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Double-Stranded RNA-Mediated Suppression of Trypsin-Like Serine Protease (t-SP) Triggers Over-Expression of Another t-SP Isoform in *Helicoverpa armigera*

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Abstract High diversity of digestive proteases is considered to be the key factor in the evolution of polyphagy in *Helicoverpa armigera*. Serine proteases (SPs) contribute ~85% of the dietary protein digestion in *H. armigera*. We investigated the dynamics of SP regulation in the polyphagous pest, *H. armigera* using RNA interference (RNAi). HaTry1, an isoform of SP, expressed irrespective of the composition of the diet, and its expression levels were directly proportional to the larval growth rate. Therefore, HaTry1 was silenced by delivering 10 and 20 μg concentrations of double-stranded RNA through semi-synthetic diet. This led to a drastic reduction in the target gene transcript levels that manifested in a significant reduction in the larval weight initially, but the larvae recovered in later stages despite continuous dsRNA treatment. This was probably due to the compensatory effect by over-expression of HaTry13 (31-folds), another isoform of SP. Phylogenetic analysis of *H. armigera* SPs revealed that the over-expressed isoform was closely related to the target gene as compared to the other tested

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isoforms. Further, silencing of both the isoforms (HaTry1 and HaTry13) caused the highest reduction in the larval weight and there was no larval growth recovery. These findings provide a new evidence of the existence of compensatory effect to overcome the effect of silencing individual gene with RNAi. Hence, the study emphasizes the need for simultaneous silencing of multiple isoforms.

Keywords RNA interference · *Helicoverpa armigera* · Serine protease · Double-stranded RNA · Insect bioassay · RT-qPCR

Introduction

Cotton bollworm, *Helicoverpa armigera* (Hub.) is a polyphagous pest, causing severe yield loss in many important crops such as chickpea, pigeon pea, okra, tomato, tobacco, cotton, and brinjal [1]. Continuous availability of host plants has resulted in heavy population buildup of this pest. As a result, it is difficult to control *H. armigera* due to its development of resistance against the commonly used insecticides [2]. Because of the difficulties in controlling this pest with commonly used insecticides, transgenic crops expressing *Bacillus thuringiensis* (Bt) Cry proteins have been deployed on a large scale for controlling this pest [3]. However, a large-scale deployment of transgenic crops might have led to development of insect resistance to Bt Cry proteins [4, 5]. Therefore, there is a need to develop transgenic crops employing alternate methods of genetic transformation of their host plants for sustainable crop production.

The dynamic regulation and the high diversity of digestive enzymes is one of the key factors involved in the evolution of polyphagy in *H. armigera* [6]. In addition to their predominant role in the digestion of plant proteins, digestive enzymes are also involved in enzyme activation and metamorphosis [7]. Therefore, it is necessary to characterize these digestive proteases for understanding the dynamics of polyphagy, which would further help in designing effective pest management strategies. The predominant digestive proteases in *H. armigera* are serine proteases (SP), namely, trypsin, chymotrypsin, and elastase [8]. There are at least 21 genes encoding trypsin-like SPs, 14 encoding chymotrypsin-like SPs, and 2 encoding elastase-like SPs in *H. armigera* [6]. The majority of these proteases belong to trypsin-like SPs [6]. An analysis of the gut proteases of 12 lepidopteran insect species indicated that trypsin-like SPs play a significant role in plant protein digestion [9]. Therefore, researchers have targeted trypsin-like SPs to block the digestive activities in *H. armigera* [10]. However, in some insects, transcriptional plasticity overcomes the loss of activity caused by the blocking of certain digestive proteases [11, 12].

RNA interference (RNAi) has provided a new avenue for functional annotation of newly identified genes by the loss of function [13, 14]. Previous studies have shown the potential of RNAi in functional genomics and insect pest management through the delivery of long double-stranded RNA (dsRNA) or small interfering RNA (siRNA) by microinjection or droplet feeding, or through the insect diet [15–17]. Among the different dsRNA delivery methods, the diet-mediated delivery is the simplest approach that akin to the field level pest management.

In the present study, the expression profiling of various genes encoding different isoforms of trypsin-like SPs was carried in different diets to identify the predominant isoform for silencing. Subsequently, we silenced predominantly expressed isoform using diet-delivered dsRNA and assessed the effect of dsRNA on target gene transcript levels and insect growth and development. Cognate dsRNA treatment manifested in the reduction of larval weight;

however, the larvae recovered from the weight loss despite the continuous administration of dsRNA. Therefore, to understand the possible factors involved in the larval growth recovery, we tested transcriptional plasticity of different isoforms of SPs by assessing the expression of some of the trypsin and chymotrypsin-like SPs of *H. armigera* in the dsRNA fed larvae. Further, simultaneous silencing of multiple isoforms was performed to overcome the compensatory effect.

Materials and Methods

Insect Culture

Neonate larvae of *H. armigera* were obtained from the Pest Control of India (PCI), Bangalore, India. The larvae were reared on chickpea-based semi-synthetic diet, which was prepared according to the composition recommended by Abbasi et al. [18]. The insect culture was maintained at 27 ± 2 °C temperature, 65 ± 5% relative humidity, and 16:8 h of light and dark cycle.

Expression Analysis of Target Gene

To assess the expression of different trypsin-like SPs in *H. armigera*, the neonate larvae fed on different diets: (i) semi-synthetic diet, (ii) cotton, and (iii) tomato leaves. The larvae were allowed to feed on the diets for 1 week (10 larvae for each treatment), and then, the total RNA was extracted. The expression of different isoforms of trypsin (HaTry1, HaTry5, HaTry8, HaTry9, HaTry13, HaTry17, HaTry19, and HaTry23) was analyzed using the above extracted total RNA. Further, the selected HaTry1 expression profiling across the developmental stages of the insect was carried out using the total RNA extracted from first-, third-, sixth-, ninth-, and 13th-day-old larva, which fed on chickpea-based semi-synthetic diet; and 3-day-old pupa; and adult moth. For estimating the extent of gene silencing, the total RNA was extracted on the fourth and seventh day of continuous dsRNA treatment using the ISOLATE II RNA Mini Kit (Bioline Reagents Ltd., UK) by following the manufacturer's instructions. Additionally, the expression of some selected isoforms of SPs (HaTry5, HaTry6, HaTry8, HaTry9, HaTry10, HaTry13, HaTry16, HaTry17, HaTry19, HaTry22, HaTry23, HaChy1, HaChy5, HaChy8, HaChy9, and Ha_pSP1) were analyzed using the above extracted total RNA. The first strand of complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the Tetro cDNA Synthesis Kit (Bioline Reagents Ltd., UK) by following the manufacturer's protocol.

