Biocatalysis and Agricultural Biotechnology 34 (2021) 102044

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Caspase gene silencing affects the growth and development of *Tuta absoluta*

Shima Rahmani [a], Ali R. Bandani [b,*]

[a] Department of Plant Protection, Science and Research Branch, Islamic Azad University, Tehran, Iran
[b] Plant Protection Department, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

ARTICLE INFO

Keywords:
RNAi
dsRNA
Tomato leaf-miner
Gene expression
Apoptosis

ABSTRACT

RNAi technology is considered an important tool to analyze gene function. In the current study, caspase-1, a vital gene in the apoptosis mechanism, was partially detected from *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) larvae and silenced using a topical assay of the dsRNA. Analysis of the gene transcript showed that the dsRNA could reduce caspase-1 mRNA, significantly 24 h post-treatment. However, the gene gradually recovered its expression, and even the treated gene expression became fairly higher than the control on day three. This indicated that the silencing effect was not persistent over time. Biological experiments showed that the caspase-1 dsRNA did not produce significant mortality. However, the development time of the third instar larvae increased significantly after treatment by the dsRNA (25, 50 and 100 ng/μl) in a dose-dependent manner. Also, knockdown of caspase-1 reduced the bodyweight of the third instar larvae as well as the emergence of the adult. These results are indicating that caspase-1 has a significant role in the growth and development of the insect.

1. Introduction

Programmed cell death (PCD), also known as apoptosis, is a normal process of physiological cell suicide and is much conserved in all multicellular organisms. Apoptosis has a vital role in the development of healthy cells, tissue homeostasis, and the removal of unwanted and damaged cells (Zhuang et al., 2011; Arguelles et al., 2019). Apoptosis machinery can conduct through one of the three following pathways, including the effect on mitochondrial function as a modulator and synchronizer of Ca^{2+} signaling and/or a place for accumulation of p53 in response to stress (Morciano et al., 2018). Thus, it would transfer the death signal directly to the apoptotic mechanisms via adaptor proteins and accumulation of calcium into a cell and triggering programmed cell death through a calcium-binding protease calpain (Zhuang et al., 2011). It seems that the Ca^{2+} dynamics of ER and mitochondria can be adjusted by the Bcl-2 proteins. Bcl-2 family members as regulators release the proteins from the space between the inner and outer membrane of mitochondria. In the cytosol, they activate caspase proteases to take cells to pieces and stimulate phagocytosis of cell corpses (Jeong and Seol, 2008; Wang and Youle, 2009). Accumulation of p53 in the cytosol or mitochondria in response to stress is a new type of transcription-independent apoptotic activities. P53 could induce permeabilization of the mitochondrial outer membrane and activate the intrinsic apoptotic pathway by triggering the release of pro-apoptotic factors from the intermembrane space (Morciano et al., 2018).

The central part of the machinery of apoptosis is caspases, which are a conserved family of aspartate-specific cysteine proteases (Hebert et al., 2009). These enzymes are divided into two groups, including inflammatory and the apoptotic caspases composing of initiators and effectors. It seems that inflammatory caspases are restricted to mammals (Chang and Yang, 2000; Kamezaki et al., 2018; Courtiade et al., 2011), although some lepidopteran caspase genes recently showed similarities to the human inflammatory caspase (Yu et al., 2019). However, during the apoptotic proteolytic cascade, initiator caspases become active, first in response to a variety of signals, and after activation through the cleavage of their long N-terminal pro-domain, they activate the effector caspases, which are responsible for the cleavage of many cellular components, resulting in the typical features of apoptotic cells (Hebert et al., 2009; Courtiade et al., 2011; Zhuang et al., 2011). Additionally, there is evidence that caspases play a significant role in the other cellular functions, including cell migration, cell shaping, immune response, spermatid individualization, etc. (Accorsi et al., 2015).

In lepidopteran order, caspase-1 is similar to human caspase-3 and its function in the apoptosis pathway has been studied more thoroughly using cell lines (Parthasarathy and Palli, 2007). The first caspase, Sf-caspase-1, with 299 amino acids, was characterized from *Spodoptera frugiperda* cell line (Sf9), which was an important target of baculovirus p35 during viral infection (Ahmad et al., 1997). The role of lepidopteran

* Corresponding author.
E-mail address: abandani@ut.ac.ir (A.R. Bandani).

https://doi.org/10.1016/j.bcab.2021.102044
Received 1 August 2020; Received in revised form 12 February 2021; Accepted 22 May 2021
Available online 27 May 2021

caspase-1 in virus-induced apoptosis (Liu et al., 2005; Hebert et al., 2009; Wang et al., 2016), entomopathogenic fungus-induced apoptosis (Tseng et al., 2008), UV-irradiation (Zoog et al., 2002; Liu and Chejanovsky, 2005; G. Liu et al., 2020), bacteria and chemicals (Yu et al., 2019), heat and cold temperatures (Lu et al., 2013), as well as development and larval-pupal metamorphosis (Yang et al., 2008, 2017) have been studied. Most of the above studies have been conducted on *Spopdoptera frugiperda*, *Spodoptera littoralis*, *Spodoptera exigua*, *Helicoverpa armigera*, *Trichoplusia ni*, and *Bombyx mori* (Cooper et al., 2009; Hebert et al., 2009; Wang et al., 2016; Yu et al., 2019). However, there is not any study about apoptosis machinery in the tomato leafminer, *Tuta absoluta* (Meyrick), one of the most destructive insect pests of the Solanaceae family (Biondi et al., 2018; Han et al., 2018; Sylla et al., 2019).

In this study, we targeted some biological traits of the tomato leafminer in which caspase-1 is involved. Previously, Yang et al. (2008) showed rises in the expression of Hearm caspase-1 in hemocytes of *H. armigera* during molting and metamorphosis. Also, the expression profiles of caspase-1 in the *Galleria mellonella* midgut during development showed that the transcript and its protein products appeared in two or more waves during metamorphosis (Khoa et al., 2012). Lu et al. (2013) reported that the expression of *Chilo suppressalis* caspase-1 was in low constitutive levels in the eggs and early larvae stages, but higher abundances were demonstrated in the last larval instars and pupae stage.

RNA interference (RNAi) is the rapid identification of in vivo gene function in which a special gene is targeted and silenced through double-stranded RNA (dsRNA) (Simmer et al., 2003). So far, vacuolar ATPase subunit A, Arginine kinase (Camargo et al., 2016), acetylcholinesterase, nicotinic acetylcholine alpha 6 receptors, and ryanodine receptors (Majidiani et al., 2019) in *T. absoluta* were silenced, and their functions explored using dsRNAs. Taking into consideration the function of cspase-1, we aimed to identify, sequence and explore the gene function in the biology of the tomato leaf-miner. Moreover, the effect of RNAi technology on the gene expression as well as survival, growth, and development of the insect examined.

