Biotechnol Lett (2015) 37:2083–2090
DOI 10.1007/s10529-015-1880-7

ORIGINAL RESEARCH PAPER

Inoculation of *Nicotiana tabacum* with recombinant potato virus *X* induces RNA interference in the solenopsis mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae)

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Received: 23 February 2015 / Accepted: 2 June 2015 / Published online: 19 June 2015

Abstract
Objectives The chitin synthase 1 (*CHS1*) gene in *Phenacoccus solenopsis* (*PsCHS1*) was evaluated as a potential target of RNA interference (RNAi) by using *Potato virus X* (PVX) as a vector (recombinant PVX) for expressing RNAi triggering elements in *Nicotiana tabacum* L.
Results RT-PCR analysis confirmed the expression of *PsCHS1* in *N. tabacum* inoculated with recombinant-PVX–*PsCHS1* (treated). RT- and multiplex-PCR further showed a reduction in mRNA levels of the target gene in mealybugs feeding on treated plants. Mortality in parent adults and emerging nymphs (21 and 29 %) exposed to the treated plants was significantly higher ($P < 0.05$) than those exposed to uninoculated (−ve control) or inoculated with non-recombinant PVX (PVX-control). The number of surviving adults and the combined number of adults and nymphs (47 and 60 %) was significantly ($P < 0.05$) lower on the treated plants than the −ve (76 %) or PVX (74 %) control. The visual observations verified the physical deformities in mealybugs exposed to the treated plants.
Conclusion chitin synthase 1 is a potential RNAi target in *P. solenopsis* and the recombinant PVX can be used as a tool to evaluate candidate RNAi triggering elements in plants.

Keywords Mealy bug · *Phenacoccus solenopsis* · *Potato virus X* · Recombinant virus · RNA interference · Solenopsis mealybug

Electronic supplementary material The online version of this article (doi:10.1007/s10529-015-1880-7) contains supplementary material, which is available to authorized users.

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Introduction

Solenopsis mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae), is a highly polyphagous insect recorded on more than 50 plant families (Hodgson et al. 2008). Cotton (*Gossypium hirsutum* L.) is a preferred host for this insect as both adults and nymphs infest all parts of the plant causing

damage by sucking phloem sap and egesting sugary material.

RNAi has emerged as a new tool for insect control with its applications in field (Hunter et al. 2010). RNAi-based plant protection against chewing insects is well documented (Chu et al. 2014) while its success against sucking insects is being debated (Christiaens and Smagghe 2014). Absence of a RNA-dependent RNA polymerase system that is required to amplify the RNAi effects by continuous transcription of RNA molecules (Sijen et al. 2001) in most insects (Price and Gatehouse 2008), makes the use of RNAi for pest control even more challenging. Production of dsRNA (in vivo) has been successful using vectors that can express dsRNA via stable transformation (Baum et al. 2007). Virus-induced gene silencing (VIGS) is an efficient and simple substitute to reduce the endogenous gene expression in plants (Lu et al. 2003) and researchers have exploited VIGS via recombinant viruses to induce RNAi in plant feeding organisms (Kumar et al. 2012). Various plant viruses have been effectively used for expression of transgenes in plants; as for example, Tobacco mosaic virus (TMV) (Khan et al. 2013), Tobacco rattle virus (Senthil-Kumar and Mysore 2014), Citrus tristeza virus (Hajeri et al. 2014) and *Potato virus X* (PVX) (Angell and Baulcombe 1999).

There is often a high variability in results due to differing dsRNA application procedures, target genes and target insects (Uryu et al. 2013). The practical dsRNA delivery for phloem-feeding hemipterans, like mealybugs, is likely to be via their host plants (Christiaens and Smagghe 2014). As for example, Wuriyanghan and Falk (2013) employed tomatillo (*Physalis philadelphica*), tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*) to infest with TMV for a successful RNAi of *β-actin* and *V-ATPase* in *Bactericera cockerelli*. Screening of genes vital for mealybug development can provide useful information to control this insect through gene suppression and interference. Moreover, development of reliable transient assay to express mealybug genes in plants can help pinpointing potential target genes in this pest for the development of transgenic plant hosts. Present study was focused on identification, expression, and analysis of a gene involved in the development of *P. solenopsis*. Furthermore, suppression of this gene in *P. solenopsis* using RNAi was evaluated by PVX, a virus only transmitted mechanically. The study provides useful information on the potential RNAi targets in solenopsis mealybug and PVX as a tool to perform transient RNAi analysis.

