INT. J. BIOL. BIOTECH., 18 (4): 673-679, 2021.

TRANSIENT POST-TRANSCRIPTIONAL GENE SILENCING IN *NICOTIANA TABACUM* FOR QUICK ASSESSMENT OF TARGET GENE EFFICACY

Imran Rauf[1]*, Raza Muhammad Memon[1], Noroza Umar[2], Rubab Zahra Naqvi[2], Imran Amin[2] and Shahid Mansoor[2]

[1]*Nuclear Institute of Agriculture (NIA) Tandojam*
[2]*National Institute of Biotechnology and Genetic Engineering (NIBGE) Faisalabad.*
*correspondence: juniper_786@hotmail.com

ABSTRACT

In herbivore insects, RNAi can be achieved by feeding target insects on plants produce double standard RNA (dsRNAs) either by stable or transient transformation. Production of dsRNAs through stable transformation is a slow and laborious process, so, here we demonstrated a quick and efficient transient method. We used Potato Virus X (PVX) as a viral vector to produce dsRNAs transiently in the host plant *Nicotiana tabacum* which cause quick gene knock-down of target gene (chitin synthase) in herbivore insect, mealybug (*Phenacoccus solenopsis).* Reverse-transcriptase-polymerase chain reaction RT-PCR validate the expression of transgene in recombinant-PVX-inoculated plants, whereas, population of mealybug was significantly reduced (30%) just after 48hrs of feeding on recombinant PVX infected plants and reached upto 78% in 7-9days. The quick knockdown of target gene and mortality of the insects feeding on recombinant PVX infected plant validate that the recombinant PVX is an effective tool for evaluating candidate RNAi effectors in plants.

Keywords: RNAi, VIGS, PVX virus, transient expression, mealybug, control

INTRODUCTION

Gene silencing in eukaryotes mainly acts as a defense system against intruding viruses. In plants, mostly gene silencing triggered with the introduction of exogenous sources like plant viruses which cause infection in plants. In response to the foreign attack, the internal defense machinery "RNAi" triggered with the help of dsRNA's production in the cytoplasm of the cell. RNAi is used as a powerful tool for the assessment of gene functions by suppressing endogenous genes and is being used for several beneficial purposes, generally in eukaryotes and specifically in plants.

The effect of silencing or knockdown of target genes expression in plants is mainly achieved by multiple ways of post transcriptional gene silencing (PTGS) like stable plant transformation and transient expression of silencing induced fragments with the help of virus vectors (VIGS). Stable plant transformation is a challenging, arduous, lengthy and time-consuming process used to produce protein on large scale or for long-term gene expression studies. Whereas, transient expression can easily provide quick output (gene expression/ gene suppression) within a short period of time.

Several plant viruses have been used as a VIGS vector to suppress the endogenous gene expression. Most of them belong to positive strand RNA with mono, bi, and tripartite genome. Examples of frequently used VIGS vectors are *Potato virus X* (PVX), tobacco rattle virus (TRV), tomato mosaic virus (TMV), cotton leaf curl virus (CLCrV) and bean pod mottle virus (BPMV) (Idris *et al.*, 2010; Khan *et al*., 2013; Meziadi *et al.*, 2017). Some vectors depend on the helper virus or satellite DNA to produce disease symptoms in the target host. VIGS vectors are binary Ti plasmids which have the capability to attain both viral genome and fragment of the target gene of the host. These vectors are inoculated into the plant system through *A. tumefaciens* infection which transfers T-DNA containing the viral genome. These viruses replicate within plant cell to produce viral symptoms as well as responsible for the production of dsRNAs of inducted fragments. These dsRNAs are cleaved by Dicer-like protein into 21-24 nucleotides (nt) short interfering RNAs (siRNA) which further processed by RNA-induced Silencing Complex (RISC) to chop down complementary RNA's (Ding and Voinnet, 2007; Robertson, 2004). The virus derived silencing signals spread all over the plant and responsible for down-regulation of the target gene (Voinnet, 2001).