Real-time quantitative PCR (RT-qPCR) assays were designed according to the Minimum Information for Publication of Quantitative RT-PCR Experiments (MIQE) guidelines [19] and performed using LightCycler 480II (Roche Applied Science, Switzerland). Primers for RT-qPCR assays were designed using the Beacon Designer 7 software program (Premier Biosoft International). To minimize the sampling errors, 18S ribosomal RNA (18S rRNA) and β-tubulin housekeeping genes were used for the normalization of RT-qPCR data of dsRNA treatment and across the developmental stages of *H. armigera*, respectively. In a previous study, 18S rRNA and β-tubulin genes exhibited least expression variations in dsRNA treatment and across the developmental stages of *H. armigera*, respectively [20]. The suitability of primer pairs for RT-qPCR was validated by calculating the PCR amplification efficiency of reference (18S rRNA) and target genes (HaTry1 and HaTry13). This assay was performed

using four (5-fold) serial dilutions, approximately 25, 5, 1, and 0.2 ng cDNA. The RT-qPCR was performed in a total reaction volume of 20 μl comprising 10 μl SYBR® Green JumpStart™ Taq ReadyMix™, 1 μl (10 μM each) of forward and reverse primer mix, and 5 μl of 1:10 diluted cDNA by following the manufacturer's instructions (Sigma-Aldrich, USA). The assays were carried out with four biological replicates of two independent experiments (two technical replicates for each biological replicate), and no template controls were also included. Primer amplification efficiency curves for reference and target genes were generated by plotting four cDNA dilutions against C_q (quantification cycle) values. The relative expression of the different trypsin-like SPs on different diets was calculated using $2^{-\Delta CT}$ method [21], while, $2^{-\Delta\Delta CT}$ method [22] was used for calculating the relative expression of the different isoforms of SPs across the developmental stages of *H. armigera* and under dsRNA treatment. Gene expression variations were statistically analyzed using *t* test with normalized values of four biological replicates (two technical replicates for each biological replicate) at $p < 0.05$.

Synthesis of Double-Stranded RNA

The region for synthesis of dsRNA was identified using the online software, *dsCheck* (http://dsCheck.RNAi), which helps in minimizing off-target effects [23]. These sequences were further scrutinized for minimizing the possible homology with other genes of *H. armigera* by the National Center for Biotechnology Information (NCBI)-BLAST analysis. For the synthesis of dsRNA template, specific primers having T7 promoter sequence at 5′ ends were synthesized (Table S1). The previously cloned recombinant plasmids harboring individual HaTry1 and HaTry13 genes of *H. armigera* [24] were used for in vitro dsRNA synthesis. Additionally, to silence both the genes, pyramided dsRNA of the both genes was used. To make a pyramided dsRNA, the selected region of HaTry1 and HaTry13 were PCR amplified and digested using flanking restriction enzymes then these fragments were sequentially cloned into pTZ57R/T vector (Thermo Scientific, India). Finally, the recombinant plasmid harboring pyramided sequences of HaTry1 and HaTry13 was used for the synthesis of template of dsRNA.

The study used 411, 474, and ~ 500 bp region of individual HaTry1, HaTry13, and pyramided HaTry1 and HaTry13 genes, respectively, for cognate dsRNA synthesis; and 423 bp of *dreb1A* gene (dehydration responsive element binding protein 1—involved in the regulation of drought stress response in plants) for non-target dsRNA control [25]. Previously, *dreb1A* was isolated from *Arabidopsis thaliana* (GenBank accession no.: DQ018385), and it has been used as a non-target control in the previous RNAi experiments in *H. armigera* [26, 27]. The template for dsRNA synthesis was amplified with suitable primers (Table S1) from respective recombinant clones, and dsRNA was prepared as described earlier [26].

Administration of dsRNA to Test Insect

Insect bioassay was performed in two sets of experiments. In the first set of experiment, the target (HaTry1) gene was silenced using two concentrations of cognate dsRNA and assessed its effect on the target and also on other isoforms transcript levels and larval growth and development. In the second set of experiment, the target (HaTry1) and over-expressed (HaTry13) isoforms were silenced individually and together using individual and pyramided dsRNA of HaTry1 and HaTry13 genes, respectively. For insect bioassay, chickpea-based semi-synthetic diet was prepared with diethylpyrocarbonate (DEPC) treated water, and 1 ml of diet

was dispensed into each cell well of the bioassay plate (Bio-Assay Tray—128 cells, White, High Intensity Poly Styrene, Bioserv). Diluted individual dsRNA of HaTry1, HaTry13, and pyramided dsRNA of HaTry1 and HaTry13 and *dreb1A* at 10 and 20 μg/100 μl per well were applied on the media surface and allowed to percolate. A single neonate larva was released per well, and there were 30 replicates for each treatment, as well as for controls (water and non-target *dreb1A* dsRNA). To ensure continuous availability of dsRNA on the media, the larvae were shifted to a fresh diet containing fresh dsRNA on every alternative day until pupation. Simultaneously, two independent experiments were performed. To investigate the effect of dsRNA on the insect, the larval weight was recorded on the first, third, sixth, and ninth day of dsRNA treatment, while the pupal weight was recorded on the fifth day of pupation. The differences in the weights of larvae and pupae among the treatments and control were statistically analyzed with ANOVA at $p < 0.05$ (GraphPad Prism v.5—GraphPad Software, Inc., USA).

Phylogenetic Analysis of SP Isoforms of *H. armigera*

Coding sequences (CDS) of 38 deposits of *H. armigera* trypsin, chymotrypsin, and putative SPs were retrieved from NCBI. These were translated into amino acid sequences by employing the ExPASy-Translate tool (http://web.expasy.org/translate/). Further, amino acid sequences were aligned using the ClustalW2 online program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic tree of SP was constructed using the MEGA v6.06 software with a neighbor-joining method using 1000 bootstrap replicates [28, 29].

