MOLECULAR IDENTIFICATION OF CYSTEINE AND TRYSIN PROTEASE, EFFECT OF DIFFERENT HOSTS ON PROTEASE EXPRESSION, AND RNAI MEDIATED SILENCING OF CYSTEINE PROTEASE GENE IN THE SUNN PEST

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Sunn pest, Eurygaster integriceps, is a serious pest of cereals in the wide area of the globe from Near and Middle East to East and South Europe and North Africa. This study described for the first time, identification of E. integriceps trypsin serine protease and cathepsin-L cysteine, transcripts involved in digestion, which might serve as targets for pest control management. A total of 478 and 500 base pair long putative trypsin and cysteine gene sequences were characterized and named Tryp and Cys, respectively. In addition, the tissue-specific relative gene expression levels of these genes as well as gluten hydrolase (Gl) were determined under different host kernels feeding conditions. Result showed that mRNA expression of Cys, Tryp, and Gl was significantly affected after feeding on various host plant species. Transcript levels of these genes were most abundant in the wheat-fed E. integriceps larvae compared to other hosts. The Cys transcript was detected exclusively in the gut, whereas the Gl

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and Tryp transcripts were detectable in both salivary glands and gut. Also possibility of Sunn pest gene silencing was studied by topical application of cysteine double-stranded RNA (dsRNA). The results indicated that topically applied dsRNA on fifth nymphal stage can penetrate the cuticle of the insect and induce RNA interference. The Cys gene mRNA transcript in the gut was reduced to 83.8% 2 days posttreatment. Also, it was found that dsRNA of Cys gene affected fifth nymphal stage development suggesting the involvement of this protease in the insect growth, development, and molting. © 2015 Wiley Periodicals, Inc.

Keywords: RNAi; gene expression; protease; Eurygaster

INTRODUCTION

Protein-digesting enzymes play important roles in many biological processes including cell proliferation (Lee et al., 2010), morphogenesis and tissue remodeling (Reichhart et al., 2011), homeostasis (Voos et al., 2013), wound healing (Karlsson et al., 2004), immunity (Gorman et al., 2000; Manoury et al., 2011), apoptosis (Zhang and Li, 2012), and food digestion (Saadati and Bandani, 2011). Insects use salivary glands and gut digestive proteases to degrade diet proteins to peptides and to amino acids (Sato et al., 2008; Dunse et al., 2010a,b).

Herbivorous insects use multiple types of proteinases as digestive enzymes. Proteinases are broadly grouped into four types including serine, cysteine, aspartate, and metallo proteases (Sternlicht and Werb, 2001). The diversity and plasticity of proteases expressed in the insect alimentary canal enables them to hydrolyze proteins into short peptides or individual amino acids. Also, these proteases enable insect to defend themselves against a variety of dietary toxins and antinutritional compounds encountered in their host plants (Bown et al., 2004; Prabhakar et al., 2007; Simpson et al., 2007; Ge et al., 2012; Chen et al., 2013; Spit et al., 2014).

Cysteine proteases are important and vital enzymes in many invertebrate groups that have different functions including functions in digestion (Goptar et al., 2012), embryogenesis (Hashmi et al., 2002), molting (Liu et al., 2006), tissue remodeling during insect metamorphosis (Wang et al., 2009), detoxification of plant defensive proteins (Koo et al., 2008), and immune responses (Zhang et al., 2013). Terra (1990) emphasized coleopterans and hemipterans utilize cathepsins to digest their food. The most extensively studied role of cysteine proteases is their functions as digestive enzymes because they are the major digestive enzymes in Coleoptera, Diptera, and Hemiptera (Zhu-Salzman et al., 2003; Cristofoletti et al., 2005; Ben-Mahmoud et al., 2015).

Trypsins are members of the serine protease family (Bown et al., 1997). Serine proteases have important roles in various physiological processes including digestion, development, and the immune response such as melanization of pathogen surfaces, hemolymph coagulation, and antimicrobial peptide synthesis (Gorman and Paszewitz, 2000; Zou et al., 2006). Midgut serine proteases, such as trypsins, are the main proteolytic digestive enzymes in Lepidoptera and Orthoptera (Gatehouse, 2011).