Different plants have been used for gene expression studies. The current study is related to evaluating the target gene expression and their knockdown effect in *N. tabacum*. The procedure of construct preparation and plant inoculation in VIGS is quite inexpensive, non-laborious and less time-consuming. Thus for the quick evaluation of gene expression, VIGS based transient expression was performed by using Potato Virus X (PVX vector; pgR107).

PVX (*Potexvirus*: *alphafexiviridae*) is a single standard RNA (ssRNA) virus. PVX vector is frequently used for VIGS based transient expression in *N. benthamiana* and other plants (Du *et al.*, 2014; Roder *et al.*, 2018).

Keeping in view the limited time frame and convenient possibilities, VIGS play an important role in developing control strategies against insect pests. The present study is designed to evaluate the efficiency of important genes by using VIGS technology before the development of transgenic plants with time-consuming stable transformation.

MATERIAL AND METHODS

PVX-based Vector Construction and Transformation

For transient expression, viral-based Potato Virus X (PVX) vector (pgR107) was used to clone target gene fragments from three sucking insect-pests i.e. whitefly (*Bemisia tabaci*), aphid (*Myzus persicae*) and mealybug (*Phenacoccus solenopsis*). A 200 bp of gene fragments (Ecdysone receptor, Cathepsin L and Chitin synthase) from each insect was selected and fused together to form a 600 bp Sense sequence (IR-RNAi-1). The sense sequence for RNAi cassette was synthesized by Eurofins Genomics, Canada. The desired sequence was amplified using gene-specific primers having restriction sites *Cla*I at 5′ end on forward primer and *Sma*I at 5′ end on reverse primer (Table 1). Amplified gene fragment was cloned in PVX vector pgR107 (Fig.1) using *Cla*I and *Sma*I restriction sites (IR-RNAi-PVX). The cloned vector was then confirmed with PCR and restriction digestion. The confirmed clone was electroporately transferred into *A. tumefecians* strain LBA4404 for plant infiltration and further insect bioassays (Leckie and Stewart, 2011).

Table 1. Primer pairs used for amplification, cloning and confirmation in VIGS vector.

Gene	Forward	Reverse	Product
IR-RNAi-PVX	5′ ATCGATAAGGATAGT TAATGCTGCAC 3′	5′ CCCGGGAGTAATGTA CACAGCCAACG 3′	600 bp
Chitin synthase	5′ TACGCTATTGGTCAC TGGCTGCAAAAA 3′	5′ AGTAATGTACACAGC CAACG 3′	200 bp
PVX - Shuttle protein (SV)	5′ TTCACTGGCCGTCGT TTTACA 3′	5′ ATCAGGCGCCATTC GCCATTC 3′	167 bp