2. Materials and methods

2.1. Plant and insect rearing

Tomato seeds, *Solanum lycopersicum*, were cultivated in a greenhouse condition (25 ± 5 °C, 16L:8D photoperiod) in seedling trays containing peat moss until the growth of the first leaf. Then, the seedlings transferred to the greenhouse and grown in pots (20 cm diameter × 20 cm height) with fertilized soil, watered three times a week (Pereyra and Sánchez, 2006; Gharekhani and Salek-Ebrahimi, 2014).

The larvae of tomato leaf-miner, *Tuta absoluta*, were collected from a commercial tomato greenhouse located in Safadasht, Karaj (Alborz province), Iran. The insect colony was kept under laboratory conditions at 25 ± 1 °C, 60–70% RH, and 16L:8D photoperiod. The colonies were provided with fresh tomato plants every two days.

2.2. RNA extraction and cDNA cloning

The RNA extraction and cDNA construction were based on Amiri et al. (2016) with slight modifications. The whole body of the fourth instar larvae of *T. absoluta* was transferred into a microtube containing 0.5 ml TRI reagent (TRIzol®, Sigma-Aldrich, Missouri, USA). TRI reagent was applied to extract the total RNA of the samples (~30 mg). To remove possible DNA contamination, total RNA samples were treated with Ambion TM DNase I (RNase-free) (Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA). The first-strand cDNA was constructed with an oligo-dT primer using *AccuPowerR_RocketScript* TM Cycle RT PreMix cDNA synthesis kit (Bioneer, Daejeon, Korea). The PCR program was as 95 °C preheating for 4 min, followed by 30 cycles of 95 °C for the 30s, 55 °C for 30s, 72 °C for 30 s, and a final extension step of 72 °C during 7 min.

Every PCR reaction contained 2.5 μl of 10 × PCR buffer, 1 μl of cDNA template, 1.5 μl of forward and 1.5 μl of reverse primers (10 pmol/μl; caspase-1 forward primer: ATGAAGGTGACGCATGGGG; caspase-1 reverse primer: CACAAAGAGCTTGCATGAACC), 1.5 μl of $MgCl_2$ (25 mmol/μl), 2 μl of dNTP (deoxynucleotide triphosphate) (2 mmol/μl each), 0.2 μl of Taq polymerase (5 U/μl), and 14.8 μl of ddH_2O. The primers were designed according to the alignment of the same sequences of other insect genes taken from the National Center for Biotechnology Information (NCBI) in Clustal omega online software (www.ebi.ac.uk/Tools/msa/clustalo/). The PCR product was analyzed on 1% agarose gel, and the target band was isolated and purified using the AccuPrep Gel Extraction Kit (Bioneer, Daejeon, Korea). The nucleotide sequence was cloned into the pTG19-T PCR cloning vector (Vivantis) and inserted into *Escherichia coli* DH5α competent cells. Colonies with recombinant plasmids were screened under standard ampicillin conditions and thus caspase-1 sequence confirmed by sequencing. The sequencing reaction was performed commercially.

2.3. Sequences and phylogenetic analysis

The sequence of the caspase-1 cDNA confirmed according to homology between other caspase-1 sequences obtained from lepidopterans in the Blast program available on the GenBank database of the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). The amino acid sequences of caspase-1 homologs from different lepidopterans were taken from the GenBank database of NCBI. Clustal Omega used for alignment of the sequences, and MEGA X software was applied for the construction of phylogenetic trees by the neighbor-joining method with a Parsimon correction model (1000 bootstrap replications to check for repeatability of the results). Also, the protein domains were predicted by NCBI Conserved Domains using the Conserved Domain Database (CDD).

2.4. dsRNAs synthesis

The double-stranded RNAs (dsRNAs) of the gene were synthesized using T7 RNA polymerase. Sequence-specific primers were constructed with a T7 promoter at the 5′-end (caspase-1- T7 forward: TAATACGACTCACTATAGGGAGAGGAGCCACGGGTTGTGTTC; caspase-1-T7 reverse: TAATACGACTCACTATAGGGAGAAAGAACAGGGGCTGGGCTAT) to create cDNA fragments of caspase-1 which contained the T7 promoter region in both sense and antisense strands. MEGAscript® RNAi kit (Ambion, Life Technologies) used for dsRNA synthesis. The purity and integrity of the dsRNAs examined by agarose gel electrophoresis, and its quantity was determined spectrophotometrically at 260 nm. The length of both dsRNAs was around 500 bp.

2.5. dsRNA topical assay

The topical assay was conducted to study the effect of Caspase-1 on the insect. The purified Caspase-1 dsRNA was diluted by acetone to obtain desired concentrations (50, 100, and 200 ng/μl) and 0.5 μl droplets of every dsRNA concentration were applied on the dorsal section of the cold larval body using a micropipette (Pridgeon et al., 2008). The control individuals were treated by acetone only. The experimental larvae were at the early age of the third instar (0-24 h-age). After drying, the larvae were put into the Petri dishes filled with tomato leaves as food. The test was replicated three times, with ten individuals in each replicate. The biological features such as survival, weight, and developmental period were calculated.

