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RNA interference of cysteine protease genes for the management of whitefly (*Bemisia tabaci*) by oral route

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The invasive whitefly (*Bemisia tabaci*) feeds on phloem sap and distributes harmful viruses, causing considerable crop losses. These insects pose a severe danger to global food security and agriculture. Whitefly management is challenging, and standard methods of control have several drawbacks. RNA interference (RNAi) appears to be a fantastic tool for pest control. This method provides a direct mode of action on specific genes, allowing altering physiological systems that affect insect growth, development or feeding behavior. The cysteine protease genes were used to create four dsRNA fragments. A simpler insect bioassay approach was established for the oral administration of dsRNA. The oral bioassay was performed by feeding 200 adult whiteflies on dsRNA mixed with an artificial solution. The dsRNA fragments were employed at three concentrations (5, 15, and 30µg/mL). Every day for four days, the mortality of adult whiteflies was reported. After 96 hours of feeding on cysteine proteases dsRNAs conc 30 µg/mL, the average mortality percentage was 73.875% for treated insects compared to 11% for the control. Quantitative PCR analysis of the treated insects revealed a substantial decrease in cysteine protease transcript levels, with mRNA levels dropping by 35, 2.11, 5.24, and 6.779 times because of cathepsin L cysteine protease genes fragments A, B, C, and D dsRNA, respectively. The expression of cysteine protease dsRNAs in transgenic plants for whitefly protection might be an intriguing use of this technology.

Keywords: *Bemisia tabaci*; RNA interference (RNAi); Cysteine protease; Whitefly

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INTRODUCTION

One of the most harmful sap-sucking insects is the whitefly. It reduces both the amount and quality of yield. Over 1000 whitefly species have been identified globally. *Bemisia tabaci* is a cryptic species complex that includes at least 24 morphologically distinct species (De Barro et al., 2011; Hu et al., 2011; Luan et al., 2011; Shadmany, Omar, and Muhamad, 2013). The whitefly (*Bemisia tabaci*) biotype B is one of the most pestiferous in this group because it spreads swiftly between field crops and weeds, excretes sticky honeydew, causes yellowing or death of leaves, and invades greenhouse vegetable crops (Pizer, 2006). The transfer of several viruses, particularly those from the geminivirus group, is the deadliest component of whiteflies. *Bemisia tabaci* is a vector of over 100 distinct begomovirus species (Alemandri et al., 2012).

Proteinases of many sorts are used as digesting enzymes by sucking insects. Proteinases are enzymes that aid in the digestion of proteins and are involved in a wide range of biological activities, including cell proliferation, morphogenesis, tissue remodeling, homeostasis, wound healing, immunogenicity, apoptosis, and food digestion (Lee et al., 2010; Reichhart et al., 2011; Voos et al., 2013; Karlsson et al., 2004; Gorman et al., 2000; Manoury et al., 2011; Zhang and Li, 2012; Saadati and Bandani, 2011). Insects use salivary gland and stomach digestive enzymes to break down food proteins into peptides and amino acids (Sato et al., 2008; Dunse et al., 2010a, b). Sucking insects use a variety of proteinases as digestive enzymes. Proteinases may be divided into four types: serine-type, cysteine-type, aspartate-type, and metallo-type. Proteinases produced in the alimentary canal of insects are diverse and polymorphic, allowing them to degrade proteins into tiny peptides or single amino acids. Insects may also self-sterilize against a wide range of dietary poisons and anti-nutritional substances contained in their host plants thanks to these enzymes (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 essential enzymes in a variety of invertebrate species, responsible for functions such as digestion (Goptar et al., 2012), embryogenesis (Hashmi et al., 2002), moulting (Liu et al., 2006), tissue remodeling (Wang et al., 2009), detoxification (Koo et al., 2008), plant defensive proteins, and immune responses (Zhang et al., 2013). Hemipterans employ cathepsin to digest food, according to Terra (1990). Cysteine proteases are the most researched digestive enzymes, as they are the principal digestive enzymes in the Coleoptera, Diptera, Hemiptera, and Hemiptera families, respectively. Cathepsins are cysteine proteases engaged in various natural processes such as protein degradation, apoptosis, and signaling, and their activity in lysosomes has been linked to disease transmission.

Whiteflies are extremely tough to control. Many physical and mechanical approaches, such as indigenous technical knowledge of biological control, plant-based goods, spray of synthetic pesticides, and biotechnology strategies, have been developed recently (Abubakar et al., 2022). The first reason for the difficulty of whitefly control is that it is difficult to target whiteflies with contact insecticides because they feed from the bottom of the leaves. Additionally, research has shown that using pesticides with broad modes of action repeatedly is costly both economically and biologically; as such methods result in losses in non-pest insects such as beneficial pollinators or natural enemies that feed on *B. tabaci* (Simmons and Jackson, 2000; Simmons and Abd-Rabou, 2005). Furthermore, *B. tabaci* has developed resistance to most pesticides (Horowitz et al., 2020). Other pest control methods, such as introducing Bacillus thuringiensis (Bt) transgenic crops, are ineffective against whiteflies and other sap-sucking insects because they lack sensitivity to Cry damaging proteins (Chougule and Bonning, 2012). Other strategies, such as biological control measures, have shown some effectiveness, but they are frequently incapable of promptly reducing pest densities to levels that minimize economic losses (Yang et al., 2011). and rely on further pesticide spraying. As a result, new control strategies are required, particularly those that may be precisely targeted against *B. tabaci*. Biotechnological Strategies for Whitefly Control RNA interference (RNAi), a genetic engineering approach, is a great pest management method.

This approach allows for a direct mode of action on specific genes, allowing for modifying several physiological systems to change insect growth, development, or feeding behavior. This gene-silencing process uses an evolutionary old strategy that cells utilize to protect the genome against RNA viruses and other genetic material (Shukla *et al*., 2016). RNAi has effectively targeted many genes in *B. tabaci*. In 2017, six whitefly genes (Trehalose transporter 1, Aquaporin 1, Heat shock protein 70, Acetylcholine receptor subunit, Trehalase1, and Alpha glucosidase 1) were isolated from the B biotype of *B. tabaci*'s alimentary tract. The effectiveness of RNAi knockdown was demonstrated in a gene-specific functional bioassay, and mortality was tracked at 24-hour intervals for six days after treatment. Real-time PCR analysis revealed that all six genes had significantly decreased gene expression. The high mortality of whiteflies was connected to the down-regulation of osmoregulation, sugar metabolism, and sugar transport-associated genes, suggesting that whitefly mortality was linked to RNAi findings (Vyas et al., 2017).