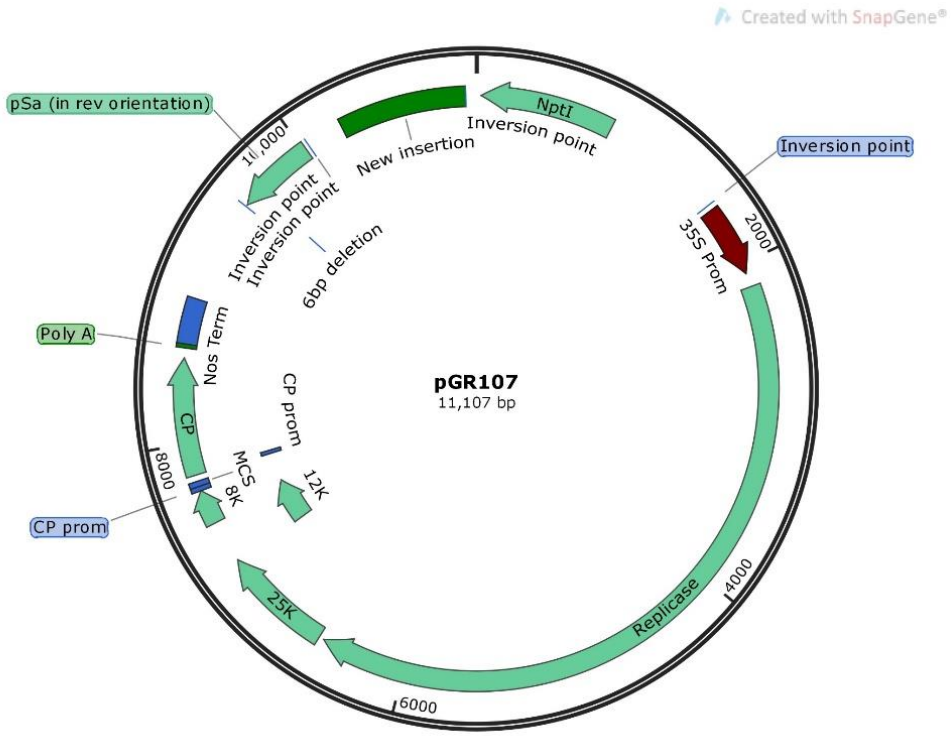

Fig. 1. pgR-107 vector map. (Source: SnapGene.com)
A PVX cloned binary vector based on pGreen0000 used for transient expression analysis.

Plant germination

N. tabacum plants were used for infiltration. Seedlings were grown by sowing *N. tabacum* seeds in 5″x 5″ plastic pots having peat moss. The pots were kept at 25 ± 2 °C temperature and 16:8 h light-dark photoperiods till germination of seeds. After germination, the selected plants were shifted into glass cages with proper ventilation in controlled conditions. When plants were on 2-3 true leaf stage, fully expanded leaves were used for infiltration.

Agro-infiltration for transient assay

For culture preparation, glycerol stocks of cloned PVX (IR-RNAi-PVX) with the gene of interest (IR-Chtn) and PVX alone in *A. tumefeciens* were used. Both cultures were streaked on plates having antibiotics (Rifampicin and kanamycin 25 µL and 50 µL, respectively). A single colony from each treatment was taken and incubated in LB liquid at 28 °C for 3-4 days with vigorous shaking (150 rpm) till the OD reach at 1. The bacterial cells obtained by centrifugation of culture at 4000 rpm were than resuspended in 10 mM $MgCl_2$. The OD of the resuspended solution was maintained at 0.7 and *acetosyringone* (150 µg/mL) was added to make final volume up to 100 µM. The prepared solution was kept at room temperature for 4-6 h before infiltration. The *N. tabacum* plants were infiltrated at room temperature with the help of 5 mL needleless disposable syringe. The solution was injected into the leaves (lower surface) with slight pressure. The infiltrated as well as non-infiltrated plants were kept in greenhouse at 16:8 h Light: Dark photoperiod and 26±1 °C temperature till bioassay (Zhu *et al*., 2012).

Insect rearing

Mealybug (*Phenacoccus solenopsis*) was selected as model insect to evaluate the transient expression of target gene in *N. tabacum* and its silencing effect on insect biological parameters. Fresh population of adults was collected from the cotton field and initially reared on cotton plants in the greenhouse at 30±2 °C at NIBGE. The secondary culture was maintained on tobacco (*N. tabacum*) on the same temperature at 70 % R.H under 16:8 (L: D) cycle. The adult females of mealybug were used in bioassays to evaluate the RNAi effect.

Insect bioassays

First instar mealybug nymphs (twenty in numbers) were released per treatment/replication. There were three treatments with five replicates of each treatment. Insect mortality was observed in all three treatments after 24 h exposure upto maturity into adults.

Statistical Analysis

The mortality data was collected and analyzed by using Statistix 8.1. Analysis of variance and Tukey's HSD was calculated to find out significant differences among treatment, whereas, Dunnet test was performed to compare treatments with control on day 9.

