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Effect of diet delivered various concentrations of double-stranded RNA in silencing a midgut and a non-midgut gene of *Helicoverpa armigera*

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Abstract

Ribonucleic acid interference (RNAi) is a sequence-specific gene silencing mechanism induced by double-stranded RNA (dsRNA). Recently, RNAi has gained popularity as a reverse genetics tool owing to its tremendous potential in insect pest management, which includes *Helicoverpa armigera*. However, its efficiency is mainly governed by dsRNA concentration, frequency of application, target gene, etc. Therefore, to obtain a robust RNAi response in *H. armigera*, we evaluated various concentrations of dsRNA and its frequency of applications delivered through diet in silencing a midgut gene, chymotrypsin and a non-midgut gene, juvenile hormone acid methyl transferase (*jhamt*) of *H. armigera*. The extent of target gene silencing was determined by employing reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). Our study revealed four significant findings: (i) single application of dsRNA elicited a delayed and transient silencing, while multiple applications resulted in early and persistent silencing of the above genes; (ii) silencing of the non-midgut gene (*jhamt*) through diet delivered dsRNA revealed prevalence of systemic silencing probably due to communication of silencing signals in this pest; (iii) the extent of silencing of chymotrypsin was positively correlated with dsRNA concentration and was negatively correlated with *jhamt*; (iv) interestingly, over-expression (15–18 folds) of an upstream gene, farnesyl diphosphate synthase (*fpps*), in juvenile hormone (JH) biosynthetic pathway at higher concentrations of *jhamt* dsRNA was the plausible reason for lesser silencing of *jhamt*. This study provides an insight into RNAi response of target genes, which is essential for RNAi design and implementation as a pest management strategy.

Keywords: *Helicoverpa armigera*, RNAi, dsRNA, chymotrypsin, *jhamt*, RT-qPCR

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Introduction

Helicoverpa armigera is a serious polyphagous pest, adversely affecting a wide variety of crops such as cotton, tomato, eggplant, potato, chickpea, pigeon pea, chilly, maize, sorghum, groundnut, soybean, sunflower, etc. (Sivakumar *et al*., 2007). Mitigation of this pest has been a challenge over the years and intensive applications of chemical insecticides

has made it more resistant, thereby leading to failure in controlling it (Bourguet *et al.*, 2000). Therefore, there is an urgent need to explore alternatives, where ribonucleic acid interference (RNAi) a sequence-specific gene silencing mechanism holds enormous potential in developing species-specific insecticides (Caplen *et al.*, 2000; Baum *et al.*, 2007; Price & Gatehouse, 2008; Whyard *et al.*, 2009; Mao *et al.*, 2011). To achieve RNAi, the cognate double-stranded RNA (dsRNA) could be delivered through diet, soaking, microinjection and transgenic plants (Turner *et al.*, 2006; Walshe *et al.*, 2009; Huvenne & Smagghe, 2010). However, among these, diet delivered dsRNA is more relevant to the field level pest management, similar to spray or dsRNA expressing transgenics. In this regard, the presence or absence of systemic RNAi deficient-1 and -2 (*sid* − 1 and -2) and RNA-dependent RNA polymerase (RdRP) are important for the uptake and amplification of silencing signal and these components determine the RNAi efficiency in target pest. However, in *H. armigera*, only *sid* − 1 is reported (Collinge *et al.*, 2006). Thus, employing RNAi as a pest management tool demanding the evaluation of factors such as concentration of dsRNA and frequency of its application is of paramount importance as it is poorly understood in insects (Belles, 2010; Terenius *et al.*, 2011; Garbutt *et al.*, 2012).

In this study, we evaluated the effects of various concentrations of dsRNA in single and multiple applications in silencing two important targets: a midgut gene (chymotrypsin) and a non-midgut gene (juvenile hormone acid methyl transferase (*jhamt*)) of *H. armigera*. Chymotrypsin is a proteolytic enzyme, predominantly expressed in the larval midgut (Terra & Ferreira, 1994; Zhan *et al.*, 2011), while *jhamt* is specifically expressed in the neural tissue corpora allata (CA) (Shinoda & Itoyama, 2003; Griebler *et al.*, 2008). *Jhamt* is a key enzyme in the maturation of juvenile hormone (JH), a unique sequi-terpenoid hormone that plays an important role in embryonic development, repression of metamorphosis and vitellogenesis (Zhang *et al.*, 2010). Specifically, *jhamt* catalyses the conversion of farnesoic acid (FA) to methyl farnesoate (MF) by transferring a methyl group from *S*-adenosyl-L-methionine to the carboxyl group of FA, the precursor molecule of JH (Belles *et al.*, 2005). Silencing of chymotrypsin impairs digestion, which often leads to larval mortality (Brackney *et al.*, 2008); whereas, silencing of *jhamt* causes precocious metamorphosis, which was shown in the red flour beetle, *Tribolium castaneum* (Minakuchi *et al.*, 2008). This kindled the interest on *jhamt* silencing in *H. armigera*. Our study provided more information leading to a better understanding of the various target genes response to dsRNA concentration and frequency of its application. This information is having greater implications in designing effective RNAi-mediated management strategies for a number of commercially important pests, including *H. armigera*.