In 2021, a study of three genes (BtCht10, BtCht5, and BtCht7) in the moulting of *B. tabaci* nymphs revealed a decreased percentage of survival when these three genes were targeted with dsRNA using a nanomaterial-promoted RNAi approach. Furthermore, the silencing of BtCht10 occurred throughout a longer period of development in comparison to control nymphs (Zhengke Peng *et al.*, 2021). Three TPS genes were found and cloned from *B. tabaci* MED, and scientists determined that the BtTPS1 and BtTPS2 genes expressed more than the BtTPS3 gene. Adult whiteflies' expression of associated genes involved in energy metabolism and chitin production was then altered by RNA interference (RNAi) of BtTPS1 and BtTPS2, which resulted in considerable mortality. Finally, knocking down BtTPS1 or BtTPS2 resulted in limited hatchability, slowed growth in whitefly nymphs, and 90% mortality and decreased fecundity in adult whiteflies. Transgenic tobacco plants influenced *B. tabaci* as well. Furthermore, transgenic tobacco with combined RNAi against BtTPS1 and BtTPS2 was more effective against whiteflies than individual silencing (Gong et al., 2022). Furthermore, dsRNA targeting heat shock protein 70 (hsp70) and fasciclin 2 (fas2) of *B. tabaci* Asia II 1 was first evaluated under controlled conditions via oral delivery. The previously mentioned study demonstrates for the first time that a topical spray of naked double-stranded RNA (dsRNA) on chilli plants causes mortality and the inability to acquire and transmit ChiLCV in *B. tabaci*. Hsp70 and fas2 dsRNA killed up to 82.22% and 72% of *B. tabaci*, respectively, while mRNA levels were reduced by 12.4 and 8.5 times (Chakraborty, 2022).

Scientists discovered in 2022 that silencing BtGR11 with RNA interference prevented whiteflies from sensing sucrose. BtGR11 is a candidate taste receptor gene that regulates taste sensitivity to sucrose. This study provides information on a critical chemoreceptor gene that can be used to understand the gustatory sensing mechanisms whiteflies use as a deterrent (Li et al., 2022). Adult *B. tabaci* were substantially more likely to die from TLR3 and TOB1 silencing as compared to the untreated control. ChiLCV copies in *B. tabaci* were drastically reduced after exposure to TLR3 and TOB1 dsRNAs. TLR3 and TOB1 silencing reduced *B. tabaci*'s ability to transmit ChiLCV (Thesnim et al., 2023). Scientists also used VIGS to target five Bemisia tabaci (whitefly) HTGs,

proving that knocking down part of these genes altered whitefly survival (Feng et al., 2023). Because cysteine protease genes play essential roles in insect digestion and development (Goptar et al., 2012; Zhang et al., 2013; Ben-Mahmoud et al., 2015), they can be used to build novel tools for integrated pest control. Following sequence identification, this investigation chose cathepsin-L cysteine protease and cathepsin-L-like cysteine proteases as target genes for RNAi gene silencing.

MATERIALS AND METHODS

Maintenance of whitefly cultures

B. tabaci (biotype B) whiteflies were reared in colonies on plant leaves. Whiteflies were maintained in insect-proof cages in a growth chamber at 26 °C with a 16 h photoperiod.

RNA isolation

Total RNA was extracted from whiteflies using a total RNA isolation kit (GeneDirex; Cat. no. NA021-0100), using instructions provided by the manufacturer. To remove potential DNA contamination, total RNA samples were treated with Promega DNase I (Cat. no. z358b).

First-strand cDNA synthesis

For reverse transcription of RNA 1 µg of total RNA, 2.5 µl of 20 µM Oligo dT primer stocks was added (final concentration 2.5 µM) to the RNA sample and added RNase-free H_2O to a final volume of 11.5 µl. The mixture was then heated at 70°C for 3 min and immediately cooled on ice. After that, the reaction was centrifuged briefly, and 4 µl 5X first-strand buffer, 2 µl dNTP mix, 2 µl 100 mM DTT a 0.5 µl SMART M MLV RT (Clonetech, USA, Cat# K1622) was added and incubated at 42 °C for 60 min and the reaction was terminated by heating at 90 °C for 10 min.

PCR amplification and cloning of cysteine protease genes

The template cDNA was denatured at 95 °C for 5 min followed by 30 cycles of heating at 95 °C for 30 secs, annealing for 30 secs, and extension at 72 °C for 30 secs and the reaction ended by 7 min at 72 °C. The amplification reaction was performed by Go Taq flexi (Promega, USA, Cat# M891A) and was comprised of 5 µl go Taq buffer (5x), 2 µl 25 mM $Mgcl_2$, 2 µl 2.5 mM dNTPs, 2.5 µl of 10 pmole primer set and 1.25 unit go Taq polymerase. Primers were designed from highly conserved regions of other insect genes using Vector NTI® program software (Life Technologies, USA); we used primer 3 (online website primer design) to make the 3 specific primers; the primers sequence and the expected amplified fragments are presented in (Table 1). The three amplified fragments were cloned at the multi-cloning site in the pDrive cloning Vector (QIAGENE, Cat# A137A231124). The ligation reaction was comprised of 5 µl of 2X buffer, 3 units of T4 DNA ligase, 3 µl of the PCR product, and 1 µl vector overnight at 4°C. The ligation mixture was then transformed into DH10β competent cells prepared by CaCl2.

Sequencing of the cysteine protease genes

The cloned fragments were analyzed sequentially using the Big TriDye sequencing kit (ABI Applied Biosystems) at the Macrogen, Korea facility. The sequences of the three fragment cysteine protease genes were confirmed by a homology search of other cysteine protease gene sequences known within the Blast programs of the National Center for Biotechnology (NCBI), USA (WWW.ncbi.nlm.nih.gov/Blast).

Selecting and preparation of dsRNA (RNAi) fragments

The selection of the dsRNA fragment sequence depended totally on the conserved regions of the gene sequence because the dsRNA primers were designed based on these conserved regions. The length of the dsRNA fragment was 300-700 bp. Four dsRNA fragments were prepared to target the cysteine protease genes using the following primer sets " Fragment A FDS/ Fragment A RDS", " Fragment B FDS / Fragment B RDS", " Fragment C FDS / Fragment C RDS", "Fragment D FDS / Fragment D RDS" These fragments were 443, 311, 623, 448 bp in size, respectively. All the dsRNA primer sequences contained the T7 promoter on their 5' end, as shown in (Table 2). The cloned vectors from the previous step were used as templates for PCR amplification. The template was heated at 95 ºC for 5 min, followed by 25 cycles, including 95 ºC for 30 seconds, annealing at 60 ºC for another 30 seconds and 72 ºC for 30 seconds, and ending at 72 ºC for 7 min.

Purification of PCR product

The four PCR products were purified using a QIAquick PCR purification kit following the manufacturer's instructions. The purified DNA product was quantified by measuring its absorbance at 260 nm using the Nanodrop and visualization on 1% agarose gels using safe green staining and U.V. trans-illumination to

ensure that the bands were sharp, and the quantity of the purified DNA was compatible with the nanodrop readings.