Extraction of Midgut Proteases from *H. armigera* Larvae

To assess the effect of silencing, midgut proteases were extracted by dissecting midgut of *H. armigera* larvae on the fourth and seventh day of continuous dsRNA treatment and control by following the method described by Parde et al. [30]. Briefly, the larvae were starved for 4 h and then immobilized by placing at − 20 °C for 30 min. A total of 10 larval midguts were dissected out from each treatment and control larvae and homogenized separately in 0.1 M glycine-NaOH buffer (pH 10). The homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were transferred to fresh tube and used as a source of protease enzyme and these were stored at − 20 °C until use. The concentration of the total proteins in the gut extract was estimated by Lowry's method [31].

Assay of Midgut Proteases

Total protease activity in the gut extract of dsRNA treated and control larvae was determined using azocasein as a substrate [32]. The midgut enzyme extract was mixed with azocasein and incubated for 30 min at room temperature. The reaction was terminated by adding 300 μl of 5% TCA (trichloroacetic acid) and centrifuged. To the supernatant, an equal volume of 1 N NaOH was added and absorbance was recorded at 410 nm using UV-visible spectrophotometer (Hitachi U-2900, Japan). Total protease activity units (UA) was calculated similarly as described by Visweshwar et al. [33].

Trypsin, chymotrypsin, elastase, and aminopeptidase activities were measured using N-α-benzoyl-DL-arginyl-*p*-nitroanilide (BApNA) [34], *N*-succinyl-alanine-alanine-*p*-nitroanilide [35], *N*-succinyl-alanine-alanine-alanine-*p*-nitroanilide [36], and L-leucine-*p*-nitroanilide

(LpNA) substrates [33], respectively. All these substrates were procured from Sigma-Aldrich (India). The gut extract was mixed with respective substrates in 0.1 M glycine-NaOH buffer (pH 10), and incubated for 20 min at room temperature. The reaction was terminated by adding 300 μl of 30% acetic acid. The assay mixture was centrifuged, and the absorbance was measured at 410 nm. One unit (U) of enzyme activity was defined as the amount of pNA released by the enzyme per minute at room temperature.

Zymogram Analysis of Midgut Proteases

The gut protein extracts were resolved on 10% polyacrylamide gel under non-reducing conditions. After electrophoresis, the gel was treated with Triton X-100 for 10 min to remove SDS. The gel was incubated with 1% casein in 0.1 M glycine-NaOH buffer (pH 10) for 60 min, then stained using Coomassie brilliant blue and finally destained. Protein bands were visualized using BioRad gel documentation unit.

Results

Expression of Various Trypsin Isoforms in the *H. armigera* on Different Diets and across Developmental Stages

All the tested trypsin isoforms showed variable expression patterns in the larvae fed on different diets (Fig. 1a); HaTry1 exhibited the highest expression, while HaTry13 and HaTry17 have shown the lower expression on different diets. The relative expression levels of HaTry1 in larval, pupal, and adult stages indicated that the expression of HaTry1 was evident only during the larval stage. The highest expression level of HaTry1 was recorded in the fifth instar larvae. We did not observe the amplification peak of HaTry1 even after 40 cycles in the pupae and adults (Fig. 1b).

Effect of dsRNA on the Expression of Target and Non-Target Proteases

The 18S rRNA, HaTry1, and HaTry13 isoforms showed PCR amplification efficiencies of 94, 97, and 96%, respectively. The expression of HaTry1 and HaTry13 was significantly altered in cognate individual and pyramided dsRNA treatments (Fig. 2a). There was 65 and 81% silencing of HaTry1 on the fourth day in 10 and 20 μg of HaTry1 dsRNA treatments, respectively. On the seventh day of treatment, silencing of HaTry1 increased to 70 and 87% in 10 and 20 μg of HaTry1 dsRNA treatments, respectively. Similarly, we observed increased silencing of HaTry13 on the seventh day compared to the fourth day in 10 and 20 μg of HaTry13 dsRNA treatments. The HaTry13 dsRNA has no effect on the expression of HaTry1 (Fig. S1). Notably, pyramided dsRNA treatment simultaneously silenced both the genes. On the fourth day, there was 62 and 43% silencing of HaTry1 and HaTry13, respectively in 10 μg of pyramided dsRNA treatment, while 79 and 71% silencing of HaTry1 and HaTry13, respectively, was recorded in 20 μg of pyramided dsRNA treatment. Similarly, on the seventh day, there was 61 and 55% silencing of HaTry1 and HaTry13, respectively, in 10 μg of pyramided dsRNA treatment, while, 83 and 72% silencing of HaTry1 and HaTry13, respectively, was observed in 20 μg of pyramided dsRNA treatment (Fig. 2a).