RESULTS AND DISCUSSION

Clone confirmation

Cloning of target fragment (IR-RNAi-PVX) of 600 bp was confirmed with PCR by using gene-specific primers (Table 1) and restriction digestion with *Cla*I and *Sma*I enzymes. The amplification of 600 bp fragment with both PCR (Fig. 2A) and restriction analysis (Fig.2B) confirmed the integration of target fragment into pgR-107 VIGS vector.

RT-PCR: Detection of Mealybug mRNA in Inoculated Plants

Presence of *IR-Chtn* mRNA in inoculated leaves was detected by RT-PCR by using gene-specific primers, whereas, PVX shuttle protein primers were used as internal control to detect the inoculation of pgR-107 vector into plant system (Table 1). Total RNA was isolated from inoculated and non-inoculated leaves by using TRIzol® reagent (Ambion, Cat No. 15596-018) and further used for cDNA synthesis. Random hexamer primers were used to synthesize First Stand cDNA synthesis and further analyzed by RT-PCR for the confirmation of mRNA of both target mealybug target gene (chitin synthase, *IR-Chtn*) and PVX internal control gene (shuttle protein). Amplification of 167 bp fragment of internal control (shuttle protein) confirmed the integration of PVX vector in inoculated tobacco plants (PVX + Sense and PVX only) (Fig.3A), whereas, amplification of 200 bp target gene (*IR-Chtn*) ratified the presence of target gene only from PVX –Sense infiltrated plants (Fig. 3B).