Preparation of dsRNA (RNAi) fragments

According to the manufacturer's instructions, four dsRNA fragments were generated using the MEGA script® RNAi Kit (Ambion Cat#AM1626). The transcription reaction was assembled from 2 µg of linear template DNA possessing T7 promoter sequences on both ends using T7 RNA polymerase. The reaction was processed in 20 µl, including 2 µg linear template DNA, 1X T7 reaction buffer, 3.75 mM of ATP, UTP, GTP, CTP solutions and 2 µl T7 enzyme. The reaction was incubated overnight at 37°C. This transcription reaction should produce RNA products that typically hybridize during the reaction. The DNA and ssRNA were removed by DNase/RNase treatment. A volume of 20 µl of dsRNA (prepared in the previous step) was treated with 2 µl DNase I (4 units), 2 µl RNase, and 1X digestion buffer. The digestion reaction was incubated at 37 °C for 1 h. The dsRNA was then purified from proteins and free nucleotides by adding binding buffer mix and filter cartridge, followed by centrifugation. Before starting the purification process, the elution buffer was heated to 95 °C for 5 min. The binding reaction was assembled in 500 µl total volume by adding 50 µl dsRNA, 1X binding buffer, and 250 µl 100% ethanol. The reaction was gently mixed by pipetting up and down. The binding mix was applied to the filter cartridge and drawn by centrifugation at maximum speed for 2 min. The flow-through was discarded. The dsRNA bound to the Filter Cartridge was washed twice with 500 µl of wash solution followed by centrifugation. After discarding the wash solution, the filter cartridge was spun down to remove any traces of the wash solution. The dsRNA was recovered twice by incubating for 2 min at 65 °C with preheated nuclease-free water and centrifugation at maximum speed for 2 min. The elution step is repeated to retain as much dsRNA as possible. The dsRNA product was quantified by measuring its absorbance at 260 nm by the Nanodrop as well as by visualization on 1% agarose gels and U.V. trans-illumination to ensure that the bands were sharp, and the quantity of synthesized dsRNA was compatible with the Nanodrop readings.

Bioassay of the dsRNA fragments

The feeding method delivery was used to determine the efficacy of Cysteine protease dsRNA fragments on whitefly insects. The dsRNA was mixed with the diet (20% sucrose solution). Three different concentrations of each dsRNA fragment were separately mixed with the diet, i.e., 5, 15, and 30 µg/mL diets. The mortality of adult whitefly insects was recorded after 24, 48, 72, and 96 hours (Figure 1).

Quantitative measurement of targeted gene transcripts

To ensure the reliability of our results, the number of transcripts of the targeted gene was measured by quantitative Real Time-PCR (qRT-PCR). Following the manufacturer's instructions, this involved a meticulous RNA extraction process from the whole whitefly using a total RNA isolation kit (GeneDirex; Cat. no. NA021-0100). The SMART™ MMLV Reverse Transcriptase Kit (Clonetech, USA, Cat# K1622) synthesized cDNA using oligo dt primers. Equal volumes of the same concentration from the RNA samples were used to eliminate concentration differences in the templates. RNA concentrations in the products of RNA extraction were measured, and proper dilutions with ultra-pure water were made to ensure 1000 ng of RNA in the 10 µl cDNA reaction solutions. Quantification of the targeted gene by real-time PCR. The qRT-PCR was performed in an AB7300 real-time PCR system® (Applied Biosystems, USA) using SYBR® *Premix ExTaqII*™ from TAKARA (Catalog no. RR82SW, TAKARA, Japan). Specific primers were designed to amplify the targeted gene using amplify software and the NCBI Primer-BLAST. The fragments were amplified using the primer sets (Table 3) in Whitefly to compare the gene expression in the control. The thermal cycler program was as follows: the first step (as a hot start step) at 95 ºC for 30 seconds, followed by the second step, which consisted of 43 cycles at 95ºC for 5 seconds, 60 ºC for 34 seconds, then the melting curve step of 95 ºC for 15 seconds.

RESULTS

RNA isolation and cDNA synthesis

Total RNA was isolated from adult whiteflies and the total RNA was determined by nano-drop analysis. In addition, the extracted RNA was visualized on 1% agarose gels to ensure that the quality and quantity of the extracted RNA from adult whiteflies were suitable (Figure 2). The gel showed no degradation of the ribosomal RNA bands confirming the integrity of the isolated RNA and then cDNAs were synthesized. Three specific primers were used in the PCR reaction. To amplify different fragments of the cysteine protease genes from adult whiteflies.

Table 1. The names, nucleotide sequences and annealing temperatures of the primers used for cloning the cysteine protease genes, and the expected size of the amplified fragments.

Name of primer	Sequence		Expected Band Result	Ann. Temp. (°C)
Cys 1	F	TTCCGGCAACAAATGGAAGG	1001bp	57 °C
	R	AGCGATTAGCGCCCCTTTTC		
Cys 2	F	TGCAGCCAACTATAATACTCCTAGC	1010bp	56 °C
	R	CGGATAGCTAGCGGCAGTG		
Cys 3	F	CGCCTTGGATTTCTGCTTCAG	1000 bp	55 °C
	R	GTACCCTTTGTCGCCCCATC		
B. actin	F	TCTTCCAGCCATCCTTCTTG	175bp	55 °C
	R	CGGTGATTTCCTTCTGCATT		

Table 2. Name and nucleotide sequence of primers used for amplifying the dsRNA fragments for the cysteine protease genes and the expected size of the amplified fragments.

Primer name	Sequence and direction	Size (bp)
Fragment A FDS	5'TAATACGACTCACTATAGGGAGAAGAGCACAATGAGCGGTACA'3	
Fragment A RDS	5'TAATACGACTCACTATAGGGAGACTTGTGTCATCAAACCACCC'3	443bp
Fragment B FDS	5'TAATACGACTCACTATAGGGAGATCCTTATGAAGGAGGGGAAAA'3	311bp
Fragment B RDS	5'TAATACGACTCACTATAGGGAGAATCTCGGACCCCAGGAATTT'3	
Fragment C FDS	5'TAATACGACTCACTATAGGGAGATCAACAGGAATACAGAAGCA'3	623bp
Fragment C RDS	5'TAATACGACTCACTATAGGGAGACCACACAGCCACTTTAAGAGC'3	
Fragment D FDS	5'TAATACGACTCACTATAGGGAGACCAACGTTGTACTGCCAAGA '3	448bp
Fragment D RDS	5'TAATACGACTCACTATAGGGAGAAACTGGAAGTCTTCTGCGCT'3	

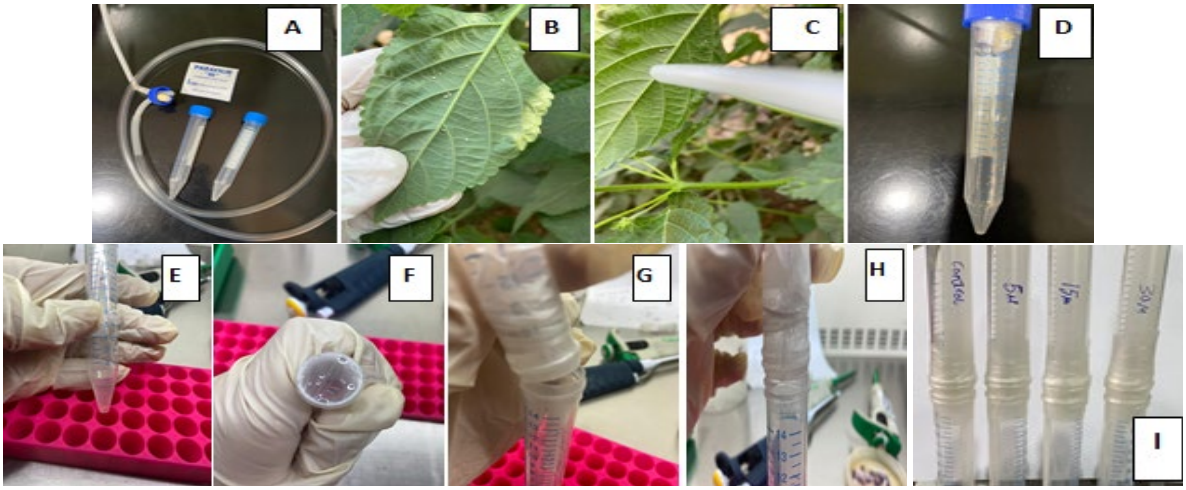

Figure 1. Collection of whiteflies and bioassay. (A) material of collection and bioassay; (B) adult whiteflies; (C&D) collection of adult whiteflies in bioassay vial from leaves; (E) preparation of diet pouch; (F) using sterilized stretched Para film on the cap of bioassay vial; (G&H) replacement of insect vial cap with the Para filmed inversed vial containing the diet; (I) whiteflies feeding on the diet.