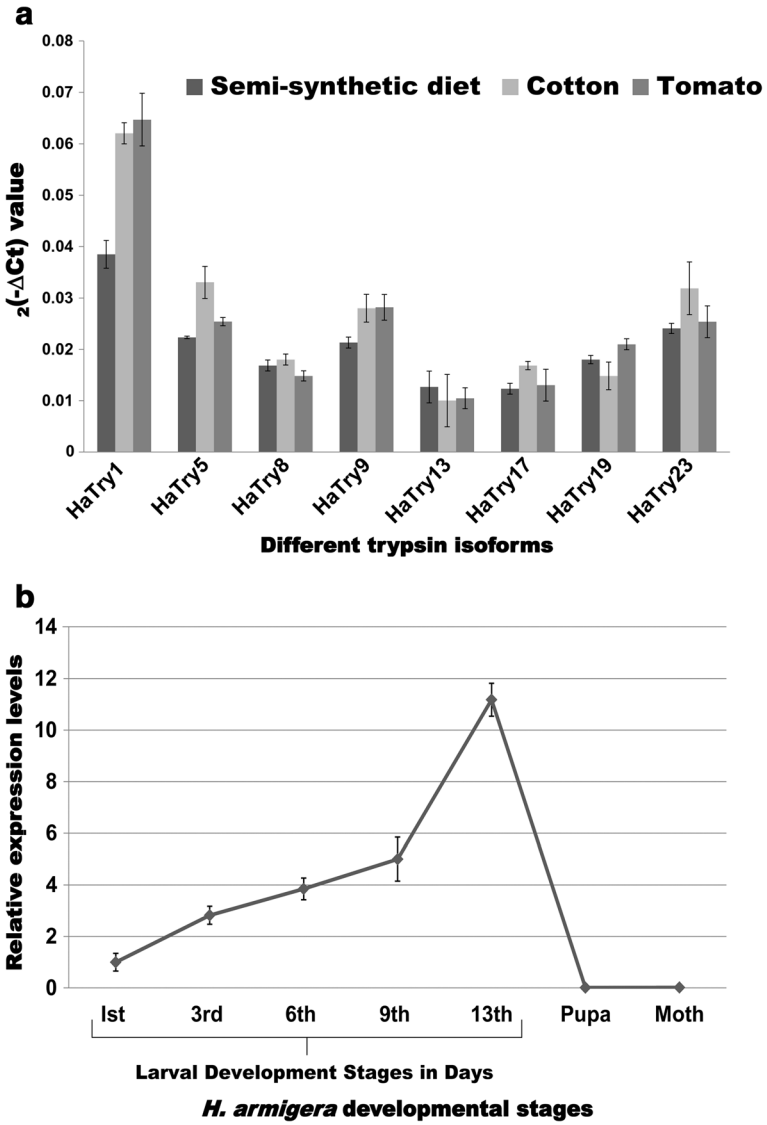

Fig. 1 Expression analysis of different isoforms of trypsin-like SPs. **a** Expression levels of different isoforms of trypsin in the larvae fed on various diets. RT-qPCR data was normalized with 18S rRNA, and the expression of different isoforms of trypsin-like SPs was calculated using $2^{-\Delta CT}$ method. **b** Relative expression of HaTry1 in the larva (at different time intervals), 3-day-old pupa, and adult of *H. armigera*. RT-qPCR data was normalized with β-tubulin. Relative expression of HaTry1 was calculated using $2^{-\Delta\Delta CT}$ method by comparing the expression of this gene on day one with the other days. Error bars indicate standard error of the four biological replicates of two experiments

Interestingly, we observed a compensatory effect by de novo over-expression of another isoform of SP (HaTry13) in HaTry1 dsRNA treatment. There was 5- and 9-fold over-expression of HaTry13 on the fourth day of 10 and 20 μg of HaTry1 dsRNA treatments, respectively. Similarly, 23- and 31-fold over-expression of HaTry13 was recorded on the seventh day of 10 and 20 μg of HaTry1 dsRNA treatments, respectively. However, there were