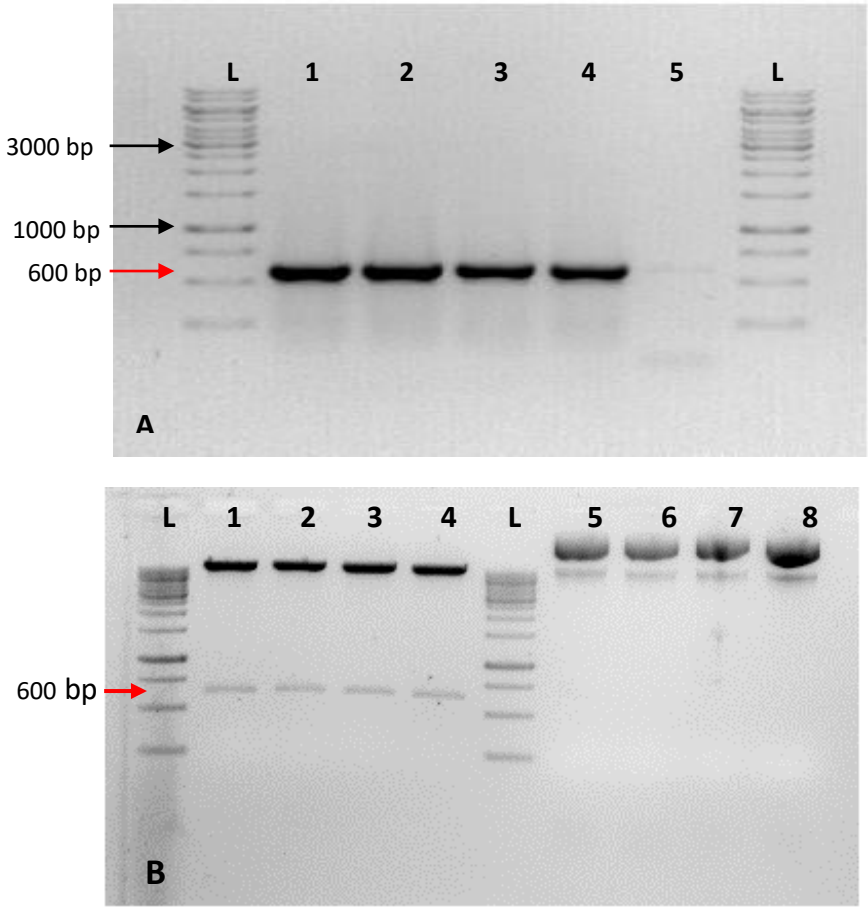

Fig. 2. Confirmation of Sense clone in pgR-107 VIGS vector.
(**A**) amplification of 600 bp fragment by PCR using gene specific primers lane 1-3 = clones , lane 4 = +ve plasmid , lane 5 = -ve water only, L = 1kb ladder (**B**) restriction analysis lane 1-4 = *Cla*I and *Sma*I restricted plasmid showing 600 bp restricted fragment and plasmid backbone, lane 5-8 = un-restricted plasmid L = 1kb ladder.

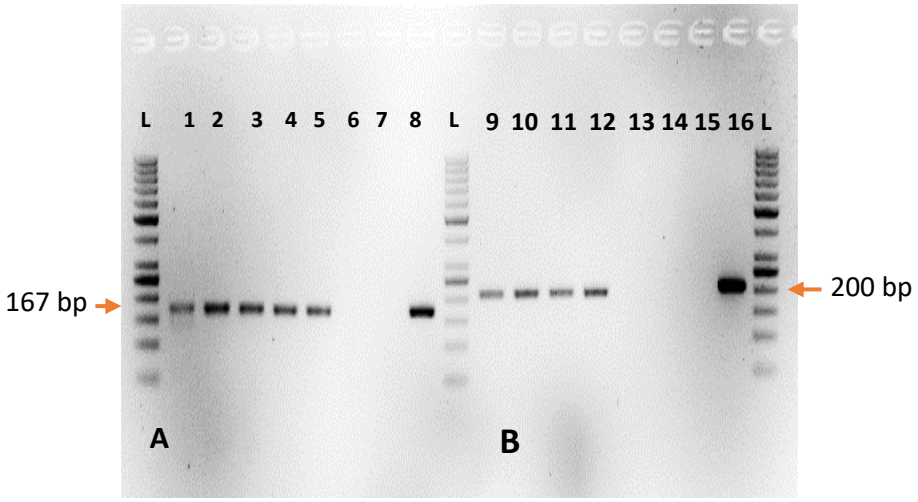

Fig. 3. PCR amplification of PVX and target gene (Chitin synthase) from infiltrated plants.
(A) amplification with PVX primers (167 bp) L= ladder 50bp, Lane 1-4 = PVX + Sense, Lane 5 = PVX alone, Lane 6 = non-transgenic, Lane 7 = Negetive control, Lane 8 = Positive control (B) amplification with gene specific primer (Chitin synthase 200bp) L= ladder 50bp, Lane 9-12 = PVX + Sense, Lane 13 = PVX alone, Lane 14 = non-transgenic, Lane 15 = Negetive control, Lane 16 = Positive control.

Insect bioassays

Cotton mealybug (*P. solenopsis*) was used to conduct bioassays of *N. tabacum* plants infiltrated with *A. tumefeciens* having modified PVX vector (IR-RNAi-PVX). Twenty, 1st instar nymphs were released on each plant. Plants were arranged in three groups and each group consists of five plants. The plants in the group first were infiltrated with modified VIGS vector (IR-RNAi-PVX), the second group was infiltrated with PVX vector (non-modified), and third groups were treated as control. The plants infiltrated with modified VIGS vector (IR-RNAi-PVX) exhibited significant population reduction of mealybug as compared to plants infiltrated with non-modified PVX and control plants. Mortality was recorded after 24 h of releasing of nymphs, whereas, data was collected on a daily basis for nine consecutive days.

Analyzed data confirmed the significant mortality of mealybug fed on plants infiltrated with VIGS based modified vector (IR-RNAi-PVX) with target gene of interest (*IR-Chtn*). Overall 78.33 % mean mortality was observed in PVX–Sense (IR-RNAi-PVX) modified vector infiltrated plants followed by 12 % in PVX only (non-modified) and 10 % in control plants (Fig.4).