Table 3. Primer sequences used for quantitative PCR analysis of cysteine protease genes with different dsRNA fragments and the internal control B.actin gene.

Primer	Sequence		Expected size
Cys 1RT	F	GCGGAACGAAACCACTTTTG	143
	R	ATAATCGCCATCACAGTCGG	
Cys 2RT	F	GTATCTTCCACTGTTTCCGGG	137
	R	AAGGTGTTTCTCAAGGAGCC	
Cys 3RT	F	CGACACTGAAATCTCTTACCCC	116
	R	TCTGATTCGCTACCTGATTCG	
B.actin	F	CGCTGCCTCCACCTCATT	129
	R	ACCGCAAGATTCCATACCC	

The three sets of primers revealed the expected fragments. Following the first set was "cys 1 F, cys 1R", amplified a PCR fragment (cysteine protease gene fragment 1) about 1001 bp in size. The second set was "cys 2F, cys 2R" amplified a PCR fragment (cysteine protease gene fragment 2) about 1010 bp in size. The third set was "cys 3F, cys 3R", amplified a PCR fragment (cysteine protease gene fragment 3) about 1000 bp in size, as described in Table 1 and shown in Figure 3. The RT-PCR products were cloned in the pDrive cloning vector. Recombinant vectors were transformed into DH10β competent E. coli cells. The recombinant plasmids were isolated from the white colonies. Then, they were confirmed by PCR using specific primers (Figure 4) and EcoRI digestion after making miniprep for them (Figure 5). Both confirmations showed the expected sizes of the three cysteine protease gene fragments.

Computational analysis of the cysteine protease gene fragments sequence

The three fragments were subjected to sequencing. Sequences of the selected three fragments were as follows: "Cysteine protease gene Fragment 1" length was 956 bp (Gene bank accession no. OR878547). "Cysteine protease gene Fragment 2" length was 839 bp (Gene bank accession no. OR882889). "Cysteine protease gene Fragment 3" length was 981 bp (Gene bank accession no. OR865753). The nucleotide sequence and the deduced amino acid sequence of each fragment was compared with previously published gene sequences from various databases in GenBank using the basic local alignment search tool (BLAST) (Altschul *et al.*, 1997). The three sequences of selected fragments showed significant homology when analyzed by Blastx and Blastn. Cysteine protease gene Fragment 1 and Cysteine protease gene Fragment 2 showed significant homology with two cathepsin L–like cysteine protease gene regions 372 to 1210 bp and 292 – 1251 bp, respectively, and the third one, Cysteine protease gene Fragment 3 showed significant homology with cathepsin L cysteine protease gene regions 278 to 1214 bp.

Preparation of the dsRNA targeting cysteine proteinase genes

Four dsRNA fragments were prepared to knock down the cysteine proteinase genes. The dsRNAs were synthesized using the PCR technique. The primer sets were designed around the conserved regions of the obtained sequences. Generally, one primer was prepared for the cathepsin L gene covering the region 313 – 760 bp. Two primers were prepared for the cathepsin L-like1 gene covering the regions 136 – 581bp and 631-941bp and one primer for the cathepsin L-like2 gene covering the region 23 – 642 bp. These primer sets contained the T7 promoter sequence at the 5' end of each primer (Table 2). Constructed vectors were used as templates, and the respective primers were used in PCR reactions to amplify the DNA fragments for RNA transcription. Figures 6-8 illustrate the PCR products resolved on an agarose gel. The resulting DNA fragments contained T7 promoters on both ends. The PCR products of the expected sizes were purified to avoid primer dimer contamination. The PCR products from the previous step were purified, and the initial template concentration was a critical step in the dsRNA preparation procedures. Therefore, the amounts of the purified DNA fragments were determined by spectrophotometer analysis at O.D. of 260 and 280 nm and visualization on 1% denaturing agarose gel (Figure 9). For dsRNA preparations, 2µg of purified DNA was used as a template. The four purified DNA fragments are used to synthesize dsRNAs. The concentrations of the dsRNA products were estimated by comparing 200 ng of the purified DNA fragments and the synthesized dsRNAs on 1% agarose gel (Figures 10-13). In addition, spectrophotometer readings at O.D. 260 nm were monitored. As shown in (Figures 10-13), the gel revealed that the dsRNA fragments were sharper and higher than the DNA fragments, assuring the success of the dsRNA preparation procedure.

Bioassay on the adult whitefly

The effect of four cathepsin L cysteine protease gene dsRNA fragments on the survival rate of adult whiteflies was evaluated by oral bioassays. Adult whitefly mortality was recorded every day for four days. As shown in Tables 4-7, the mortality percentages of the adult whitefly feeding on dsRNA fragment targeting the cathepsin L-like1 gene (fragment A) were gradually increased through all the follow-up mortality days and by increasing the concentration of dsRNA in solution. It was 1.5, 14.5, 22.5%, and 43.5% after 1, 2, 3 and 4 days, respectively, by feeding whiteflies on conc. 5µ/ml of dsRNA feeding solution, by increasing the concentration of dsRNA in feeding solution to 15µ/ml, the mortality percentages were increased up to 2, 17.5, 46.5% and 63% after 1, 2, 3 and 4 days, respectively, the most efficient concentration was 30µ/ml, it was 2, 20.5, 55.52% and 68%, after 1, 2, 3 and 4 days, respectively as shown in Table 4. The mortality ratio of adult whiteflies fed on the dsRNA fragment targeting the cathepsin L-like1

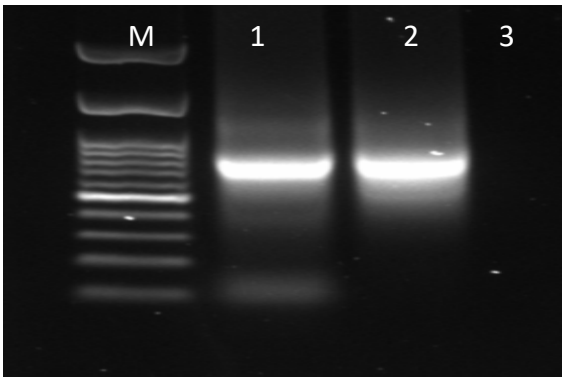

Figure 2. The total RNA from adult whitefly electrophoresed on a 1% agarose gel Amplification of cysteine protease gene fragments

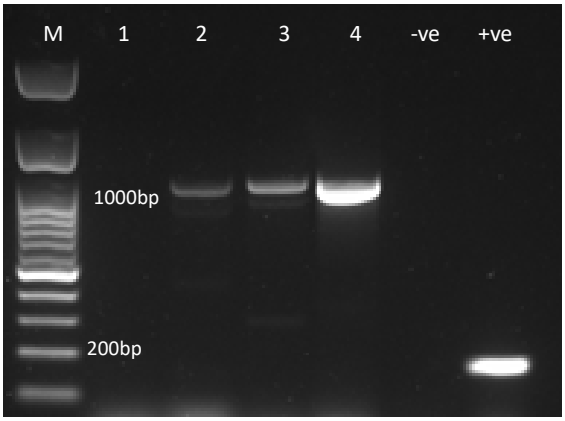

Figure 3. The RT-PCR analysis. Lane 2 – 4 represents the amplified fragments of cysteine proteinase genes. Lane 2; The primers set "cys1 F/cys1 R" revealed the expected size 1001 bp, Lane 3; The primers set "cys2 F/cys2 R" revealed the expected size 1010bp, Lane 4; The primers set "cys3 F/cys3 R" revealed the expected size 1000bp, -ve: negative control, +ve: positive control using B.actin primers and M: 100bp DNA marker (GeneDirex, Cat.No DM003-R500).