2.6. RNA isolation from dsRNA-treated larvae and qualitative gene expression assays

To analyze the effect of the dsRNAs on each target gene

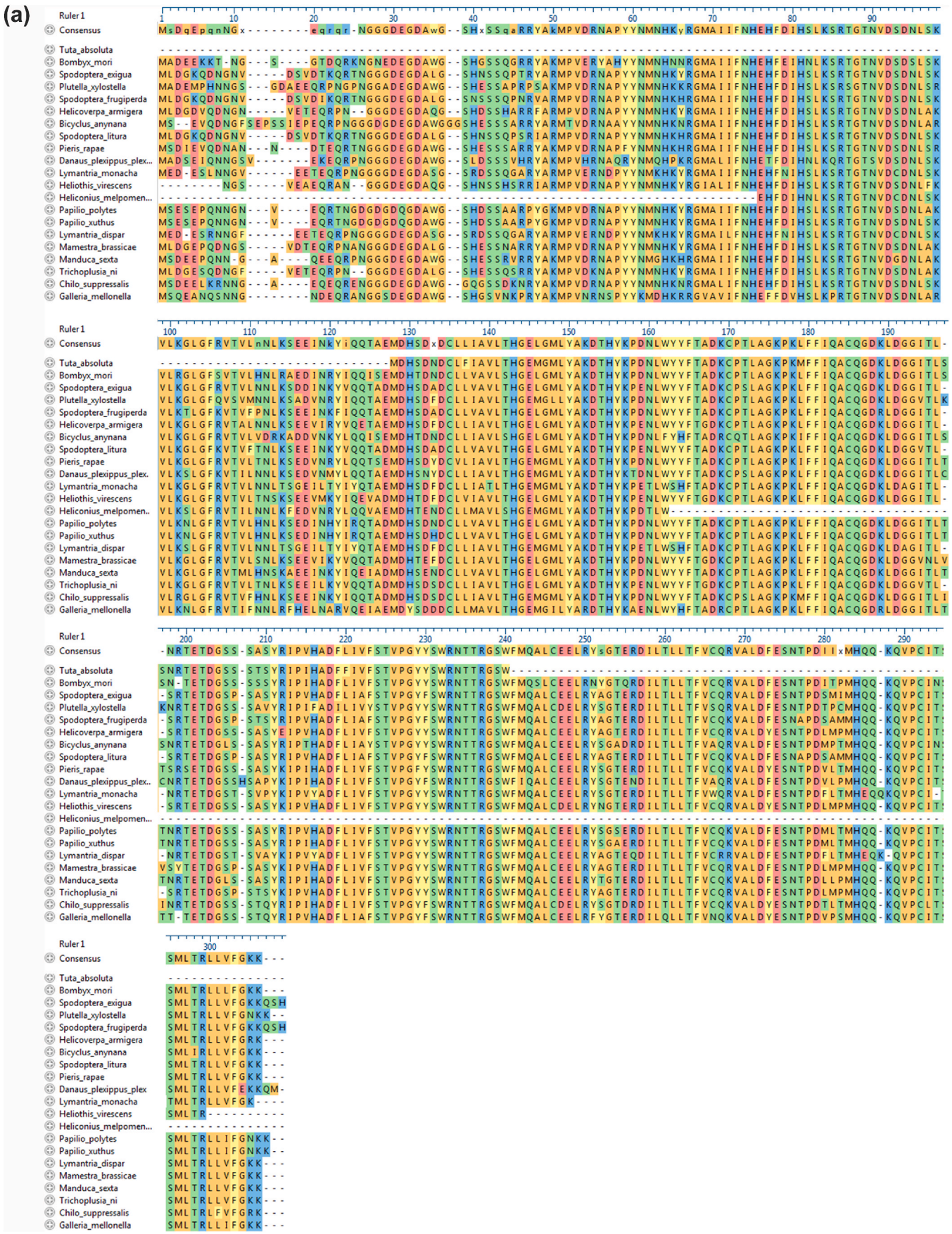

Fig. 1. Deduced amino acid sequence located between 231 and 566 nt of caspase-1 aligned with this gene belonging to *Bombyx mori* (NP_001037050.1), Spodoptera exigua (AEK12771.1), Plutella xylostella (ADK24087.1), *Spodoptera frugiperda* (AAC47442.1), *Helicoverpa armigera* (ABO93468.1), *Bicyclus anynana*

(XP_023950371.1), *Spodoptera litura* (XP_022819875.1), *Pieris rapae* (XP_022117090.1), *Heliconius melpomene amaryllis* (ACX93722.1), *Danaus plexippus* (OWR45225.1), *Lymantria monacha* (AEK20808.1), Heliothis *virescens* (AEK20807.1), *Papilio polytes* (XP_013146291.1), *Papilio xuthus* (KPJ01774.1), *Lymantria dispar* (BAM62939.1), *Mamestra brassicae* (AEK20809.1), *Manduca sexta* (AEF30493.1), *Trichoplusia ni* (ACI43910.1), *Chilo suppressalis* (AFJ97219.1), *Galleria mellonella* (AEH76885.1). The identical amino acids are shaded in same color (a). A phylogenetic tree constructed from amino acid sequences of caspase-1 of tomato leafminer and various lepidopteran insects is shown. Neighbor-joining method with genetic distance was calculated. The percentages of times that species are clustered in the bootstrap trees (10000 replicates) are indicated by the numbers above the branches. Numbers at nodes represent bootstrap *P*-values (>50%). The scale bar indicates the number of substitutions per amino acid position. MEGA5.05 was used to analyze phylogenetic test. The amino acid sequences of caspase-1 were from *Tuta absoluta* (GenBank accession no. MH651749), *Bombyx mori* (NP_001037050.1), Spodoptera exigua (AEK12771.1), Plutella xylostella (ADK24087.1), *Spodoptera frugiperda* (AAC47442.1), *Helicoverpa armigera* (ABO93468.1), *Bicyclus anynana* (XP_023950371.1), *Spodoptera litura* (XP_022819875.1), *Pieris rapae* (XP_022117090.1), *Heliconius melpomene amaryllis* (ACX93722.1), *Danaus plexippus* (OWR45225.1), *Lymantria monacha* (AEK20808.1), Heliothis *virescens* (AEK20807.1), *Papilio polytes* (XP_013146291.1), *Papilio xuthus* (KPJ01774.1), *Lymantria dispar* (BAM62939.1), *Mamestra brassicae* (AEK20809.1), *Manduca sexta* (AEF30493.1), *Trichoplusia ni* (ACI43910.1), *Chilo suppressalis* (AFJ97219.1), *Galleria mellonella* (AEH76885.1) (b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

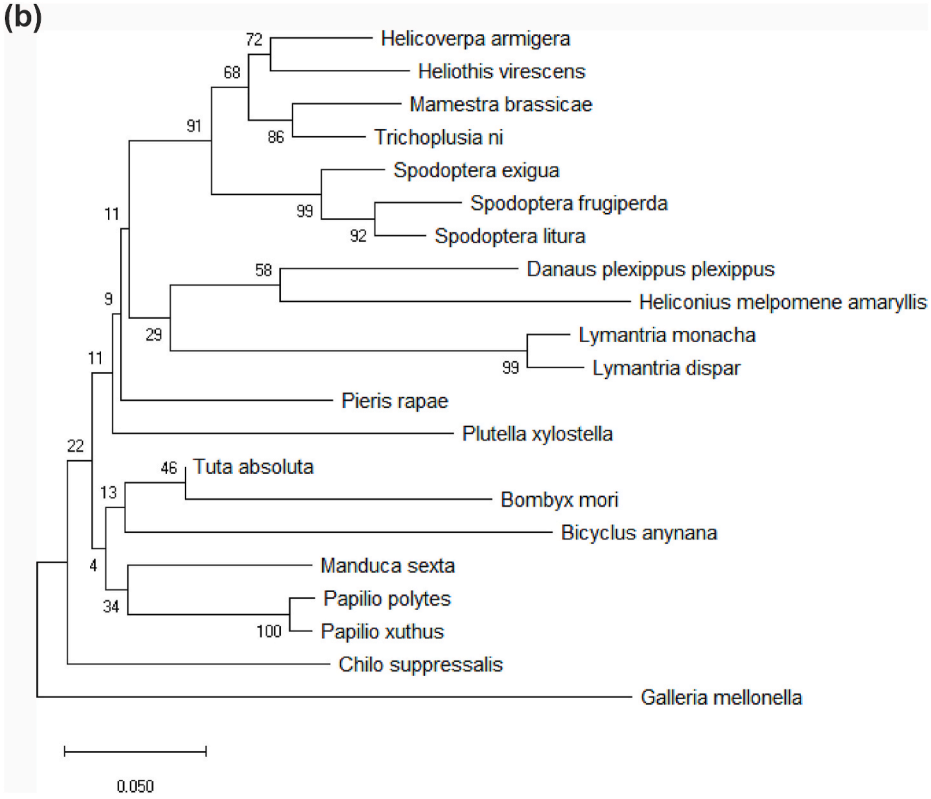

Fig. 1. (*continued*).