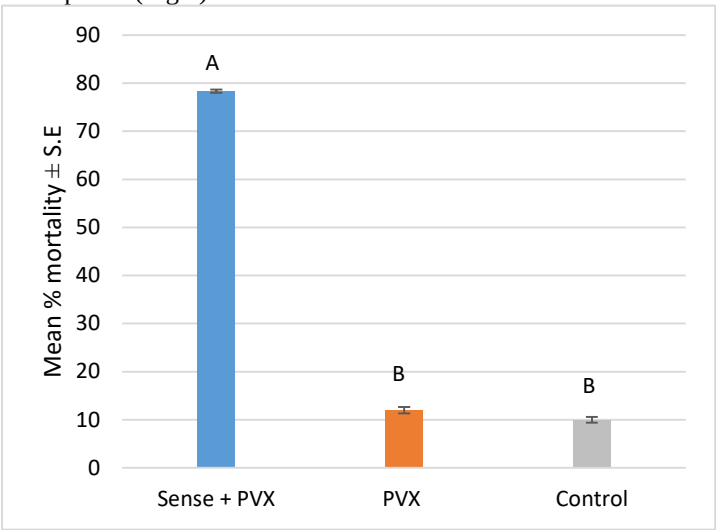

Fig. 4. Overall percent mortality of mealybug with VIGS based bioassays on *N. tabacum.*
PVX –Sense (modified VIGS vector, IR-RNAi-PVX), PVX only (Non-modified VIGS vector) and control plants. Each bar represents the mean ± S.E. Mean of N = 5, $p < 0.05$.

Significant mortality was started after day 4 (30 %) of feeding which was gradually increased and maximum mortality was observed on day 9 (78.33 %) (Fig. 5). Dennett's test confirmed the significant difference of mortality in plants infiltrated with modified VIGS vector (IR-RNAi-PVX) as compared to mock and negative control.

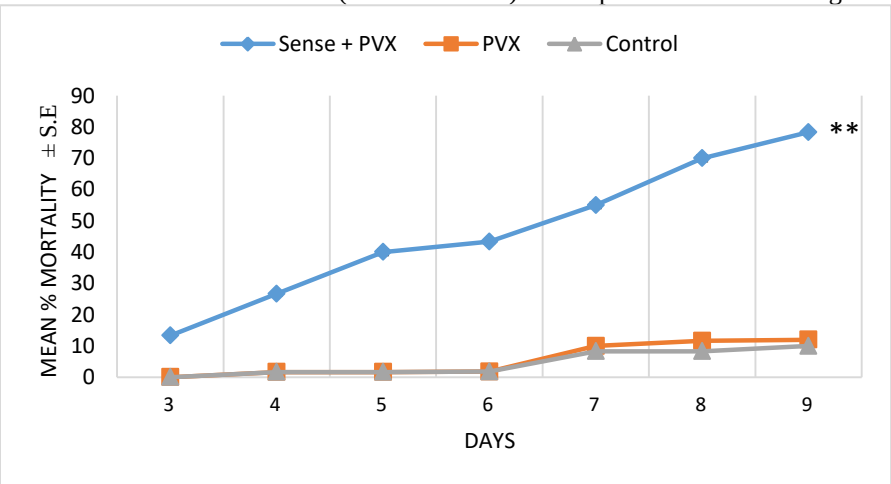

Fig. 5. Days wise mean mortality of mealybug fed on VIGS inoculated tobacco plants.
PVX –Sense (modified VIGS vector, IR-RNAi-PVX), PVX only (Non modified VIGS vector) and control plants. ** $P < 0.05$ Dunnett's test relative to control. Mean of N = 5.

RNAi based management of insects opens a new era in integrated pest management. Identification and evaluation of potential target genes play a key role in RNAi based insect management. *In planta* production of gene-specific siRNAs and dsRNAs through RNA interference ensured insect resistance (resistance of plants towards insects) (Price and Gatehouse, 2008). In order to achieve insect resistance through *in planta* expression of potential RNAi targets, virus-based transient expression is a preliminary tool used for quick assessment and evaluation of efficacy and functions of selected genes.