Eurygaster integriceps, commonly known as Sunn pest, belongs to the order Hemiptera and family Scutelleridae. Sunn pest is a serious pest of cereals that causes severe quantitative and qualitative (destruction of gluten protein) damage to crops (sometime up to 100%) by feeding on leaves, stems, and grains (Amiri and Bandani, 2013; Yandamuri
et al., 2014). Gluten hydrolase, another member of serine protease super family, has important roles in digestion of wheat gluten in the Sunn pest (Konarev et al., 2011; Dolgikh et al., 2014). During feeding, the Sunn pest injects a salivary glands secretion into seed endosperm and subsequently digests and destroys the baking property of dough (Aja et al., 2004). Proteases injected in the grains such as trypsin like gluten hydrolase damage wheat gluten and baking property of bread (Konarev et al., 2011; Dolgikh et al., 2014).

Pesticides as a major approach of insect pest control worldwide are associated with significant hazards to the environment and human health (Damalas and Eleftherohorinos, 2011). As a result, there is a need to develop more benign and ecologically safe alternative methods for pest control (Ningshen et al., 2013). For plant-feeding insects, digestive proteases have potential importance for engineering protease inhibitors and dsRNA for pest control. Huvenne and Smagghe (2010) have comprehensively reviewed mechanism of dsRNA uptake through insect gut. dsRNA is cleaved into small interfering RNA molecules (20–25 nucleotides) that incorporate into RNA-induced silencing complex and destroy the endogenous complementary mRNA. Gene silencing using RNA interference (RNAi) has been suggested as one of the new alternatives to pest control because of its specificity. RNAi has become a widely used method in order to silence genes in many eukaryotic systems such as plant (Napoli et al., 1990), nematode (Fire et al., 1998), protozoan (Kolev et al., 2011), and invertebrate species and thus could contribute to novel strategies to control agricultural pests, including a number of insect species (Garbutt et al., 2013; Christiaens et al., 2014; Deng and Zhao et al., 2014; Killiny et al., 2014; Mansur et al., 2014; Sapountzis et al., 2014; Zhou et al., 2014).

There is a great amount of variability in RNAi efficiency between different species. Regarding hemipteran species, successful RNAi experiments in the laboratory have been confirmed for several species including economically important pests such as the whitefly Bemisia tabaci (dsRNAs and siRNAs were synthesized from five different genes—actin ortholog, ADP/ATP translocase, α-tubulin, ribosomal protein L9, and V-ATPase subunit A; Upadhyay et al., 2011), brown planthopper Nilaparvata lugens (V-ATPase subunit E; Li et al., 2011), and pea aphid Acrithosiphon pisum (calreticulin and cathepsin-L; Jaubert-Possamai et al., 2007). RNAi silencing salivary sheath protein of wheat aphid, Sitobion avenae (Hemiptera: Aphididae) induced feeding suppression and affected development and survival. Feeding on transgenic plant expressing dsRNA significantly reduced target gene (Abdellatief et al., 2015). Trypsin-like serine protease gene silencing was done when the Hemipteran insect N. lugens feeding on dsRNA expressing rice plants (Zha et al., 2011). Christiaens and Smagghe (2014) summarized the RNAi of different species belonging to Hemiptera order. Cathepsin-L cysteine RNAi in the pea aphid A. pisum induced 40% decrease of gene expression. However, it did not affect the aphid growth, life span, and fecundity (Jaubert-Possamai et al., 2007). Successfully, RNAi knockdown of a Halloween gene spookier led to nymphal lethality and delayed development in the small brown planthopper Laodelphax striatellus (Hemiptera: Delphacidae; Jia et al., 2015). Killiny et al. (2014) observed that topically applied cytochrome P dsRNA could penetrate the cuticle and induce RNAi in Diaphorina citri (Hemiptera: Psyllidae). Cytochrome P dsRNA shortened the life span of D. citri. In the other hemipteran, Rhodnius prolixus, knocked down of crustacean cardioactive peptide by dsRNA had up to 84% mortality (Lee et al., 2013). Meanwhile, in this hemipteran insect, injection of chitin synthase dsRNA reduced transcript levels and oviposition, also caused reduction of ovary size, deformed eggs, and ecdysis abnormalities (Mansur et al., 2014). Ingestion of catalase dsRNA knocked down
gene expression in *S. avenae* and reduced survival rate and ecdysis index (Deng and Zhao et al., 2014).

Since cysteine proteases play critical functions in the digestion and development of the different insects (Goptar et al., 2012; Zhang et al., 2013; Ben-Mahmoud et al., 2015), they can be targets for developing new tools in the integrated pest management. So in this study, after sequence identification, cathepsin-L cysteine was chosen as a target gene for RNAi gene silencing.

Therefore, the objectives of this research were as follows: (1) molecular identification of serine and cysteine protease genes in the Sunn pest genome; (2) study of alterations in expression of salivary glands and gut protease genes in response to different host plants; and (3) silencing cysteine protease gene by using RNAi.