Materials and methods

Mealybug culture

Phenacoccus solenopsis adults were collected from pesticide-free cotton fields of the National Institute for Biotechnology and Genetic Engineering, Faisalabad. Adults were used to initiate a *P. solenopsis* colony on tobacco (*N. tabacum*) plants and maintained in glasshouse under controlled conditions at 27 ± 3 °C. Second instar nymphs were used for the bioassay studies for the evaluation of RNAi effects.

RNA extractions

RNA was extracted from whole insects and/or insect body parts using TRIzol as described previously (Khan et al. 2013). RNA was precipitated by adding two volumes 95 % ethanol and 0.1 vol 3 M sodium acetate. RNA pellet was washed with 75 % (v/v) ethanol, re-suspended in DEPC-treated SDW and stored at −80 °C. RNA/DNA was quantified by ethidium bromide/1 % (w/v) agarose gel and re-quantified by NanoDrop Spectrophotometer ND-1000 (NanoDrop, USA).

cDNA synthesis and PCR amplification

First strand cDNA was synthesized using superscript cDNA synthesis kit (Invitrogen) with oligo(dT)-primer using 500 ng RNA following manufacturer's instructions. Briefly, RNA and oligo(dT) primer were mixed and incubated at 65 °C for 5 min before adding 10 µl reaction mixture (0.1 M DTT 2 µl, 25 mM $MgCl_2$ 4 µl, 10X RT buffer 2 µl, RNase out (40 U/µl) 1 µl and SuperScript II 1 µl). The reaction was incubated at 42 °C for 50 min followed by 70 °C for 10 min and the cDNA was stored at −20 °C. A 507 bp fragment of *CHS1* was amplified using degenerate primers from Ashfaq et al. (2007) with a slight modification in PCR conditions (Supplementary Table 1). These primers are based on conserved sequences of *CHS1* from diverse insect orders (Coleoptera, Diptera, Hymenoptera, Lepidoptera) and have amplified this gene from another mealybug, *Planococcus citri* (Khan et al. 2013).

RNAi target sequence selection

A partial fragment of *CHS1* was isolated from *P. solenopsis* cDNA (200 ng/reaction) using primers and PCR conditions and outlined in Supplementary Table 1. Amplified PCR products were gel purified using Spin Prep Gel DNA kit (Novagen) following manufacturer's protocol. Amplicons were ligated into pTZ57R/T TA cloning vector and cloned into *E. coli* using standard molecular techniques, and the positive clones were sequenced commercially (Macrogen Inc., South Korea). Sequence accuracy of the genes was verified and gene-specific primers were designed (adding endonuclease recognition sites) (Supplementary Table 1) for the re-amplification of the target gene region. Vector sequences were removed and the edited sequences were reconfirmed for the identity of isolated gene using basic local alignment search tool (BLAST) at NCBI (http://blast.be-md.ncbi.nlm.nih.gov/Blast.cgi). After confirming the sequence fidelity for the gene, primers were designed using Snapdragon dsRNA design (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) (Supplementary Table 1) to amplify a 419 bp gene fragment divergent among insect species in order to reduce the off-target RNAi effects. The partial fragment of *P. solenopsis CHS1* (*PsCHS1*) was isolated by PCR amplification and used for further studies.

Transient expression of siRNA/dsRNA in plants for feeding assays

We used PVX-based vector PGR 107 (Chapman et al. 1992) that can efficiently express transgenes under the control of duplicated (CP) promoter to trigger VIGS against plant endogenous genes (Lacomme and Chapman 2008). Target genes were amplified using gene specific primers with *Sal*1 restriction site on 5′-end of the forward primer and *Xma*1 restriction site on 5′-end of reverse primers (Supplementary Table 1). Amplified gene products were cloned into pTZ57R/T. Gene fragments were excised from PTZ clones (containing *Sal*1 and *Xma*1 restriction sites) using appropriate enzymes to release the insert. The excised fragments were inserted into PGR107 vector pre-linearized using *Sal*1 and *Xma*1 restriction enzymes.