The current study validated that virus-induced gene silencing (VIGS) is a potential tool for gene knock-down studies. Potato virus X (PVX) was used to make the recombinant construct by inserting mealybug target gene (Chitin synthase). The resulted recombinant virus (IR-RNAi-PVX) was inoculated into *N. tabacum* plants. The targeted mRNA (*IR-Chtn*) was first validated by RT-PCR and further, their gene silencing efficiency was evaluated and ratify by insect bioassays. Significant mortality of mealybugs which fed on tobacco plants infiltrated with recombinant virus (IR-RNAi-PVX) confirmed the gene knockdown and silencing efficiency of VIGS. It was also noted that 50-60 % of the insect population was killed in the nymphal stage which confirmed the distinguished effect of RNAi on nymphs as compared to adults. These results are supported by previous studies (Khan *et al*., 2013; Wuriyanghan and Falk, 2013) and suggested that RNAi triggers may available in a large amount in phloem cells, and hence provide great opportunity to phloem feeder insects to suck more as compare to chewing insects. Whereas the crawlers (nymphs) or young insects are feed more prodigiously than adult ones, that's why mortality was enhanced in nymphs as compared to adult mealybug. Furthermore, our findings suggested the efficacy of chitin synthase gene as a potential candidate for RNAi to suppress the population of mealybug and these results are similar as Khan *et.al.* (Khan *et al*., 2015), which also confirmed the potential effect of same gene (*CHS1*) in PVX vector against mealybug. During experiment, it was also noted that after releasing of nymphs on infiltrated and non-infiltrated plants, initially 3-4 days, there are no adverse effects and mortality was observed in nymphs. This may be due to RNAi response that insects may take some time to accumulate siRNAs in their body.

Potato Virus X (PVX) a single standard RNA, with ˜11 kb genome, has been used as a viral vector for heterologous gene expression in plants (Dickmeis *et al.*, 2014; Mardanova *et al*., 2017; Toth *et al.*, 2001). Here we also demonstrated that PVX as a viral vector has efficiently expressed target gene (Fig. 3B) in limited time when infiltrated in *N. tabacum* and caused gene knock-down. Several studies supported our results that PVX as viral vector efficiently delivered and expressed exogenous gene and produce dsRNAs systemically in plants (Du *et al*., 2014; Khan *et al*., 2015; Mardanova *et al.*, 2017; Shuxia *et al.*, 2011).

Being an efficient delivery of target gene by using PVX viral vector into plant system, our study suggested that modified plant viruses are effective and proficient tools to preliminary investigate the RNAi targets in phytophagous insects and play a key role in eco-friendly management of insect pests.

Authors Contribution

SM conceived and designed the current project. IR conducted all experiments, statistical analysis and wrote first draft. IA and RZN were involved in construct designing. NU involved in RT-PCR. SM and RMM revised the manuscript critically. All authors have read and approved the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

Ethical approval

This article does not claim any studies with human participants or vertebrates performed by any of the authors.

Acknowledgement

The Higher Education Commission of Pakistan (HEC) is highly acknowledged for providing financial support under indigenous scholarship.

REFERENCES

Dickmeis, C., R. Fischer and U. Commandeur (2014). Potato virus X-based expression vectors are stabilized for long-term production of proteins and larger inserts. *Biotechnology Journal,* 9(11): 1369-1379. doi:10.1002/biot.201400347

Ding, S.W. and O. Voinnet (2007). Antiviral immunity directed by small RNAs. *Cell,* 130(3): 413-426.