**MATERIALS AND METHODS**

**Insect Rearing**

Sunn pest adults were collected from a wheat field during spring 2014, when feeding started, in Karaj, Alborz Province, Iran. The insects were maintained on soaked wheat kernels and water in the laboratory as described by Bandani et al. (2009) and Allahyari et al. (2010). To determine the effect of different host species on gene expression, 100 eggs for each replicate (three replicates were considered for each treatment) in each treatment were reared separately on various hosts including wheat, barley, rye, and triticale kernels. The insects were reared to fifth instar when used for sample collection. In order to establish the developmental stage and to synchronize the treated fifth instar larvae, the molt was observed every day and the newly emerged larvae were collected every 24 h and placed on fresh soaked kernels and 1 day after ecdysis, fifth instar larvae were collected for gene expression experiment. For the RNAi assays, the wheat fed newly emerged (0–24 h) fifth instar larvae were used.

**Tissue Sampling**

To obtain selected tissue samples (midgut and salivary glands), *E. integriceps* fifth instar larvae were dissected in sterile water under a stereoscopic microscope. Each tissue (midgut and salivary glands) was collected and pooled from individual insects and were transferred into a microtube containing 0.5 ml TRI reagent (~30 mg wet tissue; Sigma-Aldrich, Missouri, USA).

**RNA Extraction and cDNA Cloning**

Samples (~30 mg wet tissue) were processed for total RNA extraction by using TRI reagent using instruction provided by the manufacturer. To remove potential DNA contamination, total RNA samples were treated with Ambion™ DNase I (RNase-free) (Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA). First-strand cDNA was prepared with RNA (DNA free) and the oligo-dT primer using AccuPower® RocketScript™ Cycle RT PreMix cDNA synthesis kit (Bioneer, Daejeon, Korea). All cDNA concentrations were determined using a Nanodrop 1000 Thermo Scientific, diluted (10-fold), and used for the next PCR reactions consisting of preheating 95°C for 4 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 7 min. Each 25 μl PCR reaction contained 1 μl of cDNA template, 2.5 μl of 10× PCR (polymerase chain reaction)
Table 1. The Primer Sequences Were Used in the Study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>PCR type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>TGCGGATCTTGCTGGGCTTT</td>
<td>CCAGGAGTTCTTGACGAGCCA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Tryp</td>
<td>GTACTCACAGCTGCACATTG</td>
<td>GTCGACCACTGAATCACC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>CysT7</td>
<td>GAATTAATACGACTCATA</td>
<td>GAATTAATACGACTCATA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>GGGGATCGCGATCTGGTCTG</td>
<td>GGGGACAGGAGGAGTTCTGG</td>
<td>(dsRNA synthesis)</td>
</tr>
<tr>
<td>re-Cys</td>
<td>TCTATTCGTCAGCGTCGCA</td>
<td>GGTACAGTCGGTCCAGTGTC</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>re-Tryp</td>
<td>AGGATGGGGTTCAATTAGCGG</td>
<td>TGGTTTGTAAGCCTTGTCGCA</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>re-Gl</td>
<td>GGATTGGTTCGGTTTGGCAC</td>
<td>GGCACCCAAGAGTTCCGATT</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>18S</td>
<td>GGCCTTCGGGATCGGAGTAA</td>
<td>GCAATGCTTTCCGAGTTGA</td>
<td>Real-time PCR</td>
</tr>
</tbody>
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buffer, 1.5 μl of MgCl₂ (25 mmol/μl), 2 μl of dNTP (deoxynucleotide triphosphate) (2 mmol/μl each), 1.5 μl of forward and 1.5 μl of reverse primers (10 pmol/μl; Cys and Tryp primers, Table 1), 0.2 μl of Taq polymerase (5 U/μl), and 14.8 μl of ddH₂O. Cys and Tryp primers were designed from two highly conserved regions of other insect genes and re-Gl primers were designed according to gluten hydrolase sequence in GenBank with accession number HM579787.1 by using Primer Blast online software (Table 1). Six microliters of the PCR product was analyzed on a 1% TAE (Tris base, acetic acid and EDTA buffer) ethidium bromide gel and the target band was isolated from the gel and recovered and purified by using the AccuPrep Gel Extraction Kit (Bioneer, Daejeon, Korea), cloned into the pTG19-T PCR cloning vector (Vivantis) and transformed into Escherichia coli DH5α competent cells. Colonies with inserts were screened with blue/white under standard ampicillin conditions. Recombinant plasmids were sequenced from both directions and hereby Cys and Tryp sequences were confirmed by sequencing. The sequencing reaction was performed commercially.