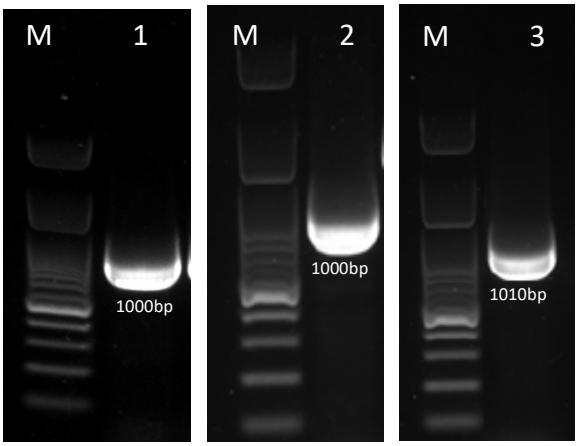

Figure 4. The PCR analysis of the cloned cysteine proteinase genes fragments. 1; cysteine proteinase gene Fragment 1, 2; cysteine proteinase gene Fragment 2, 3; cysteine proteinase gene Fragment 3. and M: 100bp DNA marker (GeneDirex, Cat.No DM003-R500).

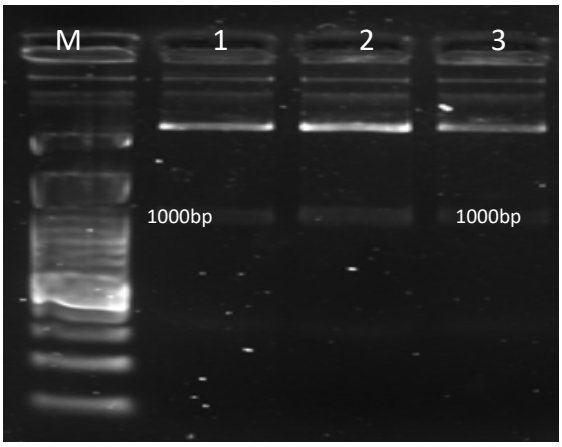

Figure 5. The restriction analysis of the cloned cysteine protease fragments. Lanes 1-3; represent the restriction of fragments 1, 2, and 3 using EcoRI restriction enzyme. The digested fragments are pointed by small red arrow. M: 100bp DNA marker (GeneDirex, Cat. No. DM003 - R500).

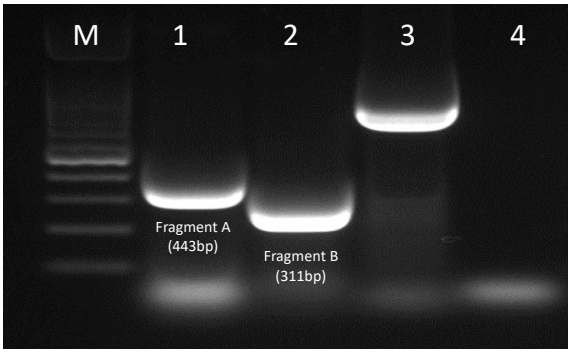

Figure 6. The PCR amplification of the two DNA fragments to serve as templates for synthesizing the dsRNA fragments for the cathepsin gene. Lane 1: the PCR products of the cathepsin L-like1 gene first fragment dsRNA (A) using dsRNA primers set (FragmentA FDS & FragmentA RDS); lane 2: the PCR products of the cathepsin L-like1 gene second fragment dsRNA (B) using dsRNA primers set (FragmentB FDS & FragmentB RDS); lane 3: the PCR products of the positive control (cathepsin L-like 1 gene with specific primers); lane 4: negative control and M: 100bp DNA ladder (GeneDirex, Cat.No DM003-R500).

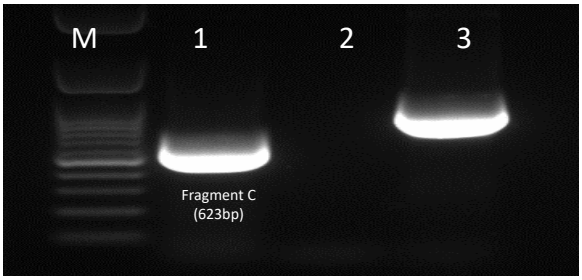

Figure 7. The PCR amplification of a DNA fragment to serve as a template for synthesizing the dsRNA fragment for the cathepsin gene. Lane 1: the PCR products of the cathepsin L-like2 gene dsRNA fragment (C) using dsRNA primers set (Fragment C FDS & Fragment C RDS); lane 2: negative control; Lane 3: the PCR products of the positive control (cathepsin L-like2 gene with specific primers) l and M: 100bp DNA ladder (GeneDirex, Cat. No DM003-R500).

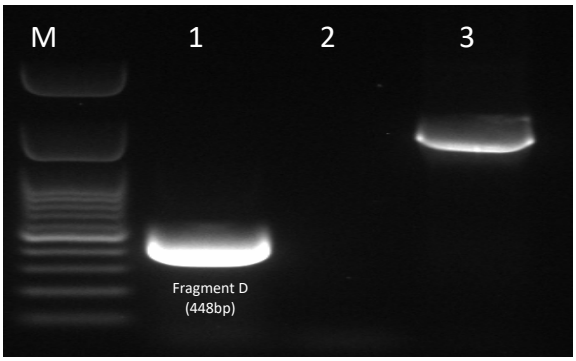

Figure 8. The PCR amplification of a DNA fragment to serve as a template for synthesizing the dsRNA fragments for the cathepsin gene. Lane 1: the PCR products of the cathepsin L gene dsRNA fragment (D) using dsRNA primers set (Fragment D FDS & Fragment D RDS); lane 2: the PCR products of the positive control (cathepsin L gene with specific primer). Lane 3: negative control; and M: 100bp DNA ladder (GeneDirex, Cat. No DM003-R500).

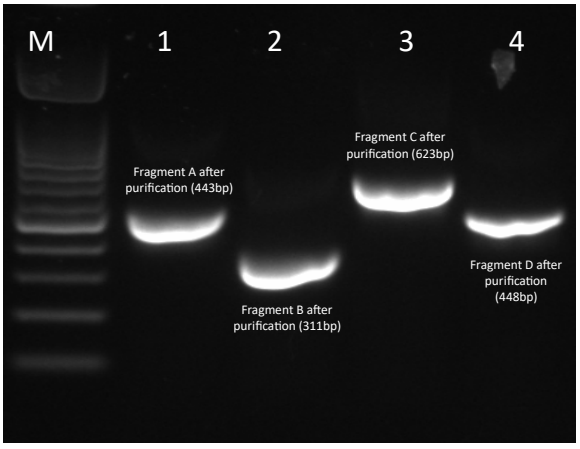

Figure 9. One microliter from the purified DNA fragments loaded on 1% agarose gel. M: 100bp DNA ladder (GeneDirex, Cat.No DM003-R500); 1: cathepsin L like 1 gene fragment A; 2: cathepsin L like 1 gene fragment B, 3: cathepsin L like 2 gene and 4: cathepsin L gene.

