toxocation in insects as previously characterized by (Manzoli-Palma et al., 2003). The toxicity symptoms of Hvt were characterized as feeding cessation; legs protruding out and disoriented locomotion of the insects might be due to paralysis of mouthparts of insects and motor muscles (Vonorax et al., 2006). The blockade and inactivation of moderate MLVA and insect HLVA with high affinity might be responsible for weakness and paralysis in test insects. The continual and disoriented antennal and cerci movements and loss of rightening reflex may be due to perturbing effect due to slower intoxication and blocking of calcium channel in CNS of the insect (Pringos et al., 2011). Mortality induced in insects by Hvt toxin (ω -Hv1a) is due to inactivation of voltage-gated calcium channels (VGCCs) in the central nervous system because P/Q-type Calcium channel widely expressed in the CNS are the primary targets of ω-Hv1a peptide toxin (King et al., 2008). The complementary results of this study indicated the substantial insecticidal activity of Hvt spider toxin against plant aphids and this study will boost the efforts to test this candidate protein against other important sucking insect species in perspective to develop insect resistant transgenic plants.

The use of agrochemicals with few target receptors has promoted the evolution of resistance to a large number of insecticides that indicated a solemn need to identify and evaluate some novel candidate insecticidal compounds/proteins with novel insecticidal targets (Windley et al., 2011). Hvt is a peptide toxin from funnel web-spider has high affinity and specificity for insect CaVα-1 channels as primary target and moderately potent blocker of putative CaV2 channels with no effect in higher vertebrates (Pringos et al., 2011; Yamaji et al., 2007). The insecticidal activity Hvt toxin is based on inhibitory cysteine knot (ICK)
Fig. 1

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *S. exigua* and *H. armigera* for 5 days feeding duration compared to control plants.

(A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plants.

of the three key amino acids Pro10, Asn27 and Arg35 of ω-Hv1a are considered to form a tight and stable toxin-target complex critical for receptor binding, inhibition of insect calcium channel, selectivity and effective insecticidal activity (Pringos et al., 2011). These residues have been proposed to bind the calcium channel pore forming ion conducting unit through hydrophobic and electrostatic interaction that perturb the Ca$^{2+}$ ion conductance of the channel and cause inactivation of Ca$^{2+}$ mediated gene transcription, gene regulation cascades and synapses transmission in P/Q type neuron cells in CNS (Pringos et al., 2011). Hvt selectively block insect P/Q type calcium channel by depolarization of the resting membrane potential and reduction in spontaneous firing to induce a depressant phenotype, a characteristic toxic symptom of ω-Hv1a in insects (Bloomquist, 2003; King, 2007). Hvt is a potential bioinsecticide combined with the desirable attributes of novel target selectivity, high potency of structural stability, target sites activity and nanomolar concentrations required for insecticidal activity than conventional agrochemicals (Windley et al., 2011). Hvt being a potential insecticidal protein with substantial insecticidal activity and insect selectivity implicated to play an eminent and competitive role in crop protection after assessments of low toxicity in non-target organisms, less persistence in environment and cost of technology transfer to farmers.

CONCLUSIONS
PVX vector was established as potential and valuable screening tool for the assessment of general efficacy of candidate insecticidal proteins against economically important phloem feeding insects in model plants. Moreover, the significant results of feeding bioassays of Hvt against cotton aphid and potato peach aphid strongly recommend the potential utilization of insecticidal characteristics of Hvt to develop insect resistant transgenic crop plants after assessment of all biosafety considerations.

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Fig. 2

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *A. gossypii* Larvae for 5 days feeding duration compared to control plants. Where, (A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plants.

Fig. 3

Insecticidal effects of Hvt spider toxin on population survival of *A. gossypii* Larvae for 5 days feeding duration compared to control plants. Where, (A)=Adult aphid population, (B)=Number of nymphs produced and survived.

Fig. 4

Transient expression of Hvt through PVX in *N. tabacum* L. plants showing insecticidal activity of Hvt spider toxin in *M. persicae* Sulzer for 5 days feeding duration compared to control plants. Where, (A)=Hvt/PVX plants, (B)=PVX empty vector and (C)=Healthy as control plant.
Insecticidal effects of Hvt spider toxin on population survival of *M. persicae* Sulzer for 5 days feeding duration compared to control plants. Where, (A)=Adult aphid Population, (B)=Number of nymphs produced and survived

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