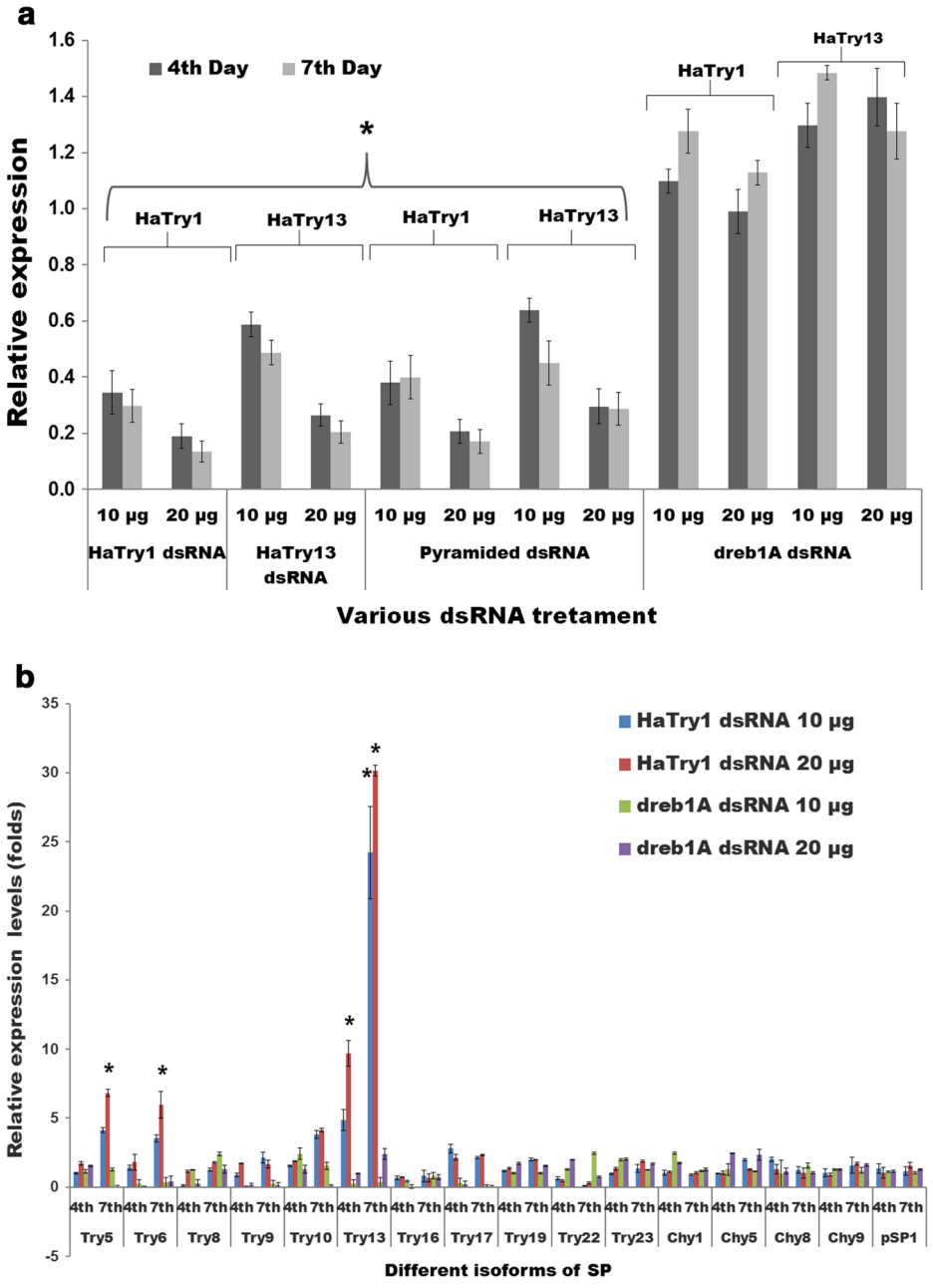

Fig. 2 Effect of dsRNA on the expression of target and other isoforms of SPs. **a** Effect of various cognate and non-target *dreb1A* dsRNAs on the expression of target gene/s. **b** Effect of target (HaTry1) dsRNA and non-target *dreb1A* dsRNA on the expression of other isoforms of SPs. According to $2^{-\Delta\Delta CT}$ method [22], the expression of the target gene in the contro1 sample is considered as "1". The relative expression of the target gene in the treated samples is compared with the control: if the target gene expression is higher than 1, it is considered as upregulated; if less than 1, it indicates downregulation. Error bars indicate standard error of the four biological replicates of two experiments. Asterisk (*) indicates the significant differences in the expression levels of tested genes analyzed using *t* test at $p < 0.05$. Try trypsin, Chy chymotrypsin, pSP putative serine protease

no significant differences in the expression of the rest of the genes (Fig. 2b). These expression levels were quite significant as the non-target *dreb1A* dsRNA treatment did not alter the expression of HaTry1 or any other isoforms of SPs. Moreover, the pyramided dsRNA has no significant effect on the expression of the other tested isoforms (Fig. S2).

Effect of Target Gene/s Silencing on Larval and Pupal Weight

Silencing of HaTry1 initially led to a significant reduction in the larval weight in the cognate dsRNA treatment as compared to that of the controls. On the third day, the highest reduction of 34 and 26% was observed in the larvae fed on the diet with 20 and 10 μg of HaTry1 dsRNA, respectively. Notably, we did not observe any significant larval weight differences between the larvae fed on the control diet and the diet with cognate HaTry1 dsRNA on the sixth and ninth day (Fig. 3a). Contrastingly, there was a persistent reduction in the weights of larvae fed on diets with HaTry13 and pyramided dsRNA. Overall, maximum larval weight reduction of 31% was observed in the larvae fed on a diet with 20 μg of pyramided dsRNA, followed by 22, 9, and 5% reduction in the weight of larvae fed on diets with 10 μg of pyramided dsRNA, 20 and 10 μg of HaTry13 dsRNA, respectively (Fig. 3a). Overall, there were no significant differences in the weights of larvae fed on diets with *dreb1A* dsRNA, and the untreated control.

Pyramided dsRNA mediated the silencing of both genes, and this eventually influenced the pupal weight, which was correlated with the larval weight (Fig. 3b). The highest pupal weight reduction of 29% was observed in the larvae fed on a diet with 20 μg of pyramided dsRNA, followed by 20 and 7% reduction in 10 μg of pyramided dsRNA and 20 μg of HaTry13 dsRNA treatments, respectively. Whereas, there were no significant differences in the pupal weight among individual (both concentrations of) HaTry1 dsRNA, 10 μg of HaTry13 dsRNA, (both concentrations of) *dreb1A* dsRNA treatments, and the control.

Phylogenetic Analysis of SPs

Unrooted neighbor-joining tree generated with 1000 bootstrap repeats of SPs showing the existence of six clades. Most of the trypsin-like SPs were grouped into clades I, II, and III, while the majority of chymotrypsin-like SPs were grouped into clade IV. Whereas, clade V contained trypsin-like SPs and putative SPs; clade VI consisted of a mixture of chymotrypsin-like SPs and one putative SP. Therefore, most of the chymotrypsin encoding genes showed relatively high homology; however, HaChy7, HaChy9, and HaChy11 were separated from clade IV (Fig. 4). Based on the distance between the isoforms in the phylogenetic tree, we selected HaTry5, HaTry6, HaTry8, HaTry9, HaTry13, HaTry16, HaTry17, HaTry19, HaTry22, HaTry23, HaChy1, HaChy5, HaChy8, HaChy9, and Ha_pSP1 isoforms for expression studies.

Activities of Midgut Total Proteases

A significant reduction of trypsin level was observed in the larvae on the fourth day of cognate (HaTry1) dsRNA treatment (Table 1). Notably, the trypsin level was on par with the control on the seventh day of individual cognate (HaTry1) dsRNA treatment. Notably, the lowest trypsin levels were recorded on the fourth and seventh day of pyramided dsRNA treatment compared to the other dsRNA treatments. However, a slight reduction in trypsin level was observed in the larvae fed on a diet with 20 μg of HaTry13 dsRNA. Total protease, chymotrypsin, elastase,