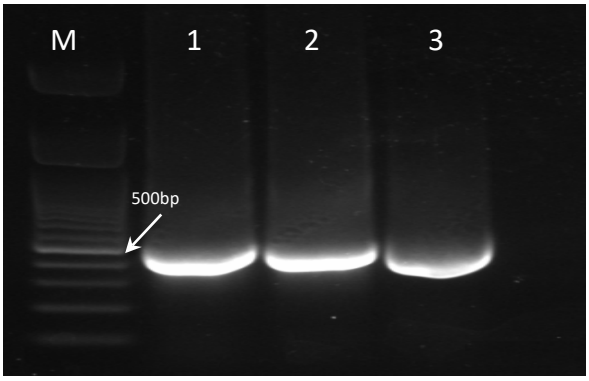

Figure 10. The electrophotetic profile of the 200 ng of the purified DNA and cathepsine L- like1 gene dsRNA (fragment A). The first and second lanes: are first and second elution from cathepsine L- like1 gene dsRNA respectively and the third lane: purified DNA fragment. And M: 100 bp DNA ladder (GeneDirex, Cat.No DM003-R500).

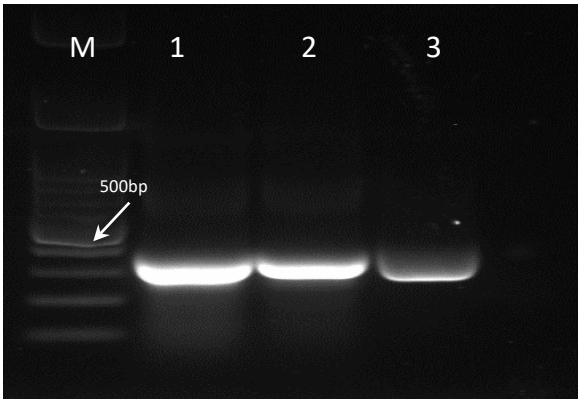

Figure 11. The electrophotetic profile of the 200 ng of the purified DNA and cathepsine L- like1 gene dsRNA (fragment B). The first and second lanes: are first and second elution from cathepsine L- like1 gene dsRNA respectively and the third lane: purified DNA fragment. And M: 100 bp DNA ladder (GeneDirex, Cat.No DM003-R500).

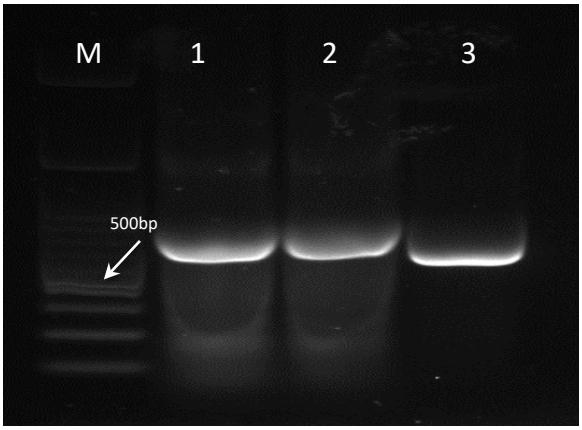

Figure 12. The electrophotetic profile of the 200 ng of the purified DNA and cathepsine L- like2 gene dsRNA. The first and second lanes: are first and second elution from cathepsine L- like2 gene dsRNA respectively and the third lane: purified DNA fragment. And M: 100 bp DNA ladder (GeneDirex, Cat. No DM003-R500).

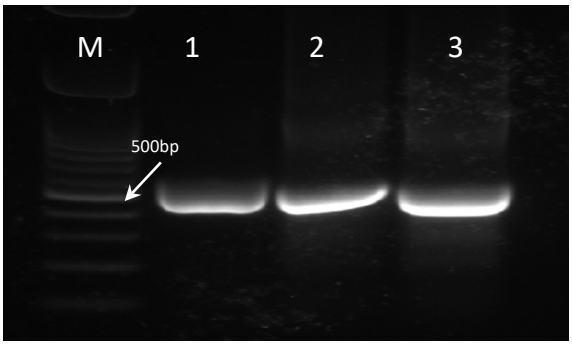

Figure 13. The electrophotetic profile of the 200 ng of the purified DNA and cathepsine L gene dsRNA. The first and second lanes: are first and second elution from cathepsine L dsRNA respectively and the third lane: purified DNA fragment. And M: 100 bp DNA ladder (GeneDirex, Cat. No DM003-R500).

gene (fragment B) was less but not far from the cathepsin L-like1 gene (fragment A), as shown in Table 5. Table 6 represents the mortality percentages of adult whiteflies feeding on dsRNA fragment targeting the cathepsin L-like2 gene. It was 2, 8, 16 and 21.5% after 1, 2, 3 and 4 days, respectively, when feeding on conc. 5µ/ml feeding solution, by increasing the concentration of dsRNA in the feeding solution to 15µ/ml, the mortality percentages were 2.5, 23.5, 27% and 55.5% after 1, 2, 3 and 4 days, respectively, also the most efficient concentration was 30µ/ml it was 4.5, 20.5, 41.5% and 82%, after 1, 2, 3 and 4 days, respectively. The mortality ratio of adult whiteflies fed on the dsRNA fragment targeting the cathepsin L gene was near to cathepsin L-like2 gene percentages (Table 7). The mortality of the control (adult whiteflies fed in a feeding solution lacking dsRNA) increased over the follow-up mortality days. It was within the normal level (≤ 8.5%) on the three follow-up mortality days (1, 2 and 3 days) and slightly increased on the fourth follow-up mortality day (after 4 days) (Tables 4, 5, 6 and 7).

Effect of silencing cysteine proteases genes on *B. tabaci* mortality and flying

Under controlled conditions, feeding on cysteine proteases dsRNAs drastically affected *B. tabaci*'s capacity to fly. Cysteine proteases dsRNAs feeding at concentrations of 5, 15, and 30 µg/mL caused death in adults of *B. tabaci*, as indicated in (tables 4, 5, 6, and 7). The percentage as avarge between four fragments was 9.75% in cysteine protease dsRNAs conc 5 µg/mL-fed *B. tabaci* 48 hr post-feeding, 10.875% in conc 15 µg/mL-fed *B. tabaci*, and 20.25% in conc 30 µg/mL-fed *B. tabaci*. Compared to *B. tabaci* fed a dsRNA-free diet (5.1%). The death rate increased further as the exposure duration lengthened. An average mortality rate of 28.37% was observed 72 hours after feeding on cysteine proteases dsRNA conc. 5µg/mL. Compared to *B. tabaci* fed on a diet lacking dsRNA (11%), the percentage rose to 40.375% in the case of conc.15 µg/mL and up to 73.875% in the case of conc.30 µg/mL.