transcription, we extracted RNAs from live larvae, and cDNAs were constructed (AccuPower® PCR premix; Bioneer, Inc., Daejeon, Korea). Survive larvae after 24, 48, 72 h post-treatment were selected for RNA extraction. RNA extraction and cDNA construction were done as described before.

For every qPCR reaction, three technical replicates were performed. *T. absoluta* specific 18s ribosomal RNA gene (18s rRNA) was used as an internal control (Majerowicz et al., 2011; Paim et al., 2012). Thus, the target genes expression levels normalized according to the control gene. The 18S primers were designed based on the leaf-miner 18s ribosomal RNA sequence in GenBank with an accession number of MH644412 by Primer Blast online software (18s rRNA qPCR forward primer: GGCCTTCGGGATCGGAGTAA; 18s rRNA qPCR reverse primer: GCAAATGCTTTCGCAGTTGTT). The reactions 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 (caspase-1 qPCR forward primer: GTCACTGTCCACGTTCGTCCC; caspase-1 qPCR reverse primer: AACACAAGAACAGGGGCTGGG) with a product size of approximately 100 bp were designed using Primer-Blast. qPCR reactions were performed based on Amiri et al. (2016).

At the end of the program, a melting curve was set up from 55 to 95 °C to ensure that only a single product was generated. The relative mRNA level of the target genes was estimated based on the *Ct* method ($2^{-\Delta\Delta Ct}$ (Pfaffl, 2001; Livak and Schmittgen, 2001)). The significant differences in the gene expression were determined by *t*-test using the Rest 2009 Software (Pfaffl et al., 2002).

2.7. Statistical analysis

The data analyzed by one-way ANOVA using SAS version 9.0 and differences between values were determined using a Tukey pairwise comparison of means. The statistical significance for results was established at $P < 0.05$.

3. Results

3.1. Identification and homologous alignment

A 642 bp partial cDNA was considered as *T. absoluta* caspase-1 based on the BLASTx analysis showed high homology with caspase-1 gene in the order of Lepidoptera such as *Pieris rapae* (Lepidoptera: Pieridae; GenBank accession no. XP_022117090.1; ~88% identity), *H. armigera* (Lepidoptera: Noctuidae; GenBank accession no. ABO93468.1; 87% identity), *Chilo suppressalis* (Lepidoptera: Crambidae; GenBank accession

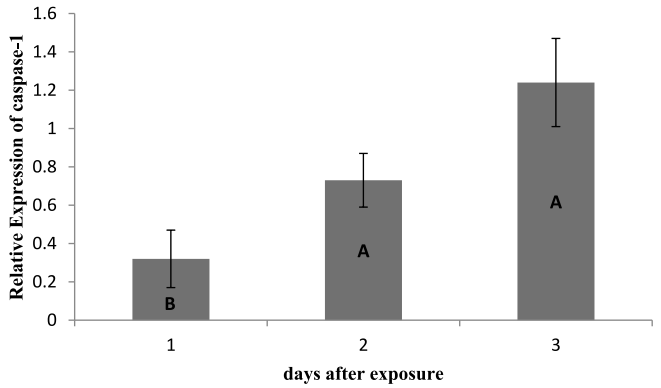

Fig. 2. Three days relative expression of caspase-1 gene in *T. absoluta* larvae were assessed after application of caspase-1 dsRNA. 18S ribosomal RNA gene (18S rRNA) was used as an internal reference gene. The relative expression was evaluated according to the value of the lowest expression which was attributed to an arbitrary value of 1. *Ct* values were first normalized to the endogenous control gene (18S rRNA), followed by normalization to the control without treatment using the $2^{-\Delta\Delta Ct}$ method. Each kinetic point was performed in triplicate. An asterisk indicates *a* significant difference ($P < 0.01$) according to the iteration test (Rest 2009 Software). The values represent averages with vertical bars indicating SE.

no. AFJ97219.1; 87% identity), *H. virescens* (Lepidoptera: Noctuidae; GenBank accession no. PCG74557.1; ~85% identity), etc. This cDNA sequence has been set in GenBank under accession number MH673894.1. This sequence consists of an open reading frame of 336 nt (start from 231 and stop to >566 nt) encoding for 111 amino acids. The calculated molecular mass of the deduced protein is 12.737 KDa, with an isoelectric point (PI) of 6.01. The domain found by using the NCBI site was named CASc super-family.

High conservation of the amino acid sequences of caspase-1 among different lepidopteran species shows that the transcriptome is extremely conserved during the evolution of these insects (Fig. 1A).

3.2. Phylogenetic analysis

Based on amino acid sequence alignment of a variety of lepidopteran species caspase-1, the phylogenetic tree was generated using MEGA5.05 (Fig. 1B). Neighbor-joining trees were constructed using sequences available in GenBank and a Poisson-corrected method with a bootstrap test of 1000 replications. Phylogenetic analysis of proteins from insect species in the order of Lepidoptera showed that the Caspase-1 seemed to have a common ancestry to *B. mori*, and *Bicyclus anynana* (Fig. 1B).

3.3. Effect of the dsRNAs on the gene transcriptome

The knockdown of the caspase-1 gene of the larvae was assessed by qRT-PCR for three-day post-treatment using a concentration of 100 ng/µ. The 24-h post-treatment results showed that the effect of dsRNA on the caspase gene expression was significant ($P < 0.05$) (the gene expression reduced about 68%) (Fig. 2). Also, results showed that as time goes by, the gene expression was recovered so that on the third day, the amount of the caspase gene expression was even more than that of control (Fig. 2).