Methods

Insects

Neonates of *H. armigera* were obtained from Bio Control Research Laboratories (BCRL) of Pest Control of India Ltd (PCIL), Bengaluru, India. They were maintained on a chickpea-based semi-synthetic diet (Gupta *et al.*, 2004) at a temperature of 27 ± 2°C, 65 ± 5% relative humidity and 16 h light/8 h dark cycle.

dsRNA synthesis

Earlier, we had cloned the two target genes (chymotrypsin and *jhamt*) from *H. armigera* (Asokan *et al.*, 2012), and used them for *in vitro* dsRNA synthesis. Off target minimised regions of approximately 500 bp were identified for the target genes using online software, *dsCheck*. This compares the target sequences with genome sequences of *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Oryza sativa* and *Rattus Norvegicus* from the database and identifies dissimilar regions (http://dsCheck.RNAi.jp/) (Naito *et al.*, 2005). Further, the sequence of the selected region was analysed for minimizing the possible homology with other gene sequences of *H. armigera* using NCBI-BLAST. The T7 promoter sites were added to the specific primers that are used for synthesizing dsRNA template (table 1). For non-target control, we selected approximately 500 bp of *dreb1A* (dehydration responsive element-binding protein 1), which is a transcription factor present in *Arabidopsis thaliana* (Liu *et al.*, 1998). Template for dsRNA synthesis was synthesized using PCR in a total reaction volume of 50 µl (38 µl of PCR grade water; 5 µl of 10 × *Taq* buffer; 2 µl of 25 mM $MgCl_2$; 1 µl of dNTP mix (10 mM); 1 µl each of forward and reverse primers (10 mM) (table 1); 2 µl 1:50 diluted respective plasmid clones as a template; and 0.8 µl of *Taq* polymerase (5 units μl^{-1}) (Fermentas Life Sciences, USA)). It was amplified by using the following cycling parameters: 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were resolved in 1.2% agarose gel and the desired size bands were excised and extracted using Nucleospin Extract II kit (Macherey-Nagel, Germany). For dsRNA synthesis, 1 µg of above-extracted product was used and *in vitro* transcription was performed using MEGAscript® T7 Kit (Life Technologies, USA) and purified according to the recommended protocol. The dsRNA's concentration was determined by NanoDrop™ 1000 (Thermo scientific, USA) and its integrity was analysed by agarose gel electrophoresis (1.2%).

Administration of dsRNA

Stock dsRNA was diluted with Diethyl pyrocarbonate (DEPC) (0.1%) treated water to yield five concentrations: 10, 20, 40, 60 and 80 µg/40 µl. For insect bioassay, chickpea-based semi-synthetic diet was prepared (Gupta *et al.*, 2004) with DEPC-treated water and 650 µl of diet was dispensed into each well of the bioassay plate (Bio-Assay Tray – 128 cells, White, High Intensity Poly Styrene, Bioserv), which was then cooled to the room temperature. In the single application method, 40 µl of each concentration of dsRNA (10, 20, 40, 60 and 80 µg/well) was separately applied on the surface of the semi-synthetic diet and allowed to completely percolate. In multiple applications, the above dsRNA concentrations were separately applied three times at consecutive time intervals (1st, 3rd and 5th day) on to a fresh semi-synthetic diet; concurrently, larvae were also shifted and the same was followed for non-target (*dreb1A*) dsRNA control. In single application, the larvae were retained on the same well until the end of the experiment and same was also followed for non-target (*dreb1A*) dsRNA control. The total duration of experimentation was 6 days. An untreated control containing only (0.1%) DEPC-treated water was also maintained. Each dsRNA concentration had eight replications (four larvae per replication).

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Table 1. List of primers used in this study.

Purpose	Gene	GenBank	F-primer (5′→3′)	R-primer (5′→3′)	Product Size (bp)
dsRNA synthesis	Chymotrypsin	GU323796.1	*TAATACGACTCACTATAGGGCTCGG* ACAGATCCCTTACCAG	*TAATACGACTCACTATAGGGG* AAATGTTGGAGTCCTGGATCAG	503
	Jhamt	GU323798.1	*TAATACGACTCACTATAGGGTGACT* AACATGCTGAAGAAATAT	*TAATACGACTCACTATAGGGCGCTTTT* CGCAAAATATGTAT	460
	18s rRNA (18S)	AJ577253.1	CACCACTAGAGGACACAGA	AACACATAACGACGGACGAG	95
Real Time PCR (qRT-PCR)	Chymotrypsin	GU323796.1	CACTCTTGATCGGTATCACCTC	TGATGTAAGACGTGACTCTGG	91
	Jhamt	GU323798.1	AGTATAAGTTGCTTGGCTGTG	CCTTCATGCCTTCAGGTAGATC	127
	Farnesyl diphospahte syntase (*fpps*)	–	CTTGCTTCCCGGACATCG	ACAGCTTAGCCAACCACTTG	82
Semi-quantitative PCR	Chymotrypsin	GU323796.1	CGTCAACCTGCCAGTGTT	AGAGTGGTCTGCTGTTCCT	158
	Jhamt	GU323798.1	ATTAGCGAGAAGATGGTGAACT	GCAGAGCGTAGAACGAGAATA	139
	18s rRNA	AJ577253.1	GCGTTGCTGGGAAGTTGA	GTGTACCCTTCTGCGTGTC	287