Confirmed clones were transformed into *Agrobacterium tumefaciens* (GV 3101) for the inoculation of tobacco (*N. tabacum*) plants following established protocol (Khan et al. 2013). Briefly, recombinant plasmid carrying single colony of *A. tumefaciens* was inoculated into 3 ml LB culture in the presence of a suitable antibiotic (rifampicin, kanamycin or tetracycline). The culture was grown at 28 °C for 48 h with vigorous shaking. 600 µl of the culture was inoculated into 30 ml LB media (containing 30 µl each of kanamycin, rifampicin and tetracycline), 6 µl acetosyringone and 300 µl 1 M MES and allowed to grow until an $OD_{600} = 1$. Cells were centrifuged at $\sim 4000 \times g$ for 10 min and pellet was re-suspended in 10 mM MES, 10 mM $MgCl_2$, and 100 µM acetosyringone to an OD600 = 1.0. The culture was placed in dark at room temperature for 2–3 h (or overnight) before infiltration. *Agrobacterium* suspension was infiltrated onto the lower side of a healthy leaf (infiltrated 2-3 leaves/plant) (treated plants) using a needleless disposable syringe (Wuriyanghan et al. 2011). PVX-control plants were inoculated with non-recombinant PVX while −ve control plants were not inoculated.

Detection of interfering RNAs in plants

After the PVX-specific symptoms were visible in the PVX–*PsCHS1* inoculated (treated) plants, the presence of transgenic mRNA in plants was confirmed through RT-PCR using plant RNA and the primers developed from the target *P. solenopsis* gene sequences (Supplementary Table 1). Plant RNA was extracted from the symptomatic leaves using TRIzol. One step RT-PCR was performed using SuperScript One-Step RT-PCR system (Invitrogen) following manufacturer's instructions. The reaction was completed at 42 °C for 30 min in a thermal cycler.

Insect bioassays and RT-PCR analyses

After confirming the presence of RNAi triggering elements in plants (7 days post-inoculation), ten 2nd instar mealybugs were placed on the symptomatic leaves of selected plants. Mealybug mortality, growth, crawler emergence and survival, and ovisac production were recorded. RT-PCR and multiplex RT-PCR were performed to analyze the mRNA levels of the target genes. cDNA was synthesized using RNA extracted from individual mealybugs pre-exposed to the PVX-inoculated plants for 12 days. Equal concentrations of cDNA were used in each RT-PCR reaction performed using primer pairs CSRTF (CTGGTCCTATGGTGTG