**Sequences and Phylogenetic Analysis**

The sequences of the Cys and Tryp cDNA were confirmed by a homology search of other cysteine and trypsin sequences known within the Blast program available on the GenBank database of National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequences of Cys and Tryp homologs from different organisms were retrieved from GenBank database. Sequences were aligned by using ClustalX, and phylogenetic trees were constructed by the neighbor-joining method with a Poisson correction model (1,000 bootstrap replications to check for repeatability of the results) using the MEGA 5.0 software.

**dsRNA Synthesis**

For silencing Cys in E. integriceps, double-stranded RNA (dsRNA) was synthesized by using T7 RNA polymerase. Sequence-specific primers were designed with a T7 promoter at the 5' end (CysT7; Table 1) to synthesize cDNA fragments of Cys which contained the T7 promoter region in both sense and antisense strands. The amplified dsDNA was used as template for dsRNA synthesis with the MEGAscript® RNAi kit (Ambion, Life Technologies). After synthesis, the dsRNA quantified spectrophotometrically at 260 nm and its purity and integrity were determined by agarose gel electrophoresis. Fragment length of Cys-dsRNA was approximately 500 bp. The concentration and quality of RNA were measured with Nanodrop.
**Topical Application of dsRNA**

Purified dsRNA was diluted using acetone to obtain desired concentrations of dsRNA. Three concentrations of dsRNA (100, 500, and 1,000 ng/μl) and a control (0 ng/μl) were used to treat 200 *E. integriceps* 1-day-old fifth larval instar. One microliter droplet of dsRNA was topically applied to the dorsal side of the abdomen (Pridgeon et al., 2008). Control insects treated only with acetone. The dead and eclosed individuals were recorded every day. Also, body weight was measured for survivors at 3 days intervals after dsRNA treatment for 10 days. One, 2, and 3 days following topical application, some treated insects were randomly selected for qPCR (quantitative PCR) analyses.

**Sampling of Total Midgut RNA From dsRNA-Treated Sunn Pest**

About 200 insects were used for RNAi. After RNAi treatments, fifth instar Sunn pest midgut was excised. Only live insects were collected for gene expression analysis. A pool of tissues from five individuals was sampled and put in a microtube containing 500 μl of TRI reagent. Insect samples were processed for RNA extraction, DNase treatment, and first-strand cDNA synthesis as described before. All cDNA products were quantified, diluted, and used for the qRT-PCR (quantitative Real-Time PCR) analysis.

**Qualitative Gene Expression Assays**

Real-time PCR was employed to determine the expression of Cys, Tryp, and Gl in various tissues of *E. integriceps*. The reactions were performed with Eva green master mix (5× HOT FIREPol® Probe qPCR Mix Plus (no ROX)) on a Rotor-Gene® Q system (QIA-Gene). Gene-specific primers (Table 1) were designed using Primer Blast. PCR amplification using forward and reverse primers resulted in an approximately 100 bp fragment within the coding region of each gene. *Eurygaster integriceps* specific 18S ribosomal RNA gene (18S) was used as internal control (Majerowicz et al., 2011; Paim et al., 2012). The 18S primers were designed according to Sunn pest 18S ribosomal RNA sequence in GenBank with accession number KP890857 by using Primer Blast online software (Amiri et al., 2015). Prior to PCR, cDNA preparations from developmental stages were quantified. For every sample, three technical replicates were performed. Each reaction was performed with 5 μl of cDNA, 0.5 μM of forward and reverse primer (re-Gl, re-Cys, re-Tryp, and 18S for separate reactions), and 4 μl of Eva green master mix in 20 μl total volumes. The PCR amplifications were performed with the following cycling conditions: one cycle at 95°C (15 min), followed by 40 cycles of denaturation at 95°C (15 s), annealing at 62°C for 20 s, and extension at 72°C for 20 s. At the end of the program, a melting curve for each primer (65–94°C read every 1°C) was acquired to ensure that only single products were generated.

The data on gene expression in different tissues of *E. integriceps* were normalized by subtracting cycle threshold (Ct) values from the corresponding 18S Ct values. The relative expression level of genes in different tissues was determined by comparative Ct method \(2^{-ΔΔCt};\) Pfaffl, 2001. The significance of differences in the gene expression was determined by t-test using the Rest 2009 Software (Pfaffl et al., 2002). The target genes expression levels were normalized relative to those of the control gene (18S). In the RNAi test, the relative expression ratio of Cys mRNA in the different tissues of individuals treated with Cys-dsRNA (topical application) was compared with the acetone-
treated control group. Also in the different host plants test, the relative expression ratio of target genes was compared with the wheat-fed Sunn pest as a control group.