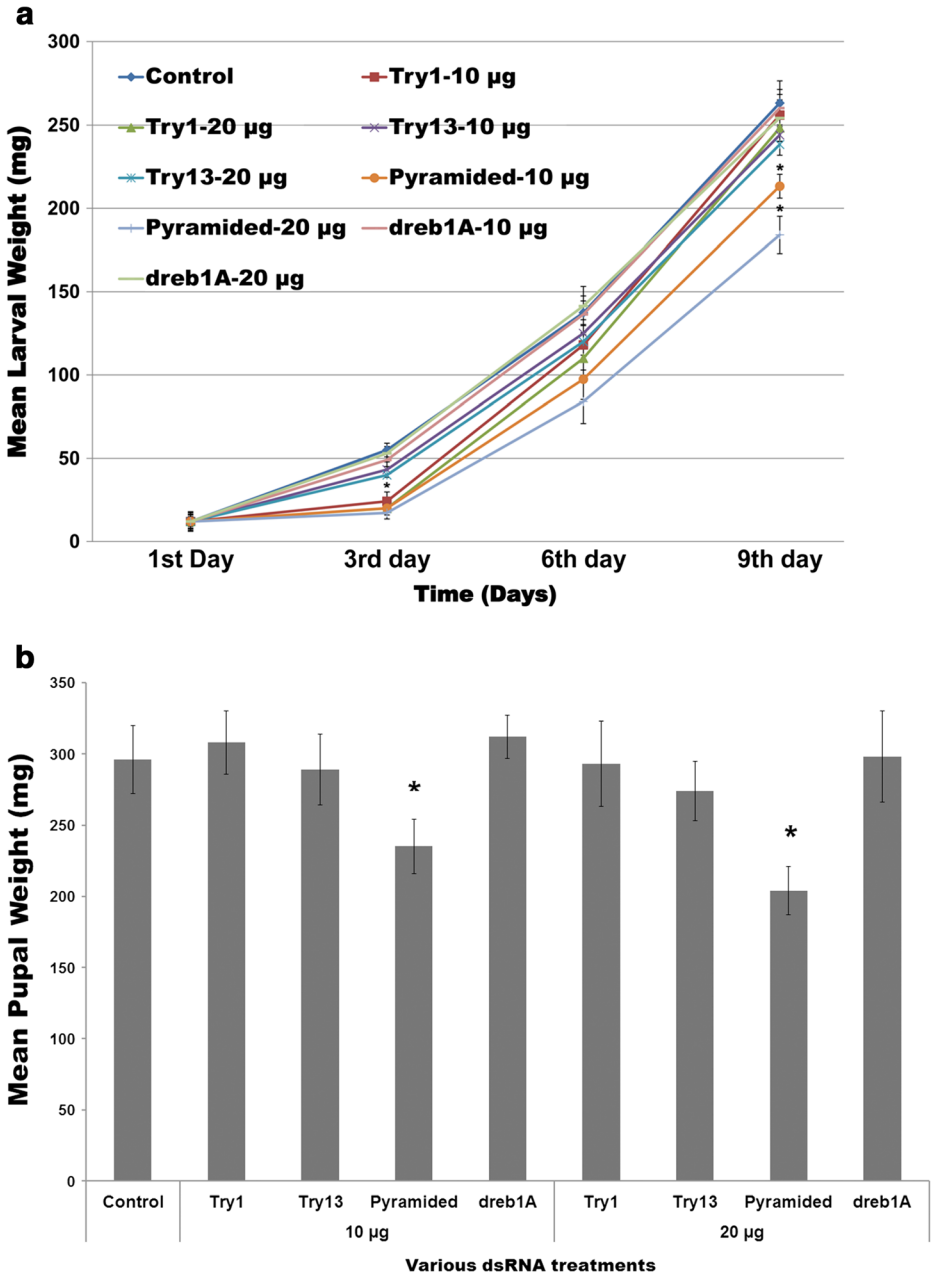

Fig. 3 Effect of various dsRNA treatments on the larval and pupal weight. **a** Larval weights were recorded on various days of different dsRNA treatments. **b** Pupal weight was recorded on the fifth day of pupation. Error bars indicate standard error of the 30 replicates. Asterisk (*) indicates the significance of the larval weight differences between treatment and control analyzed using one-way ANOVA at $p < 0.05$. *dreb1A*: non-target dsRNA

and aminopeptidase levels did not differ significantly on the fourth and seventh day of (any) dsRNA treatments as compared to the control (Table 1).