Effect of Fragment (A) dsRNA on expression of cathepsine L-like1 cysteine protease gene.

The Average fold change of cathepsine L-like1cysteine proteases gene in case of control whitefly (1.25) was higher than different concentration dsRNA- feeding whitefly (0.28, 0.07 and 0.03). It means that the transcripts of cathepsine L-like1-cysteine proteases gene in the silenced whitefly is lower than the amount in the control one.as represented in (Figure 14).

Effect of Fragment (B) dsRNA on expression of cathepsine L-like1 cysteine proteases gene

The Average fold change of cathepsine L-like1 cysteine proteases gene in case of control whitefly (1.07) was not far than concentration 5 and 15 µg/ml dsRNA- feeding whitefly (0.99 and 1.02). It means that the transcripts of cathepsine L-like1-cysteine proteases gene in the silenced whitefly that feeding on conc 5 and 15 µg/ml is somewhat as the amount in the control one. But in case of concentration 30 µg/ml dsRNA- feeding whitefly the fold change was 0.5 it means that the transcripts of cathepsine L-like1-cysteine proteases gene in the silenced whitefly reduced to half. As represented in (Figure 15).

Effect of Fragment (C) dsRNA on expression of cathepsine L – like 2 cysteine proteases gene

The Average fold change of cathepsine L – like 2 cysteine proteases gene in case of control whitefly (1.07) was higher than concentration 5, 15 and 30 µg/ml dsRNA- feeding whitefly (0.5, 0.29 and 0.20). It means that the transcripts of cathepsine L-like 2-cysteine proteases gene in the silenced whitefly is lower than the amount in the control one. As represented (Figure 16).

Effect of Fragment (D) dsRNA on expression of cathepsin L cysteine proteases gene.

The Average fold change of cathepsine L cysteine proteases gene in case of control whitefly (1.04) was higher than concentrations 15 and 30 µg/ml dsRNA-feeding whitefly (0.3 and 0.15), It means that the transcripts of cathepsine L cysteine proteases gene in the silenced whitefly is lower the amount in the control one, but it was slightly less than concentration 5 µg/ml dsRNA- feeding whitefly (1.17) as represented in (Figure 17). As shown in Figures 14-17, real-time quantitative PCR of the treated insects revealed a significant decrease in the level of cysteine protease transcripts, with mRNA levels dropping by 35, 2.11, 5.24, and 6.779 folds because of cysteine protease fragments A, B, C, and D dsRNAs after four days of whiteflies feeding.

DISCUSSION

Whiteflies usually cause major economic losses to several crops, most notably tomatoes, in various regions of the earth (Konarev et al., 2011; Malschi et al., 2012; Karimzadeh et al., 2014). Whiteflies employ gut proteases and salivary glands to break down meal proteins that play roles in growth and development as sap-sucking feeders. The bulk of protease genes are

Table 4. The effect of dsRNA cathepsine L-like1 gene (fragment A) on the whitefly as revealed by whitefly mortality detected in four days.

	Total No. of adult whiteflies	Mortality			
		1- day Dead/Alive (%)	2-day Dead/Alive (%)	3-day Dead/Alive (%)	4-day Dead/Alive (%)
Control	200	3/200 (1.5%)	16/200 (8%)	17/200 (8.5%)	24/200 (12%)
5µg/ml	200	3/200 (1.5%)	29/200 (14.5%)	45/200 (22.5%)	87/200 (43.5%)
15µg/ml	200	4/200 (2%)	35/200 (17.5%)	93/200 (46.5%)	126/200 (63%)
30µg/ml	200	4/200 (2%)	41/200 (20.5%)	111/200 (55.52%)	136/200 (68%)

Table 5. The effect of dsRNA cathepsine L-like1 gene (fragment B) on the whitefly as revealed by whitefly mortality detected in four days.

	Total No. of adult whiteflies	Mortality			
		1- day Dead/Alive (%)	2-day Dead/Alive (%)	3-day Dead/Alive (%)	4-day Dead/Alive (%)
control	200	2/200 (1%)	4/200 (2%)	5/200 (2.5%)	9/200 (4.5%)
5µg/ml	200	3/200 (1.5%)	6/200 (3%)	10/200 (5%)	11/200 (5.5%)
15µg/ml	200	6/200 (3%)	7/200 (3.5%)	15/200 (7.5%)	38/200 (19%)
30µg/ml	200	9/200 (4.5%)	31/200 (15.5%)	58/200 (29%)	127/200 (63.5%)

Table 6. The effect of dsRNA cathepsine L-like2 gene (fragment C) on the whitefly as revealed by whitefly mortality detected in four days.

	Total No. of adult whiteflies	Mortality			
		1-day Dead/Alive (%)	2-day Dead/Alive (%)	3-day Dead/Alive (%)	4-day Dead/Alive (%)
control	200	5/200 (2.5%)	10/200 (5%)	13/200 (6.5%)	21/200 (10.5%)
5µg/ml	200	4/200 (2%)	16/200 (8%)	32/200 (16%)	43/200 (21.5%)
15µg/ml	200	5/200 (2.5%)	27/200 (23.5%)	54/200 (27%)	111/200 (55.5%)
30µg/ml	200	9/200 (4.5%)	41/200 (20.5%)	83/200 (41.5%)	164/200 (82%)

Table 7. The effect of dsRNA cathepsine L gene (fragment D) on the whitefly as revealed by whitefly mortality detected in four days.

	Total No. of adult whiteflies	Mortality			
		1-day Dead/Alive (%)	2-day Dead/Alive (%)	3-day Dead/Alive (%)	4-day Dead/Alive (%)
control	200	3/200 (1.5%)	11/200 (5.5%)	27/200 (13.5%)	34/200 (17%)
5µg/ml	200	3/200 (1.5%)	18/200 (9%)	38/200 (19%)	48/200 (24%)
15µg/ml	200	3/200 (1.5%)	27/200 (13.5%)	63/200 (31.5%)	86/200 (43%)
30µg/ml	200	4/200 (2%)	49/200 (24.5%)	95/200 (47.5%)	164/200 (82%)

transcribed throughout feeding, according to Edwards et al. (2010) and Ben-Mahmoud et al. (2015), which are types of the Cysteine (cathepsins B and L) protease family.

In this study, genes encoding a cysteine protease were partially isolated and identified for the first time from the whitefly *B. tabaci*. Cysteine proteases have been recognized in the genome of the A. pisum (hemipteran insect) by Rispe et al. (2008). RNAi has a significant impact as a crop protection method compared to various pest insects. RNAi has been frequently and effectively utilized in insect gene inactivation. RNAi is a species-specific approach for insect pest management due to its sequence-dependent solid specificity (Huvenne and Smagghe, 2010). Thus, the findings presented here propose a unique *b.tabaci* regulation method based on dsRNA exclusive to the *b.tabaci* gene/s. Because cysteine protease genes play essential roles in insect ingestion, immunological response, and adaptation, they might be excellent targets for pest control via RNAi, as they do in other insects (Gruden et al., 2005; McGonigle et al., 2008). Because disrupting cysteine protease gene expression in the whitefly might have significant consequences for the insect's fitness and development, the potential of b.tabaci gene silencing was investigated in this work via oral administration of cysteine dsRNA.