Statistical Analysis

The data were given as means ± SE. One-way ANOVA analysis with at least three repeats were performed using SPSS version 19.0 to compare calculated *E. integriceps* mortality, weight, and fifth instar duration between various treatments. Post hoc pairwise comparisons between treatments were performed with Tukey’s test. Statistical significance was established as $P < 0.05$.

RESULTS

Identification and Homologous Alignment

By searching in database, the *E. integriceps* Cys and Tryp protein homologues were found in various insects. The other protein or homologue sequences used in this study were downloaded from GenBank database. Protein alignment of Cys and Tryp demonstrated a high homology to that of *Anopheles darlingi* (61% identity) and blood-sucking bug *R. prolixus* (55% identity) respectively (Fig. 1a and b).

A 500 bp partial cDNA encoded a protein was denoted as *E. integriceps* cysteine (Cys), based on the BLASTx analysis indicating homology with cathepsin-L cysteine protein from *A. darlingi* (Diptera: Culicidae; GenBank accession no. ETN64675.1; 61% identity), *Aedes*
aegypti (Diptera: Culicidae; ABE72972.1; 61%), Riptortus pedestris (Hemiptera: Alydidae; BAN20648.1; 58%), and Triatoma brasiliensis (Hemiptera: Reduviidae; ACF48469.1; 58%). This cDNA sequence has been deposited in GenBank under accession number KP295957. The next 478 bp partial cDNA encoded a protein that it is BLASTx analysis revealed homology with trypsin serine protease from R. prolixus (Hemiptera: Reduviidae; ACF70480.1; 55% identity), Bombus hypocrita (Hymenoptera: Apidae; AEN41590.1; 49%), Danaus plexippus (Lepidoptera: Nymphalidae; EHJ74921.1; 46%), Culex quinquefasciatus (Diptera: Culicidae; XP_001845947.1; 46%), A. aegypti (Diptera: Culicidae; XP_001662898.1; 45%). Therefore, it was referred to the protein as E. integriceps trypsin (Tryp). This cDNA sequence has been deposited in GenBank under accession number KP295958. High level of conservation of the amino acid sequence among different insects Cys proteins and also Tryp proteins indicates that the Cys and Tryp proteins are highly conserved during the evolution of insects.

Phylogenetic Analysis

To understand the relationship between insect cathepsin-L cysteine and trypsin serine protease and Sunn pest genes, phylogenetic analysis was performed using cDNA sequences available in GenBank. Neighbor-joining trees were constructed using amino acid sequences and a Poisson-corrected distance with bootstrap test of 1,000 replications. Phylogenetic analysis of proteins from insect species in the orders of Hemiptera, Diptera, Hymenoptera, Coleoptera, and Lepidoptera showed that Cys and Tryp from the Sunn pest E. integriceps were closely related to Lygus hesperus and R. prolixus with a bootstrap value of 61 and 84%, respectively (Figs. 2 and 3).

Sunn Pest Response to Different Host Plants

This experiment was done to investigate the effects of different hosts on the transcript levels of Cys, Tryp, and Gl genes. Wheat is the preferred host for the Sunn pest. However, the insect can also survive on barley, rye, and triticale. To gain insight on the impact of different host types on the expression of Cys, Tryp, and Gl genes, the expression patterns of the genes in the Sunn pest larvae feeding on wheat, barley, rye, and triticale kernels were examined. To determine tissue- and host-dependent expression of Cys, Tryp, and Gl, total RNA samples from the salivary glands and midgut were analyzed by real-time quantitative PCR. The results showed that these genes were differentially expressed in Sunn pest tissues and in response to different host plants kernels. The expression of these three genes was affected by host genus. In the all host plants tested, Gl and Tryp mRNA were noticeably expressed in both salivary glands and gut of the larvae of E. integriceps, however Cys was expressed only in the gut and there was not any detectable Cys transcript in the salivary glands. It was shown for wheat-fed larvae in Figure 4. Salivary glands had up to 5.68-fold higher Gl transcript level than gut, however there was no significant difference between salivary glands and gut Tryp (Fig. 4; P < 0.05). Salivary glands Gl and Tryp transcript levels decreased significantly in “rye and triticale” and “barley and rye” fed larvae, respectively, (Fig. 5a and b) comparing with that of the control group (wheat-fed larvae; P < 0.05). The expression of gut Cys mRNA decreased significantly (P < 0.05) in barley- and rye-fed larvae, whereas the gut Gl mRNA expression decreased significantly only in the rye host (P < 0.05) when compared with control group (wheat; Fig. 5c and d). For the Sunn pest larvae fed with all host kernels, gut Tryp was about significantly equally expressed (Fig. 5e). Wheat-fed larvae had higher Tryp transcript level but it was
Identification of Cysteine and Trypsin and RNAi