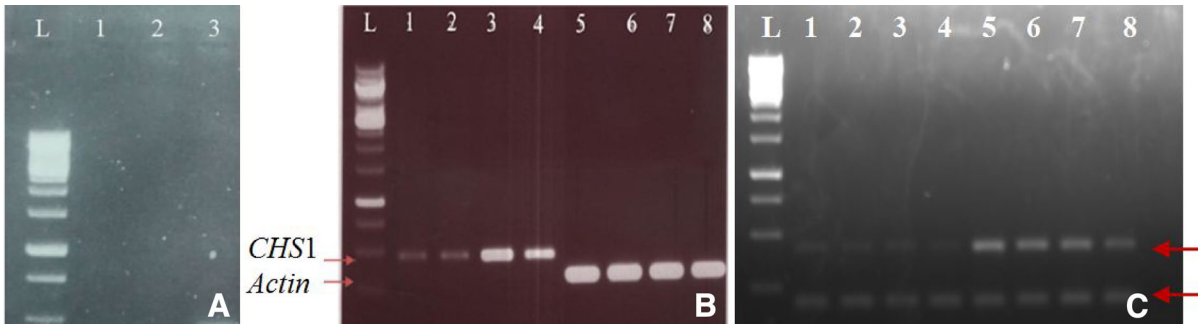

Fig. 1 PCR amplification and RT-PCR analysis of *PsCHS1*. **a** Amplification of *PsCHS1* from *N. tabacum* inoculated with non-recombinant-PVX (PVX control) (*1*), −ve control (*2*) and recombinant PVX–*PsCHS1* (*3*); **b** Two-step RT-PCR of *PsCHS1*from *P. solenopsis* exposed to PVX–*PsCHS1*-inoculated *N. tabacum* (*1–2*), PVX-control (*3*) and −ve control (*4*). Amplification of *Psβ-actin* (internal control) from the corresponding samples is shown in *5-8*; **c** Multiplex RT-PCR of *PsCHS1*from *P. solenopsis* exposed to PVX–*PsCHS1*-inoculated *N. tabacum* (*1–4*), PVX-control (*5–6*) and −ve control (*7–8*). *Psβ-actin* from the corresponding samples (*lower panel 1–8*)

GTATCAAG) and CSRTR (AACGAAAAGCATC CGGGACTA) for *Chitin synthase* 1 target and MbActinF (TCCGGTGATGGTGTATCTCA) and MbActinR (ATCACGGACGATTTCTCGTT) for *β-actin*. *β-Actin* was used as internal control in both RT-PCR and multiplex PCR.

Statistical analysis

Experimental data were analyzed to test the difference among treatments at a significance level of 0.05. Analysis of variance was performed using Statistix 8.1 following randomized complete block design. The homogenous groups were determined based on least significant difference values. Percent insect mortality was corrected using Abbott's formula.

Results

Sequence analysis

PsCHS1 (507 bp) was PCR-amplified and sequenced from *P. solenopsis* (GenBank accession KF384512) and sequence similarities with those from other insects were analyzed by GenBank BLAST. The *PsCHS1* sequence showed a maximum (87 %) nucleotide identity with that of *P. citri* (Hemiptera JX443530.1), 82 % with *Pediculus humanus corporis* (Phthiraptera XM_002423552.1), 80 % with *Aphis glycines* (Hemiptera JQ246352.1), 79 % with *Choristoneura fumiferana* (Lepidoptera EU561238.1), 78 % with *Laodelphax striatella* (Hemiptera JQ040012.1) and 77 % with *Tribolium castaneum* (Coleoptera NM_001039402.1). The BLAST search did not find an exact match for *CHS1* from *P. solenopsis* indicating that this sequence is a new addition to the GenBank.

PVX in inoculated plants express insect mRNAs

The recombinant PVX vector caused typical PVX symptoms of vein clearing and chlorotic mosaic on the leaves of inoculated *N. tabacum* plants after 18–21 days of inoculation. RNA was extracted from symptomatic leaves and RT-PCR was performed with *P. solenopsis*-specific primers to verify the presence of recombinant mRNA in plant leaves. The RT-PCR showed the expression of mealybug-specific *CHS1* mRNAs in the inoculated tobacco plants via recombinant PVX (Fig. 1a). Absence of bands smaller in size indicated that the insert remained stable in the plant system.

Plants expressing mealybug sense/antisense mRNA induce RNAi in the feeding mealybug

Recombinant PVX vector was constructed to express *PsCHS1* to verify if the ingestion of *PsCHS1* antisense RNA from inoculated plants affected the target mRNA level in the feeding mealybugs. Presence of recombinant mRNA was confirmed in the inoculated plants before providing them to the healthy mealybug nymphs (2nd instar). Total RNA was extracted from nymphs after 7 days of feeding, and RT-/multiplex-PCR were performed to detect the gene transcript. A reduction in *PsCHS1* mRNA level was observed in the

nymphs exposed to treated as compared to those fed on the healthy (−ve control) or PVX-control plants (Fig. 1b, c). Although the reduction observed in mRNA level was clear, the analysis was not properly a quantitative measurement.

Mealybug mortality on the recombinant PVX-inoculated plants

Mortality in parent mealybugs was determined after 18 days of feeding on treated or control plants. Mortality in adult mealybugs exposed to treated plants was 21 % which was significantly higher ($P < 0.5$) than those observed in adults exposed to −ve (0 %) or PVX-control (2 %) plants (Fig. 2a). The mortality in *P. solenopsis* crawlers emerging and feeding on treated plants was 29 %, which was significantly higher ($P < 0.05$) than those observed in emerging crawlers on −ve (0 %) or PVX-control (0.8 %) (Fig. 2a).

