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Influence of *Spodoptera littoralis* Multiple Nucleopolyhedrovirus miRNAs on *Spodoptera littoralis* Gene Transcription Regulation Associated with Apoptotic Pathway

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Abstract

MicroRNAs (miRNAs) are evolutionarily conserved RNAs that play a crucial role in the apoptotic process in insects. In this study, the transcriptional regulation of eight apoptotic-related genes (Fas1, Fas2, Caspase 8, Caspase 5, COX1, Apaf1, Dronc and Effector Caspase-1) in *Spodoptera littoralis* was analyzed. Using *in silico* prediction tools, 43 novel miRNAs were predicted to be encoded by *Spodoptera littoralis* nucleopolyhedrovirus (SpliMNPV). Of these, seven putative SpliMNPV miRNAs targeting the detected apoptotic genes were validated by quantitative real-time PCR (qPCR) after *S. littoralis* infection with SpliMNPV at different time points: 24, 48 and 72 h post-infection (hpi). Four miRNAs (miR1402, miR94, miR638 and miR749) were found to be unique to SpliMNPV. Gene expression analysis revealed that most of the *S. littoralis* apoptotic target genes were downregulated by viral miRNAs during SpliMNPV infection. The Dronc gene exhibited the highest level of downregulation at 72 hpi. In contrast, COX1 gene expression was upregulated at 48 and 72 hpi, respectively. Additionally, Apaf1 gene expression showed a positive correlation with most apoptotic target genes, whereas COX1 expression exhibited a negative correlation with Dronc, Effector Caspase-1, Fas1 and Fas2 gene expression. These findings suggest that the identified SpliMNPV miRNAs significantly influence the transcriptional regulation of *S. littoralis* apoptotic-related genes in response to viral infection.

Keywords: Apoptosis pathway; miRNA; *Spodoptera littoralis*; SpliMNPV; Gene regulation

Introduction

MicroRNAs are known as small endogenous non-coding RNAs of 21-24 nucleotides in length, which control gene expression at post-transcriptional level. This achieved by incomplete base pairing of miRNA "seed region" (usually 2 to 8 nucleotides) at the 5′ end to the complementary region on the messenger RNA (mRNA) the 3′-UTR (3′-untranslated regions) of the target genes, which leads for either mRNA translational inhibition or mRNA degradation (Chen and Rajewsky 2007; Motameny *et al.* 2010). In insect's complete complementarity, which results in mRNA cleavage, is rare. While partial complementarity which leads to either mRNA deadenylation and/or translation repression is more common (Bartel 2009; Huntzinger and Izaurralde 2011). The first miRNA discovery was in *Caenorhabditis elegans* (Lee *et al.* 1993; Reinhart *et al.* 2000), while the herpes virus was the first recognized viral-mediated miRNA (Pfeffer *et al.* 2004). Furthermore, Heliothis virescens ascovirus (HvAV) was determined as the first insect virus that has been recorded to encode miRNA (Hussain *et al.* 2008). Many viral encoded miRNAs have been detected, mainly from adenoviruses, herpesviruses, iridoviruses, ascoviruses, polyomaviruses and baculoviruses using computational prediction as well as small RNA sequencing approaches (Kozomara *et al.* 2019). Baculoviruses were described as the main group of insect-specific double-stranded DNA viruses, which

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predominantly infect Lepidoptera insects and have been shown to encode miRNAs (Zhu *et al.* 2013; Kharbanda *et al.* 2015; Jiao *et al.* 2019). This group of viruses are considered as the most safe and promising insect viruses to be involved in pest management strategies (Petersen *et al.* 2022; Sá *et al.* 2023). In addition, baculoviruses shown to be used as an excellent expression system in biotechnology (Contreras-Gómez *et al.* 2014; Williams *et al.* 2017). Different miRNAs have been detected from different baculovirus species such as; *Autographa californica* multiple nucleopolyhedrovirus (6 miRNAs were identified), *Spodoptera litura* nucleopolyhedrovirus (48 miRNAs were detected in which 10 miRNAs out of them were validated in Sf21 cells) (Kharbanda *et al.* 2015; Tang *et al.* 2019; Wang *et al.* 2021). Furthermore, four miRNAs were identified from Bombyx mori nuclear polyhedrosis virus (BmNPV) (Singh *et al.* 2010). The miRNAs appeared to play a crucial role in virus-host interaction, through transcription regulating of both viral and cellular genes (Asgari 2011; Mishra *et al.* 2020). A key aspect of viral infections is the presence of cellular miRNA genes that influence viral processes, along with viral miRNA genes that regulate their own expression to counter host defenses (Motta *et al.* 2024). Different reports elucidated that miRNAs are engaged in numerous physiological processes, such as apoptosis, cancer, brain development and proliferation of cells (Fullaondo and Lee 2012; Singh 2020). Apoptosis, also known as programmed cell death, is a conserved event in both vertebrates and invertebrates. It is highly essential for retaining cellular homeostasis and immune system manipulation (Liu *et al.* 2020; Bansal *et al.* 2021; Jang and Lee 2021). Apoptosis generally performed *via* extrinsic and intrinsic pathways, which link up through Caspases family activation (Liu *et al.* 2020; Kumar *et al.* 2022). In Drosophila genome, seven caspases have been discovered (Kumar and Doumanis 2000; Liu *et al.* 2020). In a previous phylogenetic study, it was reported that Caspase-1, -2 and -3 from Lepidoptera are clustered with the three caspases Drice, Decay and Dcp-1 identified in Drosophila (Courtiade *et al.* 2011). In addition, Caspase-1 has been identified in *Spodoptera frugiberda* (Ahmad *et al.* 1997) and *Spodoptera littoralis* (Liu *et al.* 2005), while Caspase-8, Caspase-7 and Caspase-6 were detected in *Spodoptera exigua* (Yu *et al.* 2020). The Dronc, an apoptotic-related gene, has been identified in several Lepidopteran insects, such as *S. frugiperda* (SfDronc), *Bombyx mori* (BmDronc), *Lymantria dispar* (LdDronc) and *S. littoralis* (SlDronc) (Suganuma *et al.* 2011; Huang *et al.* 2013; Kitaguchi *et al.* 2013; Singh and Ambi 2019; Liu *et al.* 2020). It has been reported that lepidopteran insects possess the mitochondrial-derived apoptotic pathway and/or intrinsic pathway, *e.g*., *Bombyx mori*, *S. frugiperda*, *S. litura* and *S. exigua*. Additionally, evidence revealed that the cytochrome c functions similarly in mammalian and Lepidoptera cells. Where in both cells, the activation of the caspase cascade and the mitochondrial pathway depend on cytochrome c (Kumarswamy *et al.* 2009; Liu *et al.* 2012; Wang *et al.* 2020; Kumar *et al.* 2022). *Spodoptera littoralis* (Lepidoptera: Noctuidae), also known as the Egyptian cotton leafworm, is considered one of the most destructive cotton pests in Africa, Middle Eastern and Mediterranean Europe. Moreover, *S. littoralis* has been shown to infect more than 40 plant species, including approximately 87 economically important vegetable and ornamental plants (Salama *et al.* 1971; Ellis 2004). The *Spodoptera littoralis* multiple nucleopolyhedrovirus (SpliMNPV) is an alphabaculovirus, infecting only *Spodoptera* spp. (Toprak *et al.* 2006; Takatsuka *et al.* 2007). Although, *S. littoralis* is a model organism for studying of virus-host interaction mechanisms, its apoptotic mechanism is still unclear. Therefore, this study aims to identify *S. littoralis* genes involved in apoptosis pathway, prediction and validation of SpliMNPV encoded miRNAs and subsequently identifying their role in transcription regulation of *S. littoralis* apoptotic related genes upon SpliMNPV viral infection.

Materials and Methods

Prediction of SpliMNPV miRNA sequence retrieval

The FASTA format of SpliMNPV accession no. JX454574, (Isolate AN-1956) was retrieved from NCBI. The *S. littorlis genome* is evolutionarily related to *S. litura* species, which are known as “sister species” (Li *et al.* 2021), therefore miRNAs encoded with SpliMNPV were determined using the whole genome of *S. litura* of 429.583 Mb (Accession no. PRJNA344815). Four typical characteristics are necessary for miRNA target prediction: Firstly, the complementary of 2-8 nucleotides at the 5′ end "seed match binding site" and a miRNA, which is known as Watson-Crick complementary. The second characteristic is conservation which indicates sequence maintenance throughout species. The third one is the free energy, which is used to determine the binding stability of targeted mRNA and its miRNA. Finally, the fourth characteristic which is the site availability that refers to positioning of miRNA and its binding to the target mRNA (Peterson *et al.* 2014).

Pre-miRNA prediction

Pre-miRNA candidate identification was carried out using VMir-specific *ab initio* viral pre-miRNA prediction software (v. 1.5) based on the comparing structural characteristics of identified pre-miRNA hairpins (Grundhoff *et al.* 2006). The program structure prediction is performed based on minimal free energy (MFE) through the RNAfold algorithm (Hofacker *et al.* 1994). Moreover, it classifies separate hairpins that are higher than a certain size cutoff of 45 nt, which are investigated through a scoring algorithm, in which the size of the step is set to 10 nt and the size of the window is set to 500 nt. To predict hairpin-like sequences (pre-miRNAs), the SpliMNPV genome (137.998 bp) was scanned in both orientations including the following

settings, the max size assigned Subsidiary Hairpins (SHPs) was 50, while max size assigned Repeated Hairpins (RHPs) was 50 and the calculate score was 100. To identify pre-miRNA candidates of the highest probability, the parameters were filtered by setting the following values for the hairpins; minimal score of 115, minimal window cutoffs of 35 and a hairpin length of 50 to 220 nucleotides.

Real pre-miRNA confirmation

IMiRNA-SSF web server was used, for filtering real and pseudo pre-miRNAs (Chen *et al.* 2016), based on the Minimal Free Energy (MFE); that describes an RNA secondary structure stability. The iMiRNA-SSF MFE was processed using the RNAfold program included in the Vienna RNA software (Hofacker 2003).

Prediction of mature miRNA

SpliMNPV mature miRNAs prediction was carried out using MatureBayes; a web server, which operates Naive Bayes classify for mature miRNAs prediction, depending on pre-miRNAs sequence features, in addition to the secondary structure in a provided pre-miRNA (Gkirtzou *et al.* 2010).

MiRanda program for miRNA target prediction

MiRanda program was used for viral miRNA target prediction (Enright *et al.* 2003), using the Smith-Waterman hybridization method, in which the alignment score should exceed 90 (Smith and Waterman 1981). Scaling factor should be equal to 2 and the miRNA::mRNA hybrid free energy change (ΔG) should be lower than −20 kcal/mol. Free energy; is estimated using the Vienna package through predicting miRNA : mRNA duplex folding (Hofacker *et al.* 1994).

Screening of Target hits

TargetScan is a simple method used by beginner users and consistently maintained, in which there is no need for sequence input or advanced settings configuration. Users can perform searches using miRNA names, gene names, or by selecting miRNA families that are conserved, broadly conserved, or poorly conserved across multiple species (Lewis *et al.* 2005; Grimson *et al.* 2007; Friedman *et al.* 2009; Garcia *et al.* 2011). Screening of MiRNA-target alignments was performed using the consequence guidelines: no mismatches, no gaps and only one G : U pairing is admitted at the 5′end of the miRNA; 2-8 nucleotides in the seed region. In addition, there should be no more than two gaps present in the alignment and a minimum of two matches in the final five slots (Krek *et al.* 2005; Lewis *et al.* 2005; Singh and Ambi 2019).

Secondary structure of pre-miRNAs

Precursor sequences of predicted miRNAs were isolated considering mature miRNA flanking sequences. A sliding window of 100 nucleotides (proceeding in the increment of approximately 10 nt) was obtained from the mature miRNA upstream start position which is about 80 nucleotides, till the miRNA downstream region which is also about 80 nucleotides (Singh and Nagaraju 2008). Then, for secondary structure prediction the isolated pre-miRNA sequences were screened through the Mfold program. Pre-miRNA: structure selection criteria depend on specific instructions: ΔG should be lower than −20 kcal/*M*. Furthermore, the sequences of mature miRNA should not be positioned on the unstable and the hairpin loop region.

Infection of *S. littoralis* using SpliMNPV

The *S. littoralis* larvae were provided from the provision of Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt. Larvae rearing was carried out on a semi-artificial diet, incubated at 27°C and 60% Relative humidity (RH) (Levinson and Navon 1969). For viral infection, freshly fourth instar larvae fed orally on partially purified occlusion bodies of SpliMNPV (10,000 OBs/larvae). The gut tissues from non-infected and infected larvae were isolated at different time interval of; 0, 24, 48 and 72 h pi, then instantly frozen in liquid nitrogen and finally stored at -80°C for small RNA extraction.

Small RNA extraction and reverse transcription

Total RNA was extracted from larval gut tissues of non-infected and SpliMNPV-infected fourth-instar larvae according to the general procedure of miRNA extraction method, in which frozen sample tissues were homogenized in lithium chloride and phenol (Vera-Hernández *et al.* 2019). NanoQuant (TECAN, M200 PRO) was used to measure RNA sample's concentration and purity. Thereafter, samples were treated using DNase I (Thermo-scientific) to get rid of any genomic DNA contamination. The cDNA was synthesized from purified RNA samples for target genes and miRNA detection. In the case of target genes detection, reactions were carried out using a thermos-scientific cDNA kit (#K1622, LOT no. 01112206), in which each reaction contained 100 ng/μL of total RNA, 5X reaction buffer, 10 m*M* dNTPs, 1 μL Oligo primer, 20 U·μL Ribolock RNase Inhibitor and 200 U·μL RevertAid M-MuLV RT. All reactions were incubated for 60 min at 42 °C, then 5 min at 70°C and finally held at 4°C. Whilst for miRNAs detection; specific stem-loop RT primer used for cDNAs synthesis were designed based on the predicted miRNAs mature sequences using the following website (http://www.flujogenico.cl/plantbiotech/amir319edesigner/), in which each reaction contains 11 μL total RNA (100 ng/μL), ddH_2O and 125 n*M* specific stem-loop RT primers that were firstly held for 10 min at 70°C, then maintained on ice for 2 min. Finally, the remaining reagents were added and mixed. The 20 μL total reactions were carried out in a SimpliAMP Thermal Cycler system (Applied biosystems, Thermofisher, USA) using the following reaction profile;

pre-incubation for 30 min at 16°C, followed by 30 sec at 20°C, then 30 sec at 42°C, 1 sec at 50°C, 5 min at 85°C and finally held at 4°C.

Stem-loop RT-PCR and qPCR

To measure the mRNAs and miRNAs expression levels, qPCRs were performed in a total reactions of 10 μL including the following components; 1 μL cDNA, 10 µM total concentration of each primer; and 2X SYBR Green Master Mix BioEasy (provided by BIOER, Hangzhou, China). For each time interval, three independent gut samples were pooled and three technical replicates were performed for each experiment. The relative expression of each gene was measured by applying Agilent Stratagene Mx3005p real-time PCR detection process based on the $2^{-\Delta\Delta Ct}$ technique (Schmittgen and Livak 2008) following the manufacturer's method. MiRNA-target genes expression analyses were carried out in 20 μL total volume including 10 μL of SYBR Green 2X Master Mix, 0.3 μL of 10 μM concentration for each specific forward and specific reverse primer that were designed using IDT PrimerQuest online Tool (https://www.idtdna.com/pages/tools/primerquest) and 1 μL of each diluted cDNA sample. Though miRNA expression analyses were performed in a total volume of 20 μL containing 1 μL cDNA template (1:10 dilution), 7 μL nuclease-free water, 1 μL 10 μM of the forward specific primer for each miRNA, 1 μL 10 μM of the universal reverse primer and 10 μL of 2 × SYBR Green Master Mix. The PCR reaction profile was carried out as follows: 94°C preheating for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and final extension step at 72°C for 10 min. Analysis of the qPCR product amplicon melting curve was accomplished to verify a single peak for each RT product amplification. Endogenous reference genes were applied for reaction's normalization, Elongation factor 1 (EF1) was used for mRNA target genes normalization and U6 degenerate primer was generated using genefisher2 (https://bibiserv.cebitec.uni-bielefeld.de/genefisher2) for miRNAs normalization.

Statistical analysis

Statistical data analysis was carried out using SPSS v23.0 (Chicago, IL, USA), in addition to ANOVA for one-way analysis of variance. While the significance of mean variations at $P < 0.05$ was compared using Dunnett's multiple range test. A Correlation matrix was administered and envisaged in R with the package complot.

Conservation of SpliMNPV miRNAs

It is known that the SpliMNPV genome is closely related to the following Baculoviruses genomes (Breitenbach *et al.* 2013); SpltMNPV (Li *et al.* 2021), SpeMNPV (IJkel *et al.* 1999), SfMNPV (Harrison *et al.* 2008) and AcMNPV (Ayres *et al.* 1994). However, the highest similarity is between the two genomes SpliMNPV and SpltNPV at the protein and nucleotide level (Li *et al.* 2021). Therefore, it was interesting to investigate the seven validated SpliMNPV miRNAs evolutionary conservation along with the Baculoviruses miRNAs of SpltMNPV, SpeMNPV, SfMNPV and AcMNPV genomes of Accession no.; NC_003102, NC_002169, NC_009011 and X71415 respectively. The pre-miRNAs encoded by these viruses were firstly predicted using the VMir prediction tool and then compared to the seven validated SpliMNVP miRNAs using similarity homology blastn.

Results

***In silico* prediction of SpliMNPV pre-miRNAs**

Hairpin-like sequences (pre-miRNAs) prediction of the SpliMNPV genome (137998 bp) resulted in a total number of 5713 hairpins that were identified in both orientations (2892 hairpins in forward and 2821 hairpins in reverse direction) (Fig. 1). The stem-loop was filtered to 35 minimum window counts, where a hairpin of length ranges from 50 to 220 nucleotides. Hairpins in the diagram are plotted based on their genomic site and VMir score. Only 156 hairpins were identified in both orientations, 72 forward and 84 reverse orientations (Fig. 2).

Real pre-miRNA confirmation

Hairpin sequences of 156 pre-miRNA for SpliMNPV were detected using MFold program, from which position of the hairpin apex, hairpin size; hairpin score, SHPs, RHPs, absolute window count and relative window count were predicted. Among the 156 pre-miRNA, 111 were identified as pseudo-pre-miRNAs. Whereas, 45 were confirmed as real pre-miRNA (Table S1).

Mature SpliMNPV miRNA prediction

The iMiRNA-SSF analysis predicted 43 real pre-miRNA hairpins and their apoptotic target genes. Real pre-miRNA fulfill the criteria required for miRNAs, in which the minimal free energy (MFE) should be equal to or less than 20 kcal/mol (Singh *et al.* 2010). The hairpin primary structure, MFE and P-value are presented in Table S2. Mature miRNA of 43 hairpin sequences were predicted by MatureBayes web tool (Table S3). RNAfold program (Lorenz *et al.* 2011) was performed to confirm the potential hairpin-like secondary structures of the pre-miRNA sequences (Fig. 3).

Expression validation of *S. littoralis* apoptotic target genes and their predicted candidate miRNAs using qPCR

Eight apoptotic target genes **(**Fas1, Fas2, Cas8, Cas5, Cyto-c-oxidase (COX1), Apaf1, Dronc and Effector Caspase-1), were detected from *S. littoralis* through time interval of 0, 24, 48 and 72 h pi up on SpliNPV viral infection using qRT-

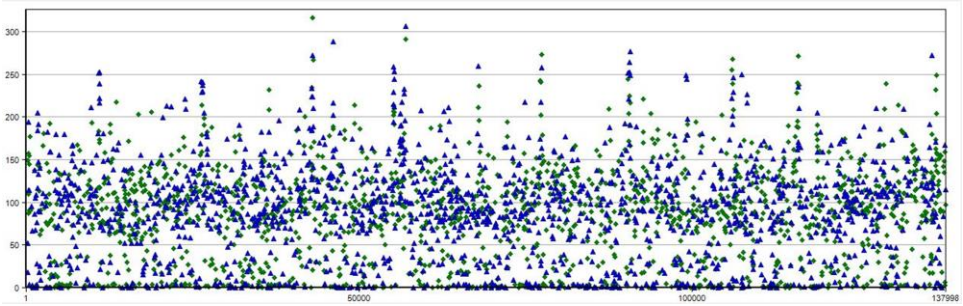

Fig. 1: SpliMNPV genome VMir analysis, demonstrates that all hairpins are broadly spread across the genome. Hairpins are mapped based on their genomic position and VMir score. Blue triangles stand for hairpins in reverse orientation and green diamonds represent hairpins reverse orientation

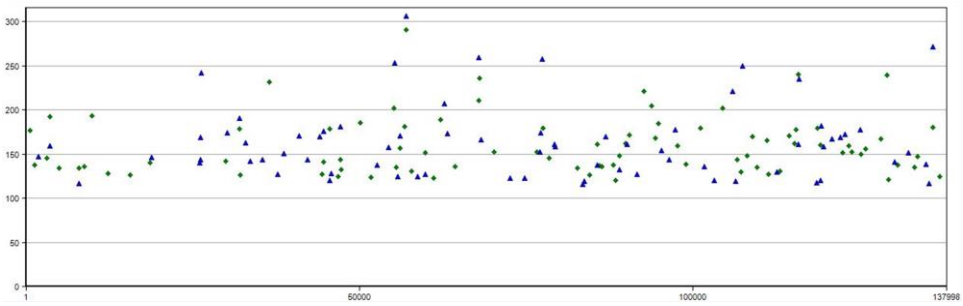

Fig. 2: Predicted SpliMNPV pre-miRNAs VMir. Blue triangles stand for hairpins in reverse orientation and green diamonds represent hairpins reverse orientation

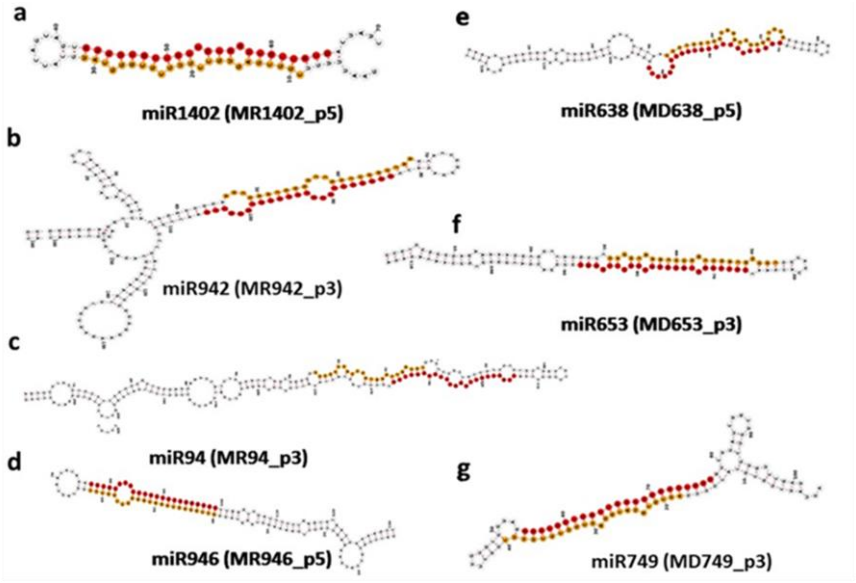

Fig. 3: Secondary structures of predicted SpliMNPV encoded miRNAs precursor of mature miRNAs. Predicted structure was performed using RNA fold. Colored sequences of the precursor miRNA on both arms indicate mature miRNAs. The red color stands for the mature 3'stem site, while orange color stands for the mature 5'stem site. Vienna package was carried out to calculate the MFE (ΔG) of optimum strand-strand interaction between the miRNA and UTR

PCR (Table S4). By performing *in silico* miRNA prediction tools, 43 SpliMNPV-encoded miRNAs were detected. Only seven miRNAs out of twelve selected putative miRNAs related to apoptotic target genes were validated using stem-loop RT-PCR (Table 1). To prepare cDNAs, stem-loop primers were employed and then miRNA-specific forward primers along with the universal reverse primer were used for amplification, as explained in the experimental section (Table S