Figure 4. Relative expression of different genes in salivary glands and gut of wheat fed fifth instar larvae of *E. integriceps*. Cysteine (a), gluten hydrolase (b), and trypsin (c). Ct values were first normalized to the endogenous control gene 18S ribosomal RNA gene, followed by normalization to the control without treatment using the $2^{-\Delta\Delta Ct}$ method. Each kinetic point was performed in triplicate on five pooled larvae. Asterisks indicate significant difference ($P < 0.01$) according to the iteration test (Rest 2009 Software). The values represent averages with vertical bars indicating SE.

not statistically significant. In conclusion, all these results indicated that the application of all-plant-based diet could decrease gene expression when compared with wheat.

Also the effect of feeding on different hosts on the larval duration was evaluated. Feeding on barley, rye, and triticale increased developmental time significantly ($P < 0.05$). The duration of the third instar stage was reduced in wheat-fed larvae (Fig. 5f) but there was no significant difference in other larval stages. The control diet (wheat) was the most favorable for larval survival.

**Effect of Topical RNAi Treatments on Cys Transcript Abundance**

The effect of Cys-dsRNA on knockdown of cathepsin-L cysteine gene of fifth instar larvae was evaluated by qRT-PCR 1, 2, and 3 days after topical application of dsRNA. No expression was detected in the salivary glands of control and Cys-dsRNA-treated Sunn pest. However, gut transcript revealed a high induction of cathepsin-L cysteine expression (61-fold) in comparison with control insects 1 day after topical application of Cys-dsRNA (Fig. 6a). Then transcript abundance of gut Cys indicated extreme reduction (83.8%) at the second day posttreatment. At third day, expression level of gut Cys increased and equaled to the control level (Fig. 6a).

**Effect of Topical dsRNA Treatment on Developmental Processes**

The effects of dsRNA on the developmental process such as weight, larval duration, adult emergence, mortality, and abnormality of fifth instar larvae were evaluated. Body
Figure 5. Impact of feeding on different host plants kernels. (a) The levels of salivary transcripts encoding gluten hydrolase and (b) trypsin serine protease. (c) Levels of gut transcripts encoding cysteine, (d) gluten hydrolase and (e) trypsin serine protease. (f) Third instar duration of the Sunn pest. The duration of the third instar was estimated from those that survived and eclosed as fourth instar larvae ($P < 0.05$). (a–e) x-Axis represents fold changes of specific transcripts in insects feeding on either barley (B), rye (R), or triticale (T) in comparison with transcript levels in insects feeding on wheat (W) kernels. Ct values were first normalized to the endogenous control gene 18S ribosomal RNA gene, followed by normalization to the control without treatment using the $2^{-\Delta\Delta Ct}$ method. Each kinetic point was performed in triplicate on five pooled larvae. Asterisks indicate significant difference ($P < 0.01$) according to the iteration test (Rest 2009 Software). The values represent averages with vertical bars indicating SE.

weight was measured for survivors up to adult emergence. The treated larvae with Cys-dsRNA tended to gain slightly higher weight 3 days posttreatment than those receiving the control treatment ($P < 0.05$; Fig. 6b). Topical application of different Cys-dsRNA concentrations decreased the time of the molt of the corresponding instar. So the duration of the fifth instar stage was reduced following treatment (Fig. 6c). Different Cys-dsRNA concentration treatments (100, 500, and 1,000 ng/μl) had the duration of 7.5, 7.8, and 7.7 days, respectively. While in control larval duration was 8.9 days. ANOVA analysis revealed that there was significant difference between control and 100 ng/μl dsRNA-treated larvae (Fig. 6c). Although all Cys-dsRNA treatments reduced adult emergence
Figure 6. Effect of Sunn pest cysteine gene (Cys) knockdown on (a) fifth instar midgut Cys expression, (b) body weight, (c) fifth instar duration, (d) adult emergence (%), (e) mortality (%), and (f) abnormality (%) of *E. integriceps*. Relative mRNA level were monitored by qRT-PCR over 3 days after treatment with 500 ng dsRNA. Ct values were first normalized to the endogenous control gene 18S ribosomal RNA gene, followed by normalization to the control without treatment using the $2^{-\Delta\Delta Ct}$ method. Each kinetic point was performed in triplicate on five pooled larvae. Asterisks indicate significant difference ($P < 0.01$) according to the iteration test (Rest 2009 Software). The values represent averages with vertical bars indicating SE. The duration of the fifth instar was estimated from those that survived through all experimental periods and eclosed as the adult.