Growth retardation and physical deformities in RNAi-affected mealybug

Daily observations were made on the development of mealybugs feeding on the *N. tabacum* (inoculated or controls) plants. Percentage of surviving adults on treated plants was 47 % as compared to 76 % on −ve and 74 % on PVX-control (Fig. 2b). The number of alive individuals (3rd instar + adult) was significantly ($P < 0.05$) lower on treated plants (60 %) than on −ve (76 %) or PVX-control (74 %) (Fig. 2b). After 18-day post-exposure, most surviving nymphs on the treated plants were 3rd instar, while all the surviving nymphs on control (−ve or PVX) plants had reached to the adult stage (egg-laying) (Fig. 2c). Mealybugs with physical abnormalities, shrunken body and disrupted or extra-thin (hemolymph visible) cuticle were counted as "deformed". Number of deformed individuals on treated (PVX–*PsCHS1* inoculated) plants and −ve or PVX-control is shown in Fig. 2c. The typical deformities in mealybugs feeding on treated plants are presented in Fig. 3. Mealybug individuals with physical deformities did not survive more than 2 days. Further, in the case of PVX–*PsCHS1*-exposed symptomatic individuals the cuticle looked delicate and almost transparent. Deformities in the nymphs feeding on *PsCHS1*-inoculated plants coincided with the decreased mRNA levels of *PsCHS1*. None of the mealybugs exposed to control plants showed physical

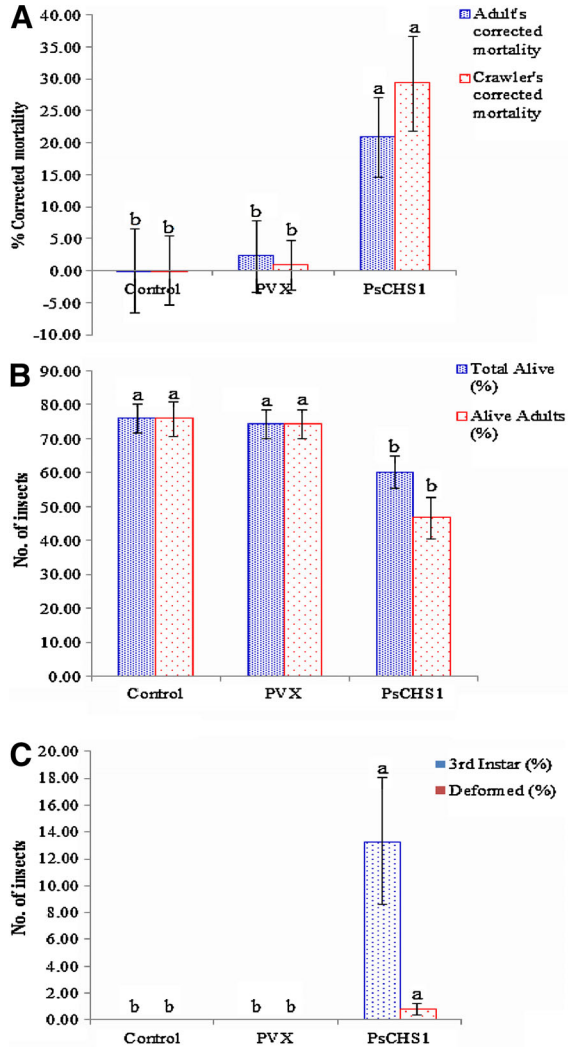

Fig. 2 Mortality (**a**), survivorship (**b**), and physical deformities (**c**) in *P. solenopsis* exposed to PVX–*PsCHS1*-inoculated *N. tabacum*. Data was recorded after 18 days of insect exposure to the inoculated or control plants. *PsCHS1* = inoculated with PVX–*PsCHS1*; PVX = inoculated with non-recombinant PVX (PVX-control); Control = uninoculated. *Bars* with same *letters* across similar *colors*/pattern show homogeneous group at $p < 0.05$

deformities. A general view of the mealybugs on PVX–*PsCHS1*-inoculated and uninoculated *N. tabacum* plants is presented in Fig. 4.