Note: Italicized nucleotides are the T7 RNA polymerase promoter sequence.

RT-qPCR

In both single and multiple application approaches, the target genes silencing was assessed at daily intervals for a period of 5 days from second day to sixth day of dsRNA application. From the four larvae of *H. armigera*, the total RNA was extracted using ISOLATE RNA Mini Kit (Bioline Reagents Ltd, UK) according to the manufacturer's instructions. DNA contamination was removed by treating the extracted RNA with RNase-free DNase I (Fermentas Life Sciences, USA) and purified by phenol–chloroform (25:25) extraction (Sambrook & Russell, 2001). The concentration and quality of the RNA samples were determined by NanoDrop™ 1000 (Thermo Scientific, USA). First-strand cDNA was synthesised in 20 µl reaction volume comprising 0.3 µl (60 units) reverse transcriptase, 1 µl of oligo-dT (10 mM) primer and 1 µg total RNA; reverse transcription was performed according to the manufacturer's instructions (Fermentas Life Sciences, USA). Subsequently, the 20 µl of cDNA was diluted ten-fold and 5 µl of diluted cDNA was used for PCR amplification employing gene-specific primers (table 1).

All the real-time PCR assays were designed according to the MIQE guidelines (Bustin *et al*., 2009) and the primers used for detection of silencing were designed according to the Herbert *et al*. (2011) guidelines. 18S rRNA was used as an internal reference gene for normalisation, which was found to be a suitable reference gene in a previous study (unpublished data). RT-qPCR was used to quantify transcript levels of three genes: chymotrypsin, *jhamt* and farnesyl diphosphate synthase (*fpps*). PCR amplification efficiency was determined employing five cDNA concentrations: 25, 2.5, 0.25, 0.025 and 0.0025 ng. RT-qPCR was performed in a total reaction volume of 20 µl comprising 10 µl of SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, USA), 0.5 µl (10 mM) each of forward and reverse primers (table 1) and 5 µl of cDNA template with the following parameters: 94°C for 4 min followed by 40 cycles at 94°C for 15 s, and 60°C for 1 min for annealing and extension, which was performed using LightCycler 480II (Roche Applied Science, Switzerland). All RT-qPCR assays were carried out in triplicates and no template controls were included in each assay. PCR amplification efficiency curves were generated by plotting five cDNA dilutions against C_q (quantification cycle) values. The relative gene expression data were analysed using the $2^{-\Delta\Delta C}$T method (Livak & Schmittgen, 2001) and the normalised values were expressed as percent silencing compared with the untreated sample.

Statistical analysis

The effect of silencing was statistically analysed by using *t* test with C_q values of three replicates using GraphPad Prism v.5 (GraphPad Software, Inc., USA) at $P<0.05$. Pearson's correlation (r) and coefficient of correlation (r^2) values were calculated for dsRNA concentrations and percent gene silencing at 95% confidence intervals (GraphPad Prism v.5).

Results

In this study, we evaluated the effect of five concentrations of dsRNA applied in single and multiple applications in silencing two target genes, chymotrypsin and *jhamt*. Our results, based on observed C_q values revealed that the expression levels of the two target genes differed significantly,

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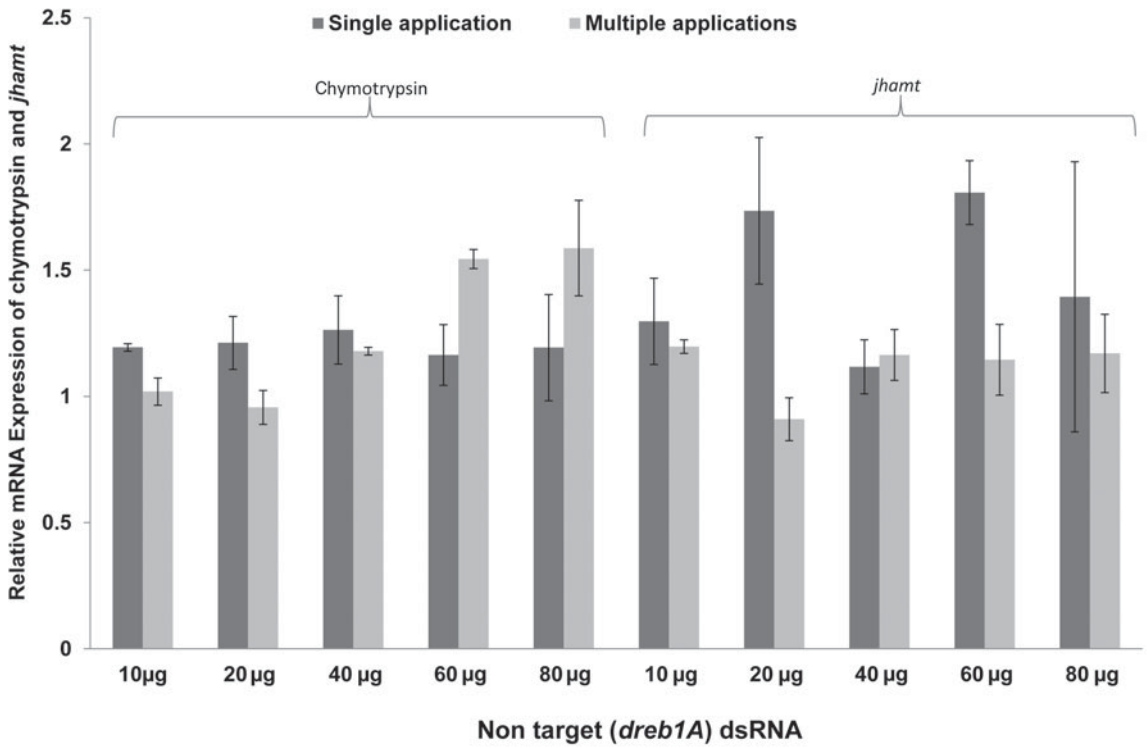

Fig. 1. Effect of unrelated non-target dsRNA on target gene expression. *Y*-axis represents relative expression of chymotrypsin and *jhamt* genes in larvae fed with *dreb1A* dsRNA (mean of five dsRNA concentrations) and control larvae. Values are averages of three independent experiments; error bars indicate SEM of the triplicate experiments.

where the expression of chymotrypsin was 30-fold higher than that of *jhamt*.

Determination of PCR efficiency and specificity

The PCR amplification efficiency of 1.902 (95.1%) for 18S rRNA, 1.924 (96.2%) for chymotrypsin and 1.888 (94%) for *jhamt*, was observed. The melt curve analysis of amplicons exhibited a single melt peak, indicating single amplicon. Further, resolving of RT-qPCR products on agarose gel confirmed the presence of single band of target and reference genes in the respective samples and no band was detected in the no-template control. The sequence obtained from sequencing of RT-qPCR products reconfirmed the specificity of amplicons.

Assessing target gene silencing

The two target genes exhibited significantly different levels of silencing. An unrelated, non-insect gene, *dreb1A* dsRNA application did not alter the target gene expression significantly as compared with untreated control (fig. 1).

Silencing of chymotrypsin

In single application, silencing was observed only on the 5th day; highest silencing of 98% was observed in 80 µg (fig. 2). A strong correlation ($r = 0.91$) was observed between the silencing effect and dsRNA concentration (table 2). No silencing was observed in the five concentrations on 2nd, 3rd, 4th and 6th day post treatment. The lower dsRNA concentrations (10, 20 and 40 µg) did not yield silencing in any of the five days of observation. In multiple applications, silencing was observed from 4th day onwards; maximum silencing (72%) was observed with 80 µg concentration (fig. 2). Similar trend of increased silencing effect of higher dsRNA concentration was evident on 5th and 6th days and a strong a correlation ($r = 0.97$) was observed (table 2). Throughout the experimental period, the lower dsRNA concentrations of 10 and 20 µg did not result in target gene silencing.

Silencing of jhamt

In single application, highest (43%) silencing was observed in lowest dsRNA (10 µg) concentration on 4th and 5th day (fig. 3). The silencing effect was negatively correlated ($r = -0.98$) with dsRNA concentration (table 2). In multiple applications, silencing was observed from 4th day onwards where highest silencing of 78% was recorded in 10 µg dsRNA. While on 5th and 6th day highest silencing was observed in 20 µg dsRNA (fig. 3). Overall, in *jhamt* the silencing and dsRNA concentrations were negatively correlated (table 2), lower dsRNA concentrations of 10 and 20 µg elicited higher silencing.

Over expression of fpps

The expression of an upstream gene *fpps* in the JH biosynthetic pathway was assessed. It revealed 5.199, 15.49

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Table 2. Correlation coefficient, '*r*' and coefficient of determination, r^2 values of two target genes, viz. chymotrypsin and *jhamt* in single and multiple dsRNA applications.

Target gene	dsRNA application	Pearson correlation coefficient '*r*'	Coefficient of determination 'r^2'	95% confidence interval	*P* value summary
Chymotrypsin	Single	0.9141	0.8356	0.1641 to 0.9944	*
	Multiple	0.9764	0.9533	0.6788 to 0.9985	**
Jhamt	Single	−0.9825	0.9404	−0.9989 to −0.7529	**
	Multiple	−0.9654	0.8843	−0.9962 to −0.3410	*

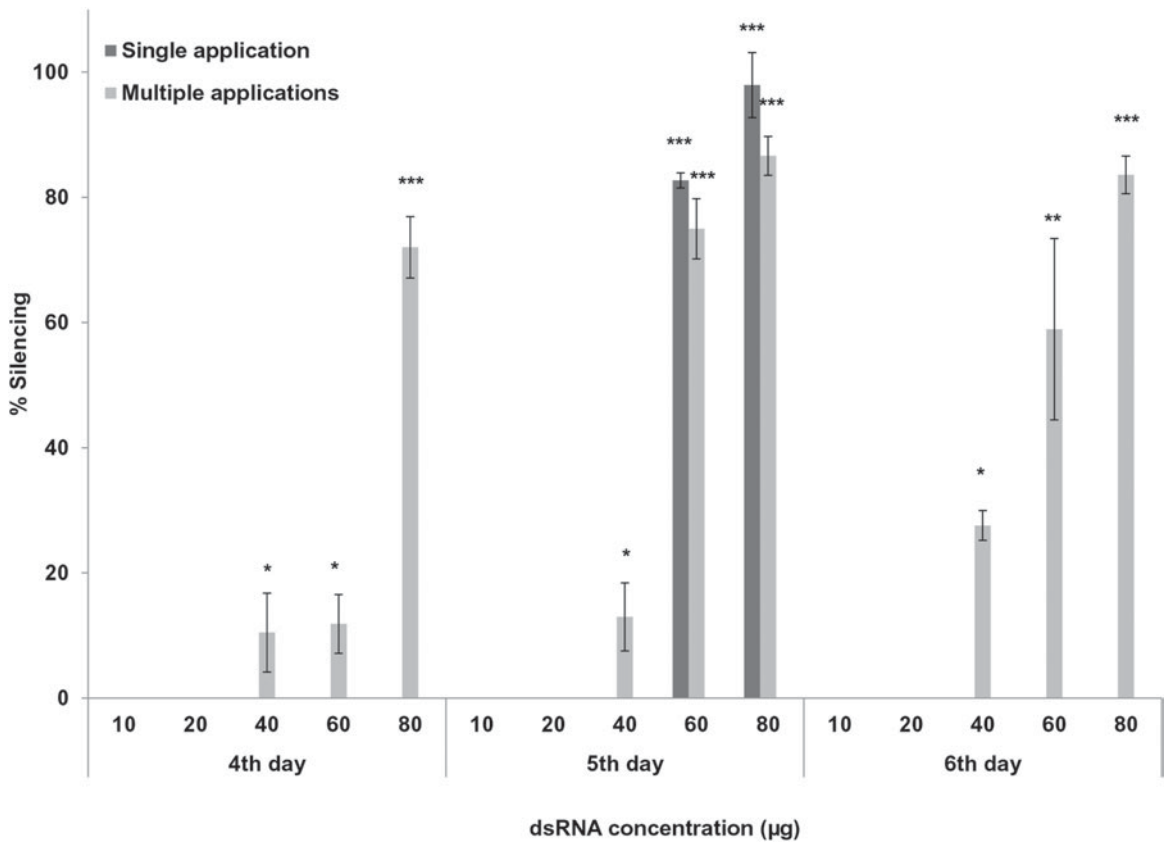

Fig. 2. Percent of silencing after chymotrypsin dsRNA application. Expression was monitored for 5 days; samples of each group were collected on day 2–6. Values are averages of three independent experiments; Error bars indicate SEM of the triplicate experiments. *P, 0.05, **P, 0.01, ***P, 0.005; t-test.

and 17.92 folds over-expression in 40, 60 and 80 µg *jhamt*-dsRNA treated samples, respectively. The expression of *fpps* in chymotrypsin and non-target *dreb1A* treated samples was on par with untreated control (fig. 4).

Discussion

Currently, there is lack of information on the response of target genes to different concentrations and frequency of application of diet delivered dsRNA in insects (Terenius *et al.*, 2011). In addition to the above, absence of RdRP in insects as against *C. elegans* and tick (Gordon & Waterhouse, 2007), it would be interesting to compare the silencing of midgut-expressed gene and non-midgut gene. In this study, we observed that the two target genes (chymotrypsin and *jhamt* of *H. armigera*) whose expression was markedly different, responded variably to the dsRNA concentrations and frequency of application.

Diet delivered dsRNA successfully induced silencing of both chymotrypsin and *jhamt* genes and silencing was specific as non-target dsRNA did not alter the transcript levels of target genes. Similarly, feeding of dsRNA through artificial diet induced target gene silencing in western corn rootworm, *Diabrotica virgifera virgifera* (Baum *et al.*, 2007). Evaluation of target gene silencing through diet-mediated delivery of dsRNA is relevant to the field level pest management either by spray or transgenic crop. Silencing of *jhamt* revealed the prevalence of systemic silencing in *H. armigera*. This study is significant as this is the first report in *H. armigera* to show that the primary silencing signals might have been communicated from midgut to the CA, which led to silencing of *jhamt*. Similarly, in light brown apple moth (*Epiphyas postvittana*),

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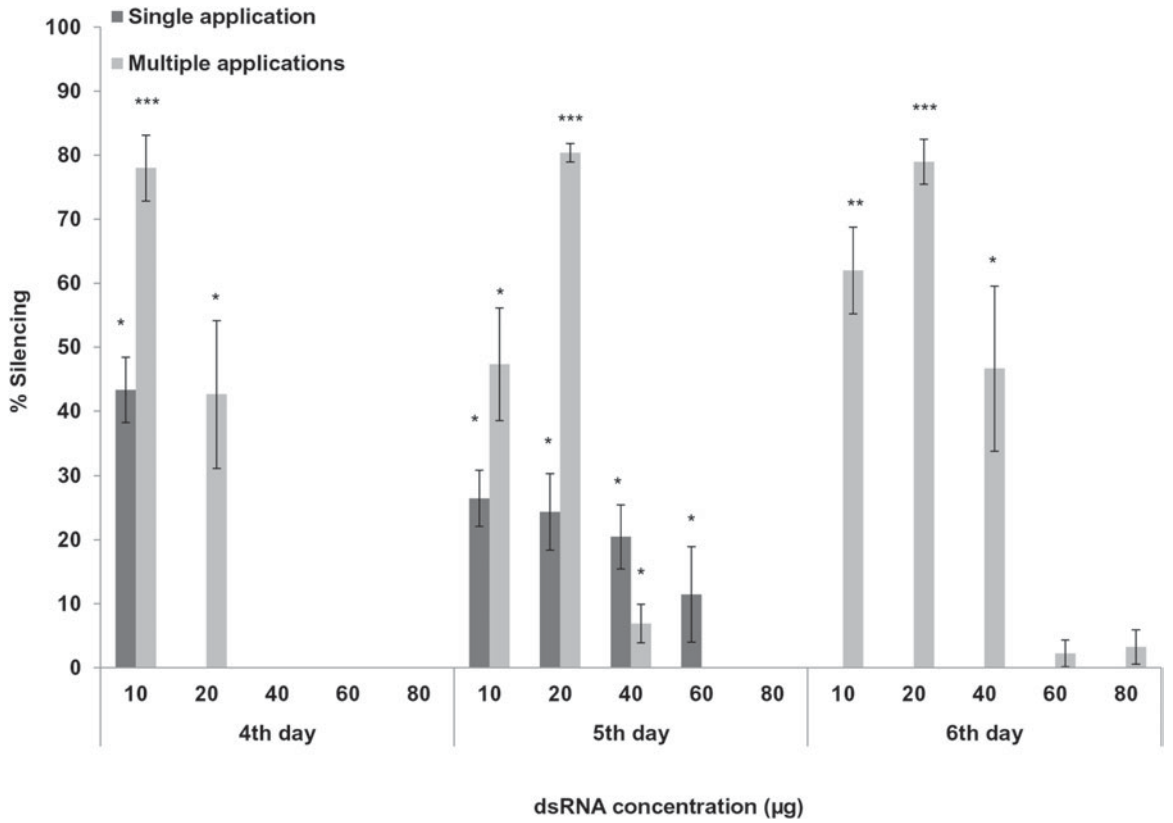

Fig. 3. Percent of silencing after *jhamt* dsRNA application. Expression was monitored for five days; samples of each group were collected on day 2–6. Values are averages of three independent experiments; Error bars indicate SEM of the triplicate experiments. **P*, 0.05, ***P*, 0.01, ****P*, 0.005; *t* test.

oral delivery of dsRNA caused silencing of the pheromone-binding protein gene (*EposPBP1*) expressed in antenna and silencing persisted for 2 days after pupal formation, revealing systemic silencing (Turner *et al*., 2006). Similarly, in *T. castaneum*, the components involved in systemic silencing were identified and the authors showed the occurrence of systemic silencing in this insect (Tomoyasu *et al*., 2008). Thus, the systemic silencing of non-midgut gene (*jhamt*) observed here may kindle interest in the selection of non-midgut genes as targets for effective management of this pest.

With regard to the effect of various concentrations of dsRNA on target gene expression, there was a marked difference in the silencing pattern between chymotrypsin and *jhamt*, which indicated that the extent of silencing was influenced by the quantity of dsRNA, type of target gene and tissue where it is expressed. In the case of chymotrypsin, 60 and 80 µg concentrations of dsRNA resulted in significantly higher silencing compared to 40 µg. This indicated that the extent of silencing was directly proportional to the dsRNA concentration and significant correlations of 0.91 and 0.98 were observed between the chymotrypsin dsRNA concentration and percent silencing in both single and multiple applications, respectively (table 2). Contrastingly, silencing of *jhamt* revealed that the extent of silencing was inversely proportional to the dsRNA concentration. Lower concentrations of 10 or 20 µg dsRNA induced a significantly high extent of silencing compared with 60 and 80 µg; and significant negative correlations of −0.98 and −0.94 were observed between the dsRNA concentration and percent of silencing in both single and multiple applications, respectively. Similarly, in fall armyworm (*Spodoptera frugiperda*), injection of 2 µg dsRNA for silencing neural tissue-specific allatostatin caused 60% mortality, but 3 µg caused only 21.3% mortality of the treated larvae (Griebler *et al*., 2008). These results indicated that higher dsRNA concentrations do not always result in increased silencing. Thus, the effect of dsRNA concentration in silencing was highly variable and target gene dependent. Silencing of chymotrypsin required relatively higher concentrations of dsRNA, perhaps due to higher transcript level as compared with that of *jhamt*.

However, in the current experiment, we have not observed any phenotypic effects on the treated larvae while silencing of chymotrypsin and *jhamt*. On the other hand, in another experiment, continuous (till end of larval stage) administration of cognate dsRNAs through a semi-synthetic diet, in silencing glutathione-*S*-transferase, cytochrome P450, trypsin, chymotrypsin and *jhamt*, revealed that prolonged dsRNA treatment had pronounced effect on larval and pupal weight and pupation (under review), which necessitate the requirement of time lag to observe RNAi-induced phenotypic changes.

With regard to frequency of dsRNA application, single application of all the concentrations for both target genes, resulted in silencing only on the 5th day after application (except for 10 µg on the 4th day for *jhamt*). Similarly, single application of dsRNA through thoracic injection resulted in

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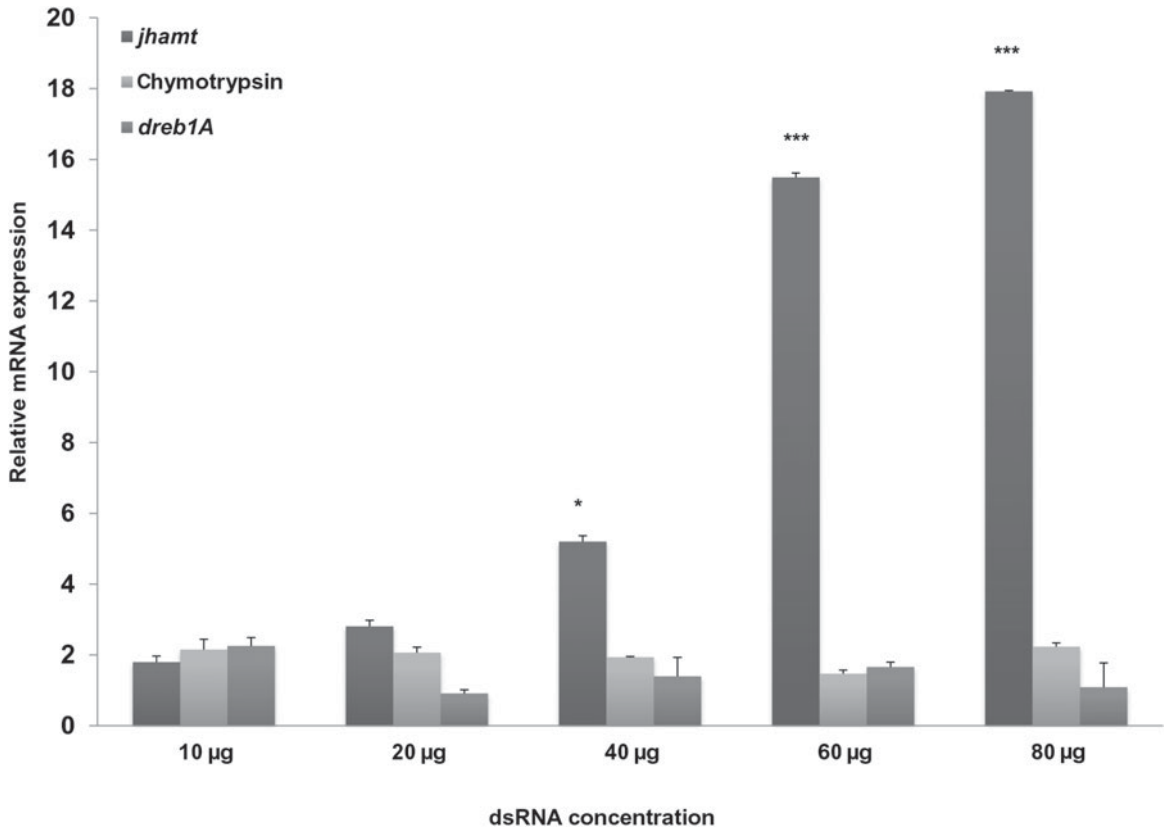

Fig. 4. Expression analysis of *fpps*. Expression of *fpps* at various concentrations of *jhamt* and non-target (*dreb1A* and chymotrypsin) dsRNA treated samples. Error bars indicate SEM of the triplicate experiments. *P, 0.05, ***P, 0.005; t test.

40–50% of calreticulin and cathepsin-B gene silencing on the 4th day after treatment in the brown plant hopper (*Nilaparvata lugens*); further, the extent of silencing declined in subsequent days and it was nil on 8th day post treatment (Liu *et al*., 2010). In another study, single injection of dsRNA in Fall Armyworm (*Spodoptera frugiperda*) resulted in 55.2% silencing of allatotropin gene on the 4th day post treatment, which later dropped drastically (Griebler *et al*., 2008). These results indicated that, irrespective of the mode of dsRNA delivery or the target genes, single application failed to induce sustained silencing. In contrast, multiple applications induced an earlier onset and sustained silencing from 4th day to till the end of the experiment of both target genes. Similar trend was observed when two periodic injections of dsRNA enhanced the extent of *N1β2* gene silencing in *N. lugens* as compared with single injection (Liu *et al*., 2010). Our study showed that multiple applications are most effective in silencing and should be considered while designing RNAi-mediated pest management strategy.

As observed above, lesser silencing of *jhamt* at higher concentrations could be due to the following reasons: (i) suppression of gene silencing by various factors; (ii) target gene over-expression; and (iii) over-expression of an upstream gene. With regard to the first hypothesis, administration of higher siRNA concentrations (40, 60 and 80 µg) resulted in an enhanced expression of ADAR-1 and ERI-1 proteins, which specifically bound to siRNAs that ultimately reduced RNAi efficiency in mouse (Hong *et al*., 2005). With regard to the second hypothesis, over-expression of target gene, where in the case of tephritid fruit fly (*Bactrocera dorsalis*) continuous dsRNA application resulted in over-expression of the target (fatty acid elongase-*noa*) gene (Li *et al*., 2011). Similarly, in Oak Tussah moth, *Antheraea pernyi*, silencing of haemolin gene through RNAi triggered the over-expression of target gene (Hirai *et al*., 2004). Similarly, in the case of humans, transfection of siRNA resulted in activation of Jak-STAT pathway and up-regulated the IFN-stimulatory genes (Sledz *et al*., 2003). In mammals, during virus attack (dsRNA as a genetic material) specific dsRNA-dependent protein kinase triggered the inflammatory response (by enhancing immune-related genes expression) (Nakamura *et al*., 2010).

With regard to the third hypothesis, understanding the responses of the biological system to RNAi is a major challenge. In the current experiment, to elucidate possibility of less effectiveness of higher concentrations of dsRNA in silencing *jhamt*, the expression of *fpps* (an upstream gene in the JH biosynthetic pathway) was assessed. We found that the higher concentrations of dsRNA: 40, 60 and 80 µg led to a significantly over-expression of *fpps* by 5.199, 15.49 and 17.92 folds, respectively, compared with control. The lower concentrations (10 and 20 µg) did not induce *fpps* over-expression. We also confirmed that over-expression of *fpps* was specific to *jhamt* and it was not observed in the non-target *dreb1A* and chymotrypsin dsRNA-treated samples. Maintaining JH titre is crucial for the growth and development of larvae and it is probably maintained by the over-expression of the

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upstream gene(s) (e.g., *fpps*, which might have compensated the silencing at higher dsRNA concentrations). Thus, further studies are necessary to elucidate this mechanism. The observed over-expression may be a compensatory mechanism to overcome the effect of gene silencing (over-expression of upstream genes) in response to higher dsRNA concentrations.

Conclusions

Our studies showed that diet delivered dsRNA effectively silenced both the midgut and non-midgut genes, chymotrypsin and *jhamt*, respectively. Silencing of *jhamt* revealed the prevalence of systemic silencing in *H. armigera*, possibly by transport of silencing signals from the midgut to the non-midgut tissue, CA. Single application of dsRNA resulted in delayed and transient silencing, while multiple applications led to an early onset and sustained silencing of both chymotrypsin and *jhamt*. Silencing of chymotrypsin was positively correlated with dsRNA concentration, while silencing of *jhamt* was negatively correlated. Incidentally, in silencing *jhamt*, higher dsRNA concentrations led to the over-expression of an upstream gene, *fpps*. The results showed the differential response of target genes in relation to the dsRNA concentration and frequency of application. These findings help in the proper selection of suitable genes, dsRNA concentration and frequency of application for achieving effective RNAi-mediated insect management.

Conflict of Interest

The authors declare that there is no conflicting interest.

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