Body weight was measured 3 days after topical application. Different concentrations of dsRNA (100, 500, and 1,000 ng) was used for b, c, d, e, and f. The means topped with the same letters are not statistically different at $P < 0.05$.

compared to control, however only 100 ng/$\mu$l dsRNA caused significantly lower adult emergence ($P < 0.05$; Fig. 6d). After the administration of dsRNA by topical application, we monitored insects daily to evaluate survival rates, over a 10-day period. The mortality of larvae treated with Cys-dsRNA (100 and 500 ng/$\mu$l) was significantly higher than that of control (Fig. 6e). After 10 days, a mortality rate of approximately 23, 50, 46.67, and 26.67% was observed in the control and different dsRNA concentrations (100, 500, and
1,000 ng), respectively. Also, malformed ecdysis was observed in the Cys-dsRNA treatment ($P < 0.05$; Fig. 6f). High percent of abnormality (41.9%) was observed in 500 ng dsRNA-treated insects. Abnormality occurred during larval ecdysis to adult. Deformed nymphs died during ecdysis.

**DISCUSSION**

*Eurygaster integriceps* causes serious economic losses to cereals especially to wheat and barley in wide areas of the world almost every year (Konarev et al., 2011; Malschi et al., 2012; Karimzadeh et al., 2014). *Eurygaster integriceps* larvae are greedy feeders, and their salivary glands and gut proteases digest diet proteins to utilize for growth and development. Since proteases play important roles in a wide range of physiological processes include diet digestion, it is important to study proteases to use them in insect integrated pest management. Majority of proteases are transcribed during larval feeding belong to the serine (chymotrypsins, trypsins, and serine carboxypeptidases) and cysteine (cathespsins B and L) protease families (Edwards et al., 2010; Ben-Mahmoud et al., 2015).

In this study, for the first time cDNAs encoding a cysteine and trypsin (named Cys and Tryp, respectively) were isolated and identified from the Sunn pest, *E. integriceps*, and then tissue (salivary glands and gut) differentially Cys, Tryp, and Gl (gluten hydrolase) gene expression was studied. The Cys transcript was detected exclusively in the gut, whereas the Gl and Tryp transcript were detectable in both salivary glands and gut. Serine and cysteine proteases have been reported from different Hemiptera. Partial characterization of a trypsin-like serine proteinase was reported in the salivary glands of the hemipteran, *Lygus lineolaris* and *L. hesperus* (Hemiptera: Miridae; Zeng et al., 2002a,b). Bao et al. (2014) studied serine protease gene family and expression profile analysis in the planthopper, *N. lugens*. They showed gene expression profiles revealed differential response of serine protease to bacterial infections as well as their development-, tissue- and sex-specific expressions. They reported various trypsins from different tissues such as midgut, salivary glands, ovary, the male reproductive system, fat body, and carcass. Cysteine protease genes were identified in the genome of hemipteran insect *A. pisum* by Rispe et al. (2008).

We investigated whether and how different host plants affect the transcription levels of Sunn pest various protease genes. Host plant quality can significantly influence gene expression and the insect growth (Oppert et al., 2010; Vogelweith et al., 2011; Wang et al., 2013; Spit et al., 2014; Wang et al., 2015). Likewise, in this study results clearly indicate that host plants quality can affect gene expression. So transcript levels of Cys, Tryp, and Gl were most abundant in the wheat-fed Sunn pest larvae in compare to other hosts. Such results were observed in other insects. Up to105 putative serine and cysteine protease genes and their developmental stage and tissue-specific expression profiles were identified from the genome of the gall midge *Mayetiola destructor*, another destructive pest of wheat. The expression of some trypsins and cysteine proteases were upregulated in *M. destructor* larvae feeding in resistant plants, whereas others were downregulated (Chen et al., 2013).

RNAi has potential as a crop protection strategy against important pest insects. It has been widely and successfully used for gene inactivation in insects. Since it has a high sequence-dependent specificity, RNAi is a species-specific technique for insect pest control (Huvenne and Smagghe, 2010). Thus, the results presented here suggest a novel control strategy for *E. integriceps*, using dsRNA specific to the Sunn pest gene/s. Cysteine proteases play critical roles in digestion, immune response, and regulation of insect
Identification of Cysteine and Trypsin and RNAi development. So it could be useful targets for pest management by RNAi like in other insects (Gruden et al., 2005; McGonigle et al., 2008). Since cysteine expression disruption in the Sunn pest larvae could seriously affect fitness and development of the insect, in this study possibility of Sunn pest gene silencing was studied by topical application of cysteine dsRNA.