Ovisac production and fecundity

The number of ovisac producing females on treated plants was 18 % and these numbers were significantly lower ($P < 0.05$ %) than those on −ve (52 %) or

Fig. 3 Phenotypic effects in mealybug feeding on uninoculated (*a*) and PVX-*PsCHS1*-inoculated *N. tabacum* (*b*, *c*)

PVX-control (54 %) plants (Fig. 5). The number of emerging crawlers was also significantly lower on PVX–*PsCHS1* inoculated plants (50 %) than the −ve (100 %) or PVX (100 %) controls (Fig. 5).

Discussion

We have analyzed the effectiveness of RNAi in mealybug via recombinant potato plant virus, PVX. PVX can be effective for delivering dsRNA and targeting plant-feeding hemipterans by RNAi, as viruses are both targets and powerful inducers of RNAi activity in plants (Carrington et al. 2001). The data presented here, and supported by previous studies (Khan et al. 2013; Wuriyanghan and Falk 2013), suggest that RNAi triggering elements were present in the phloem in sufficient amounts to induce RNAi activity in phloem feeders. Other analyses have shown that siRNAs can be recovered from phloem sap (Pitino et al. 2011); hence VIGS serves as a high throughput approach for studying genes for RNAi based insect control.

Recombinant virus constructs with the target-gene (*CHS1*) were designed to inoculate *N. tabacum* against *P. solenopsis* and retention of *CHS1* mRNA by recombinant PVX in infected plants was confirmed by RT-PCR. After confirmation of infection by symptom development and RT-PCR, these plants were used for feeding assays. Neither the difference in mRNA level nor the phenotypic effects were obvious between nymphs exposed to treated and control plants until at least 7 days of exposure. This may suggest that RNAi response induced by the ingestion of interfering RNAs requires accumulation of siRNA in the insect body. A reduced fecundity and increased mortality was observed in *P. solenopsis* crawlers, which is similar to the findings by other researchers (Khan et al. 2013; Wuriyanghan and Falk 2013). Additionally, physical deformities with fragile cuticle and stunted growth were observed in individuals feeding on the PVX–*PsCHS1* inoculated plants, and the affected mealybugs did not

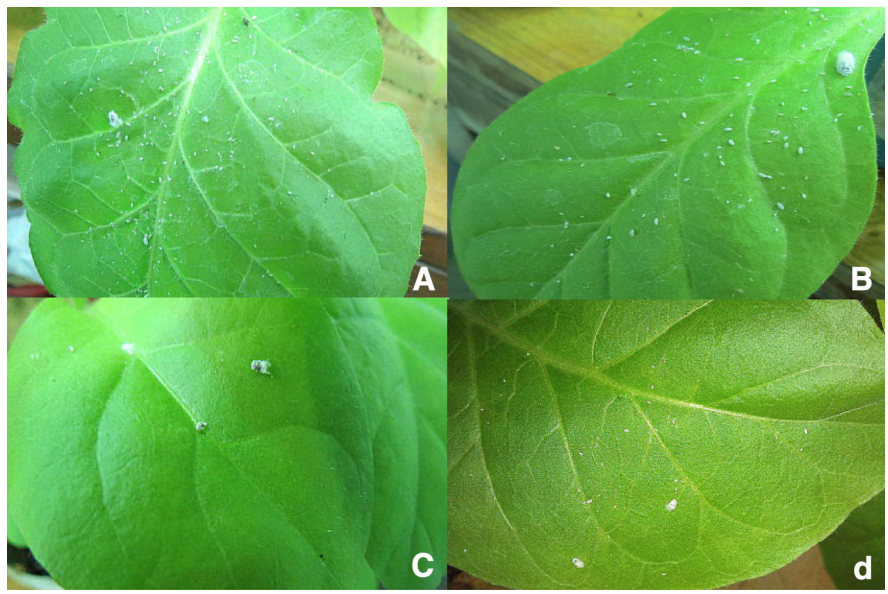

Fig. 4 *Phenacoccus solenopsis* feeding on *N. tabacum* leaves. Uninoculated (−ve control) (**a**); non-recombinant-PVX-inoculated (PVX-control) (**b**), recombinant-PVX–*PsCHS1* (**c** and **d**)