The oral administration of the four dsRNAs resulted in considerable deaths of whiteflies in this investigation. After four days, all fragments had the greatest fatality rate. 96 hours after feeding, cysteine protease dsRNAs conc 30 µg/mL-fed *B. tabaci* had a mortality rate of 73.875% compared to *B. tabaci* fed a diet without dsRNA (11%). Real-time quantitative PCR analysis of the treated insects revealed a substantial decrease in cysteine protease transcript levels, with mRNA levels dropping by 35, 2.11, 5.24, and 6.779 folds because of cathepsin L cysteine protease gene fragments A, B, C, and D dsRNA, respectively. The greatest decrease in cysteine protease gene expression was seen after four days at conc. 30µg/ml. Cysteine protease fragment (A) dsRNA was more efficient than other fragments in inducing mortality and lowering gene expression.

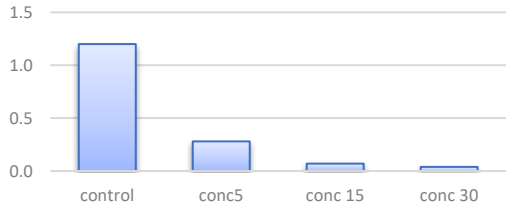

Figure 14. The relative expression levels of cathepsine L-like1 cysteine- proteases gene of *Bemisia tabaci* adults in response to cathepsine L-like1 cysteine proteases gene (fragment A) that following RNAi treatment by feeding after 96 hr. This diagram summarizes the results of the qRT-PCR experiments of three concentrations (5, 15, 30µg/ml) compared to the control one.

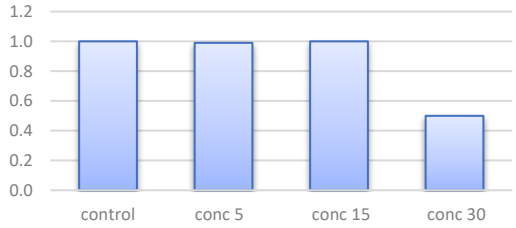

Figure 15. The relative expression levels of cathepsine L-like1 cysteine- proteases gene of *Bemisia tabaci* adults in response to cathepsine L-like1 cysteine proteases (fragment B) knockdown that following RNAi treatment by feeding after 96 hr. This diagram summarizes the results of the qRT-PCR experiments of three concentrations (5, 15, 30µg/ml) compared to the control one.

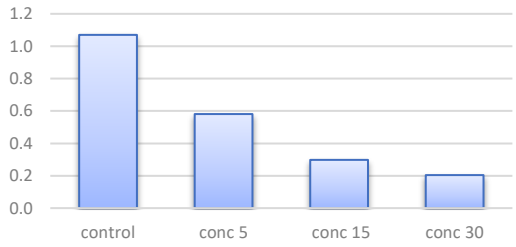

Figure 16. The relative expression levels of cathepsine L-like2 cysteine- proteases gene of *Bemisia tabaci* adults in response to cathepsine L-like2 cysteine proteases (fragment C) knockdown that following RNAi treatment by feeding after 96 hr. This diagram summarizes the results of the qRT-PCR experiments of three concentrations (5, 15, 30µg/ml) compared to the control one.

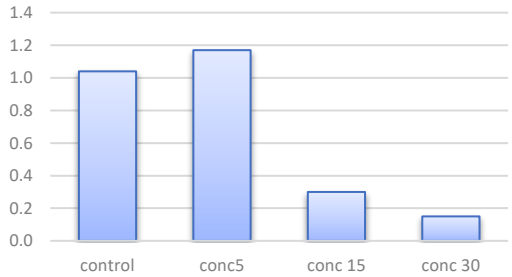

Figure 17. The relative expression levels of cathepsine L cysteine-proteases gene of *Bemisia tabaci* adults in response to cathepsine L cysteine proteases (fragment D) that following RNAi treatment by feeding after 96 hr. This diagram summarizes the results of the qRT-PCR experiments of three concentrations (5, 15, 30µg/ml) compared to the control one.

However, slightly up regulation of cathepsin L cysteine proteases gene was observed 4 days after oral delivery of cysteine proteases dsRNA of fragment D conc 5 µg/mL. Sapountzis et al. (2014) observed such a result. They found that cathepsin-L dsRNA treatment induced significant levels of cathepsin-L cysteine protease expression in the pea aphid. It did, however, diminish three days after dsRNA injection. Chu et al. (2014) also found that cysteine proteases dsRNA inhibited the gut cathepsin-L cysteine proteases in the western corn rootworm Diabrotica virgifera. They discovered that certain dsRNA-treated flies had nearly five-fold greater cysteine protease transcript abundance than controls. The consequences of oral dsRNA administration on survival and adult flight were investigated. The results demonstrated that silencing the cysteine gene had a variety of impacts on adult flying abilities, including an increase in malformed adults. These results from cysteine protease gene knockdown suggested that this gene effect serves a crucial physiological role(s) in whiteflies.

30 µg/mL dsRNA was more efficient at gene silencing and adult mortality than other doses. Asokan et al. (2014) reported comparable findings. They examined the influence of two dsRNA concentrations (10 and 20 µg) on the abundance of Helicoverpa armigera trypsin transcripts. They discovered that 20 µg dsRNA concentrations had a greater influence on gene silencing, pupation, and larval and pupal weight than 10 µg. In addition, several doses of dsRNA (1, 5, 10, 20, and 40 µg/ml) were examined in *B. tabaci*. Higher doses had a larger impact on target gene silencing and increased mortality (Upadhyay et al., 2011). In the other experiment, various P450 cytochrome dsRNA concentrations (0, 5, 10, 20, and 40 µg/ml) were introduced to the *B. tabaci* diet (Li et al., 2014). Feeding resulted in gene silencing and a high death rate. Reduced gene transcripts were seen after 4 days of dsRNA feeding for 5 µg/ml dsRNA, and gene silence occurred after 2 days of dsRNA feeding for 10, 20, and 40 µg/ml dsRNA. In addition, larger dsRNA concentrations resulted in more silencing.

Similarly, Yu et al. (2014) silenced the ecdysone receptor in the brown planthopper N. lugens and found that dsRNA concentration increased mortality. In this investigation, treated whiteflies had a considerably larger defect and a loss of flying ability than control insects, indicating that this protease is involved in insect ecdysis. Cathepsin-L is involved in many insect moulting processes (Hashmi et al., 2002; Liu et al., 2006; Zhang et al., 2013; Chen et al., 2014;

Sapountzis et al., 2014). Sapountzis et al. (2014) reported a successful RNAi of cathepsin-L in the pea aphid A. pisum. They discovered a new role for the cathepsin-L cysteine protease gene as a moulting enzyme in a hemimetabolous insect for the first time. They discovered that cathepsin-L knockdown altered aphid body appearance and increased mortality. Chen et al. (2014) also found that knocking out cysteine transcripts using dsRNA induced phenotypic alterations in Spodoptera litura at the prepubal, pupal, and adult stages.

CONCLUSION

We identified and analyzed three distinct snippets encoding cysteine protease in the whitefly pest for the first time. Then, using dsRNA, we investigated the potential for silencing cysteine genes. Cysteine protease genes silencing using an RNAi approach resulted in various impacts on insect mortality and flight; also, the knockdown of cysteine protease transcripts with dsRNA decreased insect transcripts. This is the first study to show that inhibiting cysteine protease with RNAi generates high gene transcript knockdown and insect death in whitefly pests.

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