3.4. Effect of the dsRNAs on the larval survival and development

The survival rate of the individuals treated by different concentrations of caspase-1 dsRNAs did not change significantly compared to the control (Fig. 3A). However, the silencing of caspase-1 caused a significant reduction in the bodyweight of the third instar larvae treated with different concentrations of dsRNAs ($df = 3, 8$; $F = 30.14$, $P = 0.0001$) (Fig. 3B). Nevertheless, there were not any significant differences between the treatments; namely, individuals treated with different dsRNA concentrations have almost the same bodyweight. The development period of the third instar larvae in all treatments (25, 50 and 100 ng/µl of dsRNA) changed significantly in a dose-dependent manner (Fig. 3C). Thus, the high dose greatly increased the development period of the larvae. As the figure shows, the third instar larvae developmental time when they treated by 100 ng/µl of dsRNA was 2.3 times higher than the control development time ($df = 3, 8$; $F = 3.95$; $P = 0.05$) (Fig. 3C).

The development time of the fourth instar larvae did not change significantly, although a trend showed that as the concentration of the dsRNA increased, a slight increase in development time occurred, e.g., the development time of untreated individual (the control) was four days while in the high dose treated ones were five days (Fig. 3D). Also, the pupal development time was not affected by dsRNA significantly. Pupal developmental time was evaluated to be about ten days (9.68 days in control and 9.5, 10.3, and 10.5 days in the treated ones when treated with 25, 50, and 100 ng/µl of caspase-1 dsRNA, respectively). Although slight changes in pupal development were seen in different treatments, these changes were not significant (Fig. 3E).

The appearance of the adult individuals treated with higher concentrations (50 and 100 ng/µl of caspase-1 dsRNA) was much fewer than those of control and the other two treatments ($df = 3, 8$; $F = 4.20$; $P = 0.046$) (Fig. 3F). The adult survival affected by treatments since the mortality of the adults treated with a higher concentration (100 ng/µl of the dsRNA) was much more than the control and the other two treatments.

4. Discussion

In the current study, caspase-1 was considered as a target for silencing in *T. absoluta* using RNAi technique. Therefore, RNA extraction and construction of cDNA were done using the third instar larvae. After doing sequences alignment based on a non-redundant database in the NCBI blastx, the e-values, and high percent identity between our sequence and the other lepidopteran genes, declared that the gene was caspase-1. Also, according to Fig. 1A, there was high conservation between the amino acid sequence of this gene and different lepidopteran species caspase-1. The only domain found in the *T. absoluta* caspase-1 deduced amino acid was CASc super-family. Zhuang et al. (2011) after analyzing the deduced amino acid of *Plutella xylostella* caspase-1 using SMART (http://smart.embl-heidelberg.de/), detected a big CASc domain as a proenzyme. Also, this protein possessed two domain profiles, caspase-p20 (position 61–184) and caspase-p10 (position 203–298) based on the SIB (http://exPasy.org/prosite) analysis. Thus, these two big and small catalytic domains in our sequence were in the position 34–157 (caspase-p20) and 176–213 (caspase-p10). Similarly, It had His and Cys, as two distinct amino acid residues essential for caspase enzyme catalytic site and this case was suggested for Hearm caspase-1 from *H. armigera* (Yang et al., 2008), *P. xylostella* caspase-1 (Zhuang et al., 2011), Drosophila drICE (Fraser and Evan, 1997), and human Mch4 caspase-8 (Fernandes-Alnemri et al., 1996).

In this study, the quantitative caspase-1 gene expression was examined on the treated and untreated third instar larvae. Studies by Wang et al. (2017) showed that the mRNA level of this gene was expressed in all stages of *B. mori,* including egg, larval instars, pupae, and adult moth. Also, they showed that caspase-1 existed in all of the surveyed tissues comprising integument, head, silk gland, midgut, trachea, fat body, and hemocytes.

The role of caspase-1 in growth, development, and metamorphosis has been demonstrated in *S. frugiperda* (Ahmad et al., 1997), *S. littoralis* (Liu and Chejanovsky, 2005), *H. virescens* (Parthasarathy and Palli, 2007) and *H. armigera* (Yang et al., 2008). Furthermore, the expression of caspase-1 could be up-regulated by hormones participating in development and metamorphosis, like ecdysone (H. Liu et al., 2020).

In the caspase-1 dsRNA, the gene knockdown level reduced as time passed over after treatment. Indeed, the lowest amount of the gene