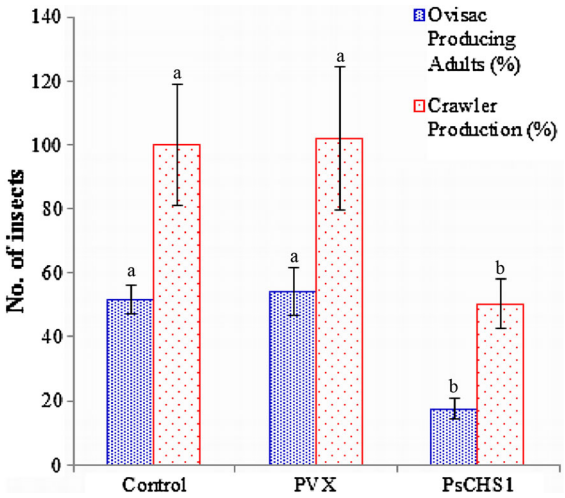

Fig. 5 Number of ovisac-producing *P. solenopsis* adults and emerging crawlers. Data was recorded after 18 days of insect exposure to the inoculated or control plants. *PsCHS1* = inoculated with PVX-*PsCHS1*; PVX = inoculated with non-recombinant PVX (PVX-control); Control = uninoculated. *Bars* with same *letters* across similar *colors*/pattern show homogeneous group at $p < 0.05$

survive to the ovisac producing adults. Similar results have been reported for *T. castaneum* (Arakane et al. 2005) and *Locusta migratoria* (Zhang et al. 2010) where physical abnormalities in test insects due to silencing of *CHS1* showed that this gene was essential for insect survival and development.

The suppression of *PsCHS1* caused mortality in both the emerging nymphs (crawlers) and parent mealybugs with a higher mortality in crawlers than the parent mealybugs which were initially released onto the inoculated plants. These results corroborate previous observations on *P. citri* (Khan et al. 2013) and *B. cockerelli* (Wuriyanghan and Falk 2013). Further, the reduced number of ovisac-producing-females and crawlers on treated plants observed in this study indicates that *CHS1* plays a role in fecundity and reproduction. Prior studies (Khan et al. 2013; Zhang et al. 2010) on *CHS1* suppression have reported similar results for *P. citri* and *L. migratoria*.

We cannot be sure if any other genes were affected (off-target effects) but, since the mRNA levels of *PsCHS1* in mealybug exposed to non-recombinant-PVX vector (against which VIGS might be induced in plants and its triggering RNAs were taken by the insects while feeding on PVX inoculated plants) were similar to those in the negative control, and expression of *β-actin* did not vary among insects from PVX–*PsCHS1*-inoculated and control plants, we suggest that mRNA reduction of *CHS1* in treated mealybug was the result of targeted gene silencing. Infection of RNA viruses in plants produces different forms of viral RNAs including dsRNA (Palukaitis et al. 1983). These dsRNAs activate the plant defense and cause the production of siRNAs (Voinnet 2001). Both dsRNA and siRNA can induce RNAi in insects (Wuriyanghan and Falk 2013). The siRNAs are reported to move through phloem (Yoo et al. 2004) and can be ingested by phloem feeders leading to trigger RNAi in the feeding insects (Wuriyanghan and Falk 2013).

We employed *N. tabacum* as PVX host as this plant has been commonly used in RNAi studies on insects. RNA plant viruses replicate in plant cell cytoplasm via viral dsRNA intermediates and plant respond to these dsRNAs by RNAi thus generating viral siRNAs. Hence plant viruses produce both dsRNA as well as siRNA (Wuriyanghan and Falk 2013). If the recombinant plant virus contains insect gene sequence then insect relevant dsRNA/siRNA will also be produced along with the plant virus dsRNA/siRNA. With an efficient delivery of *CHS1* via PVX into plant host, our study shows that recombinant plant viruses are workable and efficient tools to screen the RNAi targets *in planta* in phytophagous insects as well as in phloem feeders.

Acknowledgments This study was supported by the Higher Education Commission of Pakistan.

Conflict of interest The authors declare no financial or commercial conflict of interest.

Supporting information Supplementary Table 1—Primers and PCR conditions used in the study.

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