Du, J., H. Rietman and V. G.Vleeshouwers (2014). Agroinfiltration and PVX agroinfection in potato and *Nicotiana benthamiana*. *Journal of Visualised Experiments*, (83): e50971. doi:10.3791/50971

Idris, A. M., J.R. Tuttle, D. Robertson, C. H. Haigler and J. K. Brown (2010). Differential cotton leaf crumple virus-VIGS-mediated gene silencing and viral genome localization in different *Gossypium hirsutum* genetic backgrounds. *Physiological and Molecular Plant Pathology.* 75(1-2): 13-22.

Khan, A. M., M. Ashfaq, M. A. Khan, A. Rasool, J. Iqbal and S. Mansoor (2015). Inoculation of *Nicotiana tabacum* with recombinant potato virus X induces RNA interference in the solenopsis mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae). *Biotechnology Letters*, 37(10): 2083-2090. doi:10.1007/s10529-015-1880-7

Khan, A. M., M. Ashfaq, Z. Kiss, A. A. Khan, S. Mansoor and B. W. Falk (2013). Use of recombinant tobacco mosaic virus to achieve RNA interference in plants against the citrus mealybug, *Planococcus citri* (Hemiptera: Pseudococcidae). *PLoS ONE, 8*(9): e73657. doi:https://doi.org/10.1371/journal.pone.0073657

Leckie, B. M. and C. N. Stewart (2011). Agroinflitration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. *Plant Cell Reports*, 30(3): 325-334.

Mardanova, E. S., E.A. Blokhina, L. M. Tsybalova, H. Peyret, G.P. Lomonossoff and N. V. Ravin (2017). Efficient transient expression of recombinant proteins in plants by the novel pEff vector based on the genome of Potato Virus X. *Frontier Plant Science,* 8: 247. doi:10.3389/fpls.2017.00247

Meziadi, C., S. Blanchet, V. Geffroy and S. Pflieger (2017). Virus-Induced Gene Silencing (VIGS) and foreign gene expression in *Pisum sativum* L. using the "One-Step" Bean pod mottle virus (BPMV) viral vector. *Functional Genomics, Methods and Protocols*, 311-319.

Price, D. R. and J. A. Gatehouse (2008). RNAi-mediated crop protection against insects. *Trends in Biotechnology,* 26(7): 393-400. doi:10.1016/j.tibtech.2008.04.004

Robertson, D. (2004). VIGS vectors for gene silencing: many targets, many tools. *Annual Review of Plant Biology,* 55: 495-519.

Roder, J., C. Dickmeis, R. Fischer and U. Commandeur, (2018). Systemic infection of *Nicotiana benthamiana* with Potato virus X nanoparticles presenting a fluorescent iLOV polypeptide fused directly to the coat protein. *Biomedical Research International,* 9328671. doi:10.1155/2018/9328671

Shuxia, C., W. Xiaowu and C. Zhihui (2011). Construction of PVX virus-expression vector to express enterotoxin fusion gene LTB-ST and transformation into tobacco. *African Journal of Biotechnology,* 10(6): 1022-1028. doi: 10.5897/AJB10.002

Toth, R. L., S. Chapman, F. Carr and S. S. Cruz (2001). A novel strategy for the expression of foreign genes from plant virus vectors. *FEBS Letter*, 489(2-3): 215-219. doi:10.1016/s0014-5793(01)02091-9

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends in Genetics,* 17(8), 449-459.

Wuriyanghan, H. and B. W. Falk, (2013). RNA interference towards the Potato Psyllid, *Bactericera cockerelli*, is induced in plants infected with recombinant tobacco mosaic virus (TMV). *PLoS ONE,* 8(6): e66050. doi:10.1371/journal.pone.0066050

Zhu J. Q., S. Liu, Y. Ma, J.Q. Zhang, H.S. Qi, Z. J. Wei, Q. Yao, W. Q. Zhang and S. Li. (2012) Improvement of Pest Resistance in Transgenic Tobacco Plants Expressing dsRNA of an Insect-Associated Gene *EcR*. *PLOS ONE* 7(6). https://doi.org/10.1371/journal.pone.0038572

(*Accepted for publication October 2021*)