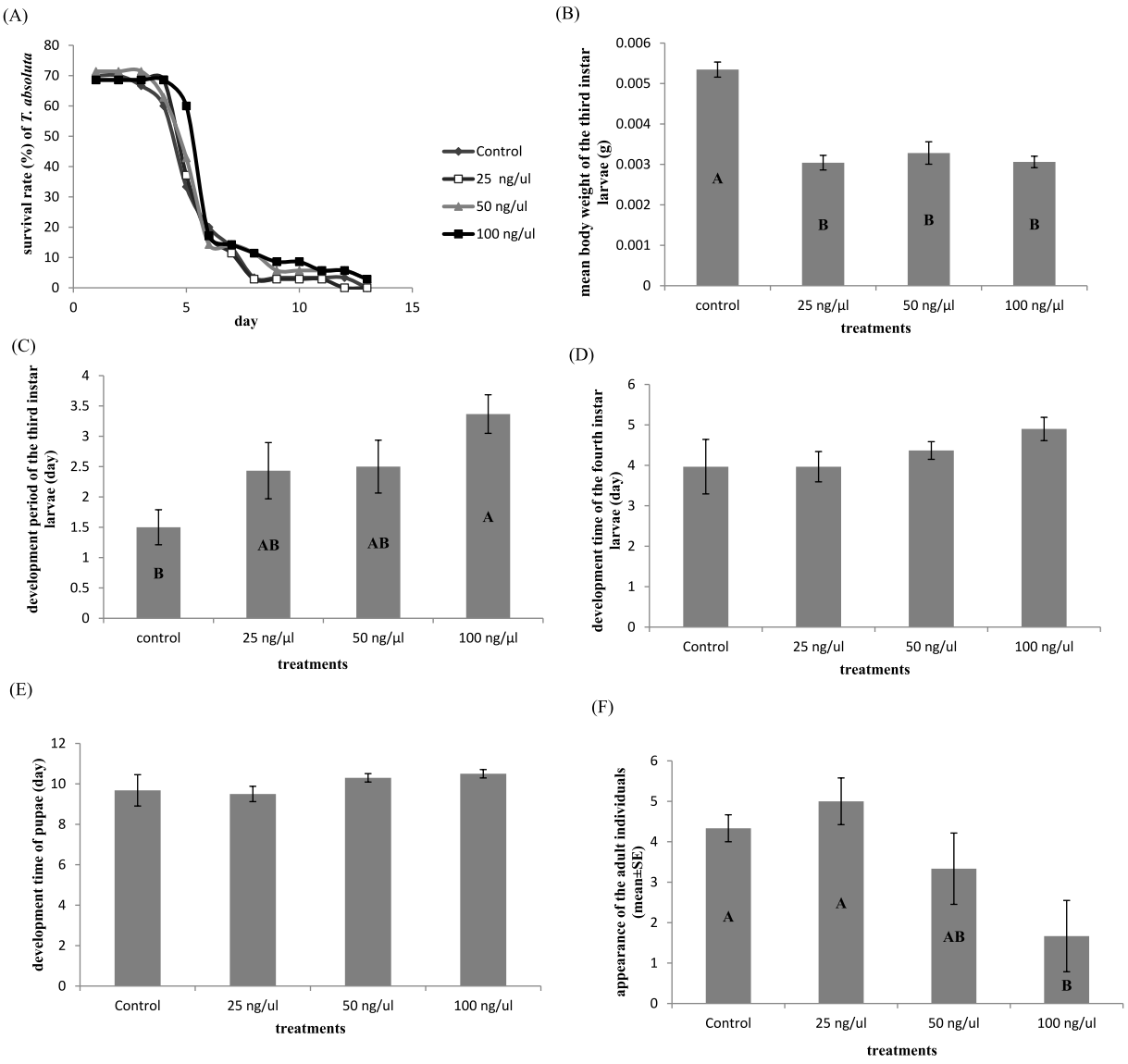

Fig. 3. The survival percentage of *T. absoluta* treated by different concentrations of caspase-1 dsRNA (25, 50 and 100 ng/µl) (a). The mean body weight (g) of the third instar larvae of *T. absoluta* treated by 25, 50 and 100 ng/µl of caspase-1 dsRNA (b). The development time of the third (c), and fourth instar larvae (d) and pupae (e) of *T. absoluta* treated by 25, 50 and 100 ng/µl of caspase-1 dsRNA. The appearance of the adult individuals treated by 25, 50 and 100 ng/µl of caspase-1 dsRNA (f). All tests are done according to Tukey test (Different letters indicate a significant difference in the level of $P < 0.05$).

expression was observed at 24 h-post treatment, while on the third day, the greatest amount of the gene expression was observed, indicating as time passed by, the gene recovery was more. The recovery of the gene expression was the reason why there was not any significant mortality when caspase-1 dsRNA was utilized in the assays. However, some biological parameters affected, to some extent showing some effect of the dsRNA on the insect.

Also, studies are showing that for the efficacy of RNAi apparatus, dsRNA constructs didn't need to have a systemic effect. For example, Amiri et al. (2020) showed that gluten hydrolase expression profile in the gut and the salivary glands of the sunn pest, *Eurygaster integriceps* was reduced 6 h after injection of the dsRNA. However, the gene knockdown was not persistent because nuclease enzyme existence in the hemolymph plasma could degrade dsRNA in the hemocoel (Garbutt et al., 2013). Therefore, the expression level was recovered after 21 h post-treatment. Nevertheless, the mortality of the sunn pest after this time (21 h post-injection) was more than 65% in both nymphs and adults. Also, Cao et al. (2018) showed that injection of 30 ng v- ATPase dsRNA in *Acyrthosiphon pisum* caused 77% mortality during 7 days post-treatment. However, the gene expression level was transient and decreased only 24 h post-injection.

One of the most important factors responsible for RNAi efficacy (specially in pest management) is the stability of dsRNA in the environment and in the insect body (Christiaens et al., 2020). Garbutt et al. (2013) illustrated that in *Manduca sexta*, dsRNA vanished in the hemolymph after 3 h of incubation. However, in *Blattella germanica*, dsRNA persisted in the hemolymph plasma after 24 h post-incubation. They concluded that the short persistence of dsRNA was due to the effect of a metal-dependent nuclease enzyme, which was responsible for dsRNA degradation in the hemolymph (Garbutt et al., 2013). Also, digestive nucleases can impact RNAi efficacy when dsRNA is ingested by the insect through an oral treatment. Giesbrecht et al. (2020) found that when two midgut-specific nucleases of *Aedes aegypti* were knocked-down, silencing efficiency increased compared to the control harboring digestive nucleases. Since the rate of degradation of dsRNA is varied among different insect species, it could explain the differing susceptibilities of insects to RNAi.

In the current study, topically applied caspase-1 dsRNAs significantly

affected the development time and the bodyweight of the third instar larvae and adult emergence. These findings were in accordance with the studies of Bento et al. (2020) who demonstrated that the larval cycle of *T. absoluta* was significantly affected when fed on a diet containing *Escherichia coli* HT115(DE3) expressing dsRNAs constructed for juvenile hormone epoxide hydrolase protein, ecdysteroid 25-hydroxylase, and carboxylesterase. However, there were no considerable changes in the weight of the pupae in all treatments. Mamta et al. (2016) illustrated that chitinase silencing in *H. armigera* larvae fed continuously with several RNAi tobacco and tomato lines, lead to reduce larval weight and increase the developmental time. Amiri et al. (2020) showed that the Sunn pest gluten hydrolase gene knockdown using a topical assay of the different concentrations of the dsRNA, reduced the fifth instar nymphal stage duration in a dose-dependent manner.

A major drawback in silencing target genes is the delivery of dsRNAs (Yamamoto-Hino and Goto, 2013). One of the well-organized methods for the practical application of RNAi technique in insects is the oral delivery of dsRNA. Camargo et al. (2016) used two methods to deliver dsRNA to *T. absoluta* larvae. In the first approach, they delivered the dsRNAs into tomato leaflets and in the second one, a system based on ''in planta-induced transient gene silencing'' was applied. Furthermore, injecting dsRNA into the insect body has high efficiency in the silencing of gene expression (Katoch et al., 2013). In this way, the exact amount of dsRNA can deliver immediately into the hemolymph and get to the target tissue easily (Yu et al., 2013). Majidiani et al. (2019) indicated that during the injection procedure, the dsRNAs could reduce the transcript levels of the *T. absoluta* targeted genes in a range of about 63–75% and caused 92% mortality at a maximum rate. In the current study, we treated the tomato leaf-miner with the dsRNAs using the topical application as a delivery method. There is some evidence that topically applied dsRNAs could lead to gene expression inhibition. Wang et al. (2011) showed that direct spray of dsRNAs constructed for DS10 and DS28 genes were effective on newly hatched *Ostrinia furnalalis* larvae proposing that dsRNA could penetrate the lepidopteran larvae integument and could affect larval development and survivorship.

5. Conclusion

The current study showed that the results of the RNA interfering mechanism against caspase-1 had, to some extent, adverse effects on the biology of the tomato leaf-miner, *T. absoluta.* These effects were the reduction of the larval body weight, the decline in the appearance of adult individuals, delay in the insect growth and development, as well as reduction of the gene transcripts showing the gene silencing. However, though the gene silencing was not persistent over time, showing promising results that RNAi techniques could be used in the IPM programs in order to combat the tomato leaf-miner in the agriculture systems.

Author contribution

Investigation, methodology, software, data gathering, data analysis, and writing the original draft of the manuscript, S. Rahmani; funding acquisition, writing, review, and editing, A.R. Bandani.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research is supported by a research grant (Grant Number 92005987) under Iran National Science Foundation, Tehran, Iran.

References

Accorsi, A., Zibaee, A., Malagoli, D., 2015. The multifaceted activity of insect caspases. J. Insect Physiol. 76, 17–23.

Ahmad, M., Srinivasula, S.M., Wang, L., Litwack, G., Fernandes-Alnemri, T., Alnemri, E. S., 1997. *Spodoptera frugiperda* caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein p35. J. Biol. Chem. 272 (3), 1421–1424.

Amiri, A., Bandani, A.R., Alizadeh, H., 2016. Molecular identification of cysteine and trypsin protease, effect of different hosts on protease expression, and rnai mediated silencing of cysteine protease gene in the sunn pest. Arch. Insect Biochem. 91 (4), 189–209.

Amiri, A., Bandani, A.R., 2020. Gluten hydrolase gene silencing using RNAi and its effect on the Sunn pest growth and development. Phytoparasitica 48 (4), 575–587. https://doi.org/10.1007/s12600-020-00821-8.

Arguelles, S., Guerrero-Castilla, A., Cano, M., Munoz, M.F., Ayala, A., 2019. Advantages and disadvantages of apoptosis in the aging process. Ann. N.Y. Acad. Sci. https://doi.org/10.1111/nyas.14020.

Bento, F.M.M., Marques, R.N., Campana, F.B., Demetrio, C.G.B., Leandro, R.A., Parra, J. R.P., Figueira, A., 2020. Gene silencing by RNAi *via* oral delivery of dsRNA by bacteria in the South American tomato pinworm, *Tuta absoluta*. Pest Manag. Sci. 76, 287–295.

Biondi, A., Guedes, R.N.C., Wan, F.-H., Desneux, N., 2018. Ecology, worldwide spread, and management of the invasive south American tomato pinworm, *Tuta absoluta*: past, present, and future. Annu. Rev. Entomol. 63 (1), 239–258.

Camargo, R.A., Barbosa, G.O., Possignolo, I.P., Peres, L.E.P., Lam, E., Lima, J.E., Figueira, A., Marques-Souza, H., 2016. RNA interference as a gene silencing tool to control *Tuta absoluta* in tomato (Solanum lycopersicum). PeerJ 4, e2673. https://doi.org/10.7717/peerj.2673.

Cao, M., Gatehouse, J., Fitches, E., 2018. A systematic study of RNAi effects and dsRNA stability in *Tribolium castaneum* and *Acyrthosiphon pisum*, following injection and ingestion of analogous dsRNAs. Int. J. Mol. Sci. 19 (4), 1079.

Chang, H.Y., Yang, X., 2000. Proteases for cell suicide: functions and regulation of caspases. Microbiol. Mol. Biol. Rev. 64 (4), 821–846.

Christiaens, O., Niu, J., Nji Tizi Taning, C., 2020. RNAi in Insects: a revolution in fundamental research and pest control applications. Insects 11 (7), 415. https://doi.org/10.3390/insects11070415.

Cooper, D.M., Granville, D.J., Lowenberger, C., 2009. The insect caspases. Apoptosis 14, 247–256.

Courtiade, J., Pauchet, Y., Vogel, H., Heckel, D.G., 2011. A comprehensive characterization of the caspase gene family in insects from the order Lepidoptera. BMC Genom. 12, 357.

Fernandes-Alnemri, T., Armstrong, R., Krebs, J., 1996. In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc. Natl. Acad. Sci. U. S. A. 93, 7464–7469.

Fraser, A.G., Evan, G.I., 1997. Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. EMBO (Eur. Mol. Biol. Organ.) J. 16, 2805–2813.

Garbutt, J.S., Bellés, X., Richards, E.H., Reynolds, S.E., 2013. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*. J. Insect Physiol. 59, 171–178.

Gharekhani, G.H., Salek-Ebrahimi, H., 2014. Life table parameters of *Tuta absoluta* (Lepidoptera: Gelechiidae) on different varieties of tomato. J. Econ. Entomol. 107 (5), 1765–1770.

Giesbrecht, D., Heschuk, D., Wiens, I., Boguski, D., LaChance, P., Whyard, S., 2020. RNA interference is enhanced by knockdown of double-stranded RNases in the yellow fever mosquito *Aedes aegypti*. Insects 11, 327.

Han, P., Bayram, Y., Shaltiel-Harpaz, L., Sohrabi, F., Saji, A., Esenali, U.T., Jalilov, A., Ali, A., Shashank, P.R., Ismoilov, K., Lu, Z., Wang, S., Zhang, G., Wan, F., Biond, A., Desneux, N., 2018. *Tuta absoluta* continues to disperse in Asia: damage, ongoing management and future challenges. J. Pest. Sci. 92 (4), 1317–1327.

Hebert, C.G., Valdes, J.J., Bentley, W.E., 2009. Investigating apoptosis: characterization and analysis of *Trichoplusia ni*-caspase-1 through overexpression and RNAi mediated silencing. Insect Biochem. Mol. Biol. 39, 113–124.

Jeong, S.Y., Seol, D.W., 2008. The role of mitochondria in apoptosis. BMB Rep. 41 (1), 11–22. https://doi.org/10.5483/bmbrep.2008.41.1.011. PMID: 18304445.

Kamezaki, M., Yokoi, K., Miura, K., 2018. RNA interference mediated knockdown of an inhibitor of apoptosis protein induces apoptosis in *Mythimna separata* (Lepidoptera: Noctuidae). Eur. J. Entomol. 115, 223–231.

Katoch, R., Sethi, A., Thakur, N., Murdock, L.L., 2013. RNAi for insect control: current perspective and future challenges. Appl. Biochem. Biotechnol. 171, 847–873. https://doi.org/10.1007/s12010-013-0399-4.

Khoa, D.B., Trang, L.T.D., Takeda, M., 2012. Expression analyses of caspase-1 and related activities in the midgut of *Galleria mellonella* during metamorphosis. Insect Mol. Biol. 21 (2), 247–256. https://doi.org/10.1111/j.1365-2583.2011.01131.x.

Liu, G., Lv, Z., Wu, Q., Zhou, Z., Zhang, G., Wan, F., Yan, Y., 2020. The *Bactrocera dorsalis caspase-1* gene is expressed throughout development and required for female fertility. Pest Manag. Sci. https://doi.org/10.1002/ps.5966.

Liu, H., Zhou, K., Yang, Z., 2020. Identification and functional characterization of SlDronc in *Spodoptera littoralis*. PeerJ 3 (8), e10329. https://doi.org/10.7717/peerj.10329.

Liu, Q., Qi, Y., Chejanovsky, N., 2005. *Spodoptera littoralis* caspase-1, a Lepidopteran effector caspase inducible by apoptotic signaling. Apoptosis 10 (4), 787–795. https://doi.org/10.1007/s10495-005-0365-x.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Ct method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lu, M.-X., Du, Y.-Z., Cao, S.-S., Liu, P., Li, J., 2013. Molecular cloning and characterization of the first caspase in the striped stem borer, *Chilo suppressalis*. Int. J. Mol. Sci. 14 (5), 10229–10241.

Majerowicz, D., Alves-Bezerra, M., Logullo, R., Fonseca-de-Souza, A.L., Meyer-Fernandes, J.R., Braz, G.R., Gondim, K.C., 2011. Looking for reference genes for real-time quantitative PCR experiments in *Rhodnius prolixus* (Hemiptera: Reduviidae). Insect Mol. Biol. 20 (6), 713–722.

Majidiani, S., Farshbaf, P.R., Laudani, F., Campolo, O., Zappalà, L., Rahmani, S., Mohammadi, S.A., Palmeri, V., 2019. RNAi in *Tuta absoluta* management: effects of injection and root delivery of dsRNAs. J. Pest. Sci. https://doi.org/10.1007/s10340-019-01097-6.

Mamta, K., Reddy, R.K., Rajam, M.V., 2016. Targeting chitinase gene of Helicoverpa armigera by host-induced RNA interference confers insect resistance in tobacco and tomato. Plant Mol. Biol. 90 (3), 281–292.

Morciano, G., Marchi, S., Morganti, C., Sbano, L., Bittremieux, M., Kerkhofs, M., Corricelli, M., Danese, A., Karkucinska-Wieckowska, A., Wieckowski, M.R., Bultynck, G., Giorgi, C., Pinton, P., 2018. Role of mitochondria-associated ER membranes in calcium regulation in cancer-specific settings. Neoplasia 20 (5), 510–523. https://doi.org/10.1016/j.neo.2018.03.005.

Paim, R.M., Pereira, M.H., Di Ponzio, R., Rodrigues, J.O., Guarneri, A.A., Gontijo, N.F., Araújo, R.N., 2012. Validation of reference genes for expression analysis in the salivary gland and the intestine of *Rhodnius prolixus* (Hemiptera, Reduviidae) under different experimental conditions by quantitative real-time PCR. BMC Res. Notes 5 (128), 1–11. https://doi.org/10.1186/1756-0500-5-128.

Parthasarathy, R., Palli, S.R., 2007. Developmental and hormonal regulation of midgut remodeling in a lepidopteran insect, *Heliothis virescens*. Mech. Dev. 124, 23–34.

Pereyra, P.C., Sánchez, N.E., 2006. Effect of Two Solanaceous plants on developmental and population parameters of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). Neotrop. Entomol. 35 (5), 671–676. https://doi.org/10.1590/s1519-566x2006000500016.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Pridgeon, J.W., Zhao, L., Becnel, J.J., Strickman, D.A., Clark, G.G., Linthicum, K.J., 2008. Topically applied AaeIAP1 double-stranded RNA kills female adults of *Aedes aegypti*. J. Med. Entomol. 45, 414–420.

Simmer, F., Moorman, C., van der Linden, A.M., Kuijk, E., van den Berghe, P.V.E., Kamath, R.S., Fraser, A.G., Ahringer, J., Plasterk, R.H.A., 2003. Genome-wide RNAi of *C. elegans* using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol. https://doi.org/10.1371/journal.pbio.0000012.

Sylla, S., Brévault, T., Monticelli, L.S., Diarra, K., Desneux, N., 2019. Geographic variation of host preference by the invasive tomato leaf miner *Tuta absoluta*: implications for host range expansion. J. Pest. Sci. 92 (4), 1–10. https://doi.org/10.1007/s10340-019-01094-9.

Tseng, Y.-K., Wu, M.-S., Hou, R.F., 2008. Induction of apoptosis in SF21 cell line by conditioned medium of the entomopathogenic fungus, *Nomuraea rileyi*, through *sf*-caspase-1 signaling pathway. Arch. Insect Biochem. 68, 206–214.

Wang, K., Peng, Y., Pu, J., Fu, W., Wang, J., Han, Z., 2016. Variation in RNAi efficacy among insect species is attributable to dsRNA degradation in vivo. Insect Biochem. Mol. Biol. 77, 1–9.

Wang, C., Youle, R.J., 2009. The role of mitochondria in apoptosis. Annu. Rev. Genet. 43, 95–118. https://doi.org/10.1146/annurev-genet-102108-134850.

Wang, Q., Ju, X., Chen, L., Chen, K., 2017. Caspase-1 from the silkworm, *Bombyx mori*, is involved in *Bombyx mori* nucleopolyhedrovirus infection. Z. Naturforsch. C. J. Biosci. 72 (3–4), 147–153.

Wang, Y., Zhang, H., Li, H., Miao, X., 2011. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PloS One 6, e18644.

Yamamoto-Hino, M., Goto, S., 2013. In vivo RNAi-based screens: studies in model organisms. Genes 4, 646–665. https://doi.org/10.3390/genes4040646.

Yang, C., Lin, X.-W., Xu, W.-H., 2017. Cathepsin L participates in the remodeling of the midgut through dissociation of midgut cells and activation of apoptosis via caspase-1. Insect Biochem. Mol. Biol. 82, 21–30.

Yang, D., Chai, L., Wang, J., Zhao, X., 2008. Molecular cloning and characterization of Hearm caspase-1 from *Helicoverpa armigera*. Mol. Biol. Rep. 35, 405–412.

Yu, H., Li, Z.-Q., Ou-Yang, Y.Y., Huang, G.H., 2019. Identification of four caspase genes from *Spodoptera exigua* (Lepidoptera: Noctuidae) and their regulations toward different apoptotic stimulations. Insects 27 (6), 1158–1172. https://doi.org/10.1111/1744-7917.12741.

Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G., 2013. Delivery of dsRNA for RNAi in insects: an overview and future directions. Insect Sci. 20 (1), 4–14.

Zhuang, H.M., Wang, K.F., Miyata, T., Wu, Z.J., Wu, G., Xie, L.H., 2011. Identification and expression of caspase-1 gene under heat stress in insecticide-susceptible and -resistant *Plutella xylostella* (Lepidoptera: plutellidae). Mol. Biol. Rep. 38, 2529–2539.

Zoog, S.J., Schiller, J.J., Wetter, J.A., Chejanovsky, N., Friesen, P.D., 2002. Baculovirus apoptotic suppressor P49 is a substrate inhibitor of initiator caspases resistant to P35 in vivo. EMBO J. 21 (19), 5130–5140. https://doi.org/10.1038/sj.emboj.7594736.