In this study, it was found that topical application of dsRNA reduced significantly expression level of Cys gene in the nymphal gut 2 days posttreatment. However, upregulation of Cys was observed 1 day after topical application of Cys-dsRNA to compensate dsRNA effect. Such result was observed by Sapountzis et al. (2014). They reported induction high levels of cathepsin-L expression in the pea aphid, 1 day after cathepsin-L dsRNA treatment. However, it decreased 3 days post-dsRNA administration. Also, Chu et al. (2014) demonstrated that cysteine dsRNA knocked down gut cysteine protease (cathepsin-L) in the western corn rootworm Diabrotica virgifera virgifera. Interestingly, they observed some dsRNA-treated insects had over fivefold higher cysteine transcript abundance than control.

The effects of topical application of the dsRNA on survival, development, and adult emergence were examined. The data showed that Cys gene silencing had diverse effects on the fifth nymphal stage development including change in duration of the nymphal stage, reduction of adult emergence, increasing deformed adult, increasing the adult weight. These effects as a result of Cys gene knockdown clearly indicated that this gene product plays important physiological role(s) during development and metamorphosis of the Sunn pest.

Results showed that 100 ng/μl dsRNA was more effective in gene silencing and nymphal stage development such as weight, mortality, and adult emergence as compared to other concentrations. Similar results have been reported by Asokan et al. (2014). They evaluated effect of two concentrations of dsRNA (10 and 20 μg) in Helicoverpa armigera trypsin transcript abundant. They reported that 20 μg dsRNA concentration had more effect on gene silencing, pupation and larval, pupal weight as compared to 10 μg. Also different concentrations (1, 5, 10, 20, and 40 μg/ml) of dsRNA were tested in B. tabaci. Higher concentrations had greater impact in target genes silencing and led to higher mortality (Upadhyay et al., 2011). In the other experiment, a series of concentrations (0, 5, 10, 20, and 40 μg/ml) of P450 cytochrome dsRNA was added to B. tabaci diet (Li et al., 2014). Feeding led to gene silencing and high mortality. For 5 μg/ml dsRNA, reduction of gene transcript was observed after 4 days dsRNA feeding, and in treatments with 10, 20, and 40 μg/ml dsRNA, gene silencing was occurred after 2 days dsRNA feeding. By the way, higher concentrations of dsRNA induced more silencing. Likewise, Yu et al., (2014) silenced ecdysone receptor in the brown planthopper N. lugens, and showed mortality increased with an increase in dsRNA concentration.

Also, in this experiment a significant higher number of abnormality was observed during ecdisis in treated bugs than in the control insects suggesting the involvement of this protease on the insect ecdisis. The involvement of cathepsin-L in different insect molting has been demonstrated (Hashmi et al., 2002; Liu et al., 2006; Zhang et al., 2013; Chen et al., 2014; Sapountzis et al., 2014). As our results, successful RNAi of cathepsin-L in the pea aphid, A. pisum, was reported by Sapountzis et al. (2014). They found for the first time in a hemimetabolous insect, new function for the cathepsin-L gene as molting enzyme. They observed cathepsin-L knockdown caused alteration of aphid body shape and induced high mortality. Also Chen et al. (2014) indicated that in Spodoptera litura knockdown of cysteine transcripts with dsRNA caused prepupal, pupal, and adult phenotypic changes.
To conclude, we have for the first time identified two different genes encoding cysteine protease, and trypsin in the Sunn pest and analyzed their expression in addition to gluten hydrolase gene in different tissues on the Sunn pest reared on various hosts. Variation in host plant strongly affects the expression of protease levels. Then we studied possibility of cysteine gene silencing using dsRNA. Cys gene silenced through RNAi system caused diverse effects on the insect growth and development, that is, it affects survival, development, and adult emergence including change in duration of the nymphal stage, reduction of adult emergence, increasing deformed adult, increasing the larvae weight. Also, knockdown of Cys transcripts with dsRNA caused reduction of nymph gut transcript. This study is the first to report that the specific inhibition by RNAi of Cys protease causes high gene transcript knockdown, insect mortality, and abnormality during Sunn pest development.

**Compliance with Ethical Standards**

I certify that we have complied with Ethical Standards in the conduct of the research presented in this manuscript. All institutional and national guidelines for the care and use of laboratory animals were followed. Also we certify that:

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**CONFLICT OF INTEREST**

All authors have no conflict of interest.
LITERATURE CITED


Archives of Insect Biochemistry and Physiology

Identification of Cysteine and Trypsin and RNAi