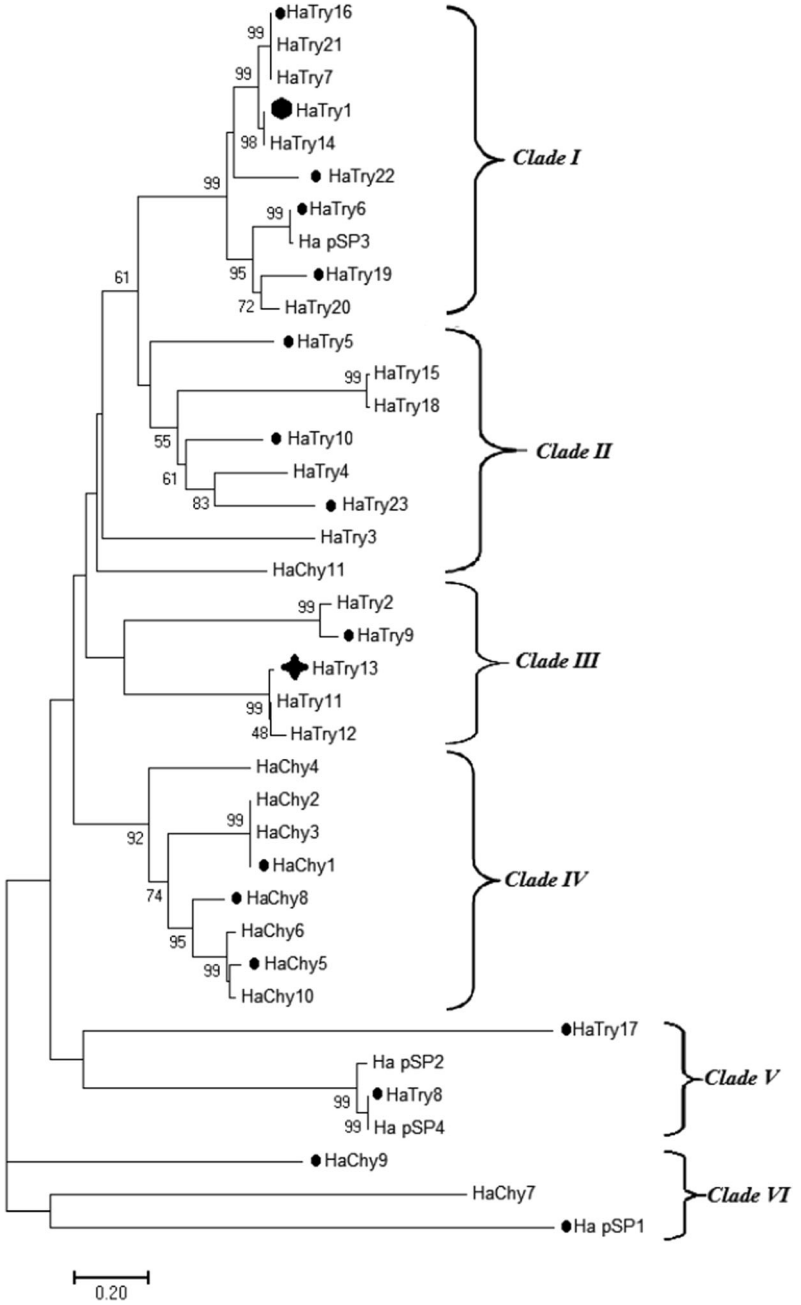

Fig. 4 Phylogram of trypsin and chymotrypsin-like SPs of *H. armigera*. Coding sequences of trypsin and chymotrypsin of *H. armigera* were translated into amino acids using the ExPASy-Translate tool, and these were aligned using ClustalW2. The unrooted phylogenetic tree was generated with a neighbor-joining method using 1000 bootstrap replicates. Details of different isoforms of SPs used in phylogenetic tree construction are provided in Table S2. HaTry *H. armigera* trypsin, HaChy *H. armigera* chymotrypsin, Ha_pSP *H. armigera* putative serine protease

Table 1 Activities of midgut proteases in *H. armigera* larvae fed on the various dsRNAs

Treatment	Total protease (UA)	Trypsin (U)	Chymotrypsin (U)	Elastase (U)	Aminopeptidase (U)
Fourth day of dsRNA treatment					
Control	0.319 ± 0.006	0.187 ± 0.004	0.103 ± 0.010	0.105 ± 0.006	0.041 ± 0.004
HaTry1 @ 10 μg	0.234 ± 0.004	0.137 ± 0.006[a]	0.088 ± 0.004	0.087 ± 0.002	0.047 ± 0.002
HaTry1 @ 20 μg	0.325 ± 0.003	0.121 ± 0.003[a]	0.114 ± 0.013	0.111 ± 0.003	0.056 ± 0.002
HaTry13 @ 10 μg	0.310 ± 0.003	0.213 ± 0.002	0.108 ± 0.004	0.102 ± 0.001	0.010 ± 0.006
HaTry13 @ 20 μg	0.280 ± 0.001	0.146 ± 0.002[a]	0.113 ± 0.002	0.091 ± 0.002	0.039 ± 0.003
Pyramided @ 10 μg	0.244 ± 0.004	0.126 ± 0.008[a]	0.095 ± 0.018	0.087 ± 0.001	0.036 ± 0.002
Pyramided @ 20 μg	0.239 ± 0.006	0.103 ± 0.005[a]	0.101 ± 0.016	0.080 ± 0.002	0.034 ± 0.003
dreb1A @ 10 μg	0.289 ± 0.004	0.177 ± 0.005	0.113 ± 0.025	0.099 ± 0.004	0.049 ± 0.004
dreb1A @ 20 μg	0.304 ± 0.003	0.168 ± 0.007	0.106 ± 0.021	0.103 ± 0.003	0.051 ± 0.005
Seventh day of dsRNA treatment					
Control	0.230 ± 0.008	0.088 ± 0.001	0.155 ± 0.006	0.053 ± 0.003	0.057 ± 0.004
HaTry1 @ 10 μg	0.241 ± 0.004	0.098 ± 0.001	0.151 ± 0.004	0.059 ± 0.004	0.067 ± 0.001
HaTry1 @ 20 μg	0.239 ± 0.090	0.099 ± 0.001	0.145 ± 0.004	0.067 ± 0.002	0.061 ± 0.004
HaTry13 @ 10 μg	0.246 ± 0.040	0.196 ± 0.001	0.169 ± 0.006	0.062 ± 0.003	0.063 ± 0.001
HaTry13 @ 20 μg	0.315 ± 0.015	0.071 ± 0.018	0.233 ± 0.003	0.063 ± 0.005	0.062 ± 0.002
Pyramided @ 10 μg	0.274 ± 0.010	0.067 ± 0.010[a]	0.144 ± 0.005	0.062 ± 0.001	0.080 ± 0.008
Pyramided @ 20 μg	0.240 ± 0.012	0.052 ± 0.002[a]	0.115 ± 0.001	0.062 ± 0.002	0.068 ± 0.001
dreb1A @ 10 μg	0.242 ± 0.050	0.094 ± 0.019	0.155 ± 0.003	0.053 ± 0.003	0.057 ± 0.006
dreb1A @ 20 μg	0.222 ± 0.070	0.098 ± 0.021	0.151 ± 0.004	0.059 ± 0.005	0.067 ± 0.004

[a] Indicates significant differences in the activity compared to control as analyzed with one-way ANOVA at $p < 0.05$ (GraphPad Prism v. —GraphPad Software, Inc., USA)

The data represented as mean ± standard error of triplicates. Here: 10 and 20 μg are the concentrations of respective dsRNA treatment

UA units of protease activity, *U* μmol/mg/ml

Zymogram Analysis of Midgut Proteases

Zymogram analysis revealed that HaTry1 dsRNA treatment inhibited one protease activity (first band from bottom of the gel) on the fourth and seventh day, whereas, over-expression of another protease isoform (second band from bottom of the gel) was observed on the seventh day of HaTry1 dsRNA treatment, compared to the control (Fig. 5a, b). Notably, inhibition of both the over-expressed (second band from the bottom of the gel) and the earlier inhibited proteases (first band from the bottom of the gel) was observed in the larvae fed on the diet with pyramided dsRNA (Fig. 5c).

Discussion

Expression analysis of different isoforms of trypsin showed variable expression patterns. HaTry1 exhibited consistently higher expression in *H. armigera* larvae fed on all three diets

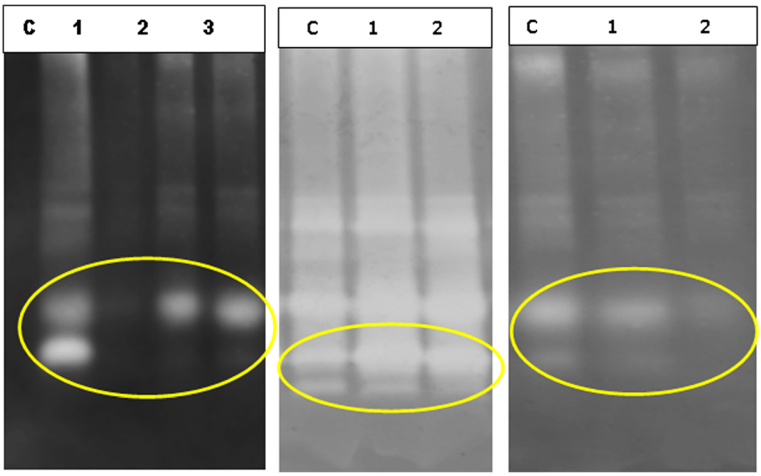

Fig. 5 Zymogram analysis of midgut proteases of *H. armigera* larvae fed on dsRNA. Midgut proteases were collected from different dsRNA treated and control larvae. Proteases were resolved on 10% polyacrylamide gels under non-reducing conditions. **a** Zymogram of total proteases on the fourth day of HaTry1 dsRNA treatments. Here: C: control, 1: overflowed control, 2–3: 10 and 20 μg of HaTry1 dsRNA treated samples, respectively. **b** Zymogram of total proteases on the seventh day of HaTry1 dsRNA treatments. Here: C control, 1–2: 10 and 20 μg of HaTry1 dsRNA treated samples, respectively. **c** Zymogram of total proteases on the seventh day of pyramided dsRNA treatment. Here: C: control, 1–2: 10 and 20 μg of pyramided dsRNA treated samples, respectively. Here, we assume that upper bands are HaTry13 and lower bands are HaTry1 in the marked circle, since these have shown band intensity variation in HaTry1, and Pyramided dsRNA of HaTry1 and HaTry13 treatments

(chickpea-based semi-synthetic diet, cotton, and tomato leaves) compared to other isoforms. In addition, HaTry1 expression was predominant during the larval stages, which was correlated with the larval growth. Therefore, we silenced HaTry1 isoform to reduce the larval feeding and inhibit the growth and development of *H. armigera* larvae. Feeding of cognate dsRNA led to successful silencing of the target gene, while the non-target *dreb1A* dsRNA did not cause target gene silencing. These results are similar to our previous reports, which suggest that delivery of dsRNA through semi-synthetic diet was successful in silencing the target genes in *H. armigera* [26].

Cognate dsRNA feeding resulted in sustained silencing of HaTry1, and the highest silencing of 87% was observed on the seventh day in the larvae fed on diet with 20 μg HaTry1 dsRNA. To assess the effect of gene silencing on the larval growth and development, the larval weights were recorded on the first, third, sixth, and ninth day of dsRNA feeding. The larval weight gain was significantly lower during the initial few days of cognate dsRNA treatment (Fig. 3a); however, the larvae recovered from the weight loss during later stages despite continuous silencing of the target gene. We hypothesized that recovery of larvae from weight loss might be because of compensation of target gene silencing by over-expression of other isoforms of the digestive proteases to cope with RNAi induced adversity. Therefore, we assessed the expression of other isoforms of SPs to identify the isoforms involved in the compensation of gene silencing effect. In this regard, the phylogenetic analysis was performed with the available SPs encoding gene sequences of *H. armigera* in order to select the isoforms for expression analysis. The phylogenetic tree revealed the presence of six clades and a division between trypsin and chymotrypsin encoding gene sequences. These results are congruent with the earlier reports on SPs of *H. armigera* [6]. Based on the phylogenetic tree, a minimum of one isoform was randomly selected from each phylogenetic clade to study their expression levels.

The RT-qPCR analysis showed that one isoform of trypsin (HaTry13) had significant levels of (27–33-folds) over-expression in the larvae fed on diet with HaTry1 dsRNA (Fig. 2b). The over-expression of HaTry13 was found to be induced due to the depletion of HaTry1 transcript caused by cognate dsRNA, and it was not elicited with the non-target *dreb1A* dsRNA. Similarly, the adverse effects of dietary protease inhibitors (PI) on the insect were overcame by over-expression of inhibitor-insensitive enzymes to compensate the loss of protease function [37]. In this regard, beet armyworm, *Spodoptera exigua* showed resistance to chymotrypsin/trypsin-specific potato proteinase inhibitor II by over-expressing a new range of trypsins that are not inhibited by PIs [38]. In the present study, silencing of HaTry1 might have caused the nutritional stress in the *H. armigera* larvae, resulting in elicitation of over-expression of another isoform of SP through compensatory mechanisms.

To alleviate the compensatory effect, both the isoforms (target, HaTry1, and over-expressed HaTry13) were simultaneously silenced by feeding the larvae on a diet supplemented with pyramided dsRNA. The results revealed that the simultaneous silencing of multiple genes causes maximum reduction of larval weight thus can able to alleviate the compensatory effect induced by the over-expressed isoform. Midgut protease assays and zymogram analysis corroborated the above observations that the reduced trypsin activity and inhibition of one of trypsin (HaTry1) isoform on the fourth and seventh day and over-expression of another (HaTry13) isoform with equivalent total trypsin activity to that of control on the seventh day in the larvae fed on diet supplemented with HaTry1 dsRNA. Whereas, larvae fed on a diet supplemented with HaTry13 dsRNA exhibited a slight reduction in the trypsin activity. Inhibition of both HaTry1 and HaTry13 isoforms was observed in the larvae fed on a diet supplemented with pyramided dsRNA. These results demonstrated that the simultaneous silencing of multiple genes can be used to tackle the plasticity of digestive proteases in *H. armigera*.

Conclusion

HaTry1 expressed irrespective of the composition of the diet and its expression levels were increased with the development of the larvae. Feeding the larvae on 10 and 20 μg of cognate dsRNA has led to efficient silencing of HaTry1. To the best of our knowledge, this is the first report on RNAi-mediated silencing of one isoform (HaTry1) of SP, causing the over-expression of another isoform of SP (HaTry13) that restored the normal growth of larvae. These studies could be useful in identification of the isoforms of SPs involved in the compensation of silencing effect. An understanding of the compensatory effect is an essential step in designing an effective gene silencing strategies for the management of *H. armigera*. Compensatory effect could be alleviated by simultaneous silencing of multiple isoforms that are able to tackle the plasticity of digestive proteases; which, in turn, may reinforce the effect of RNAi in the management of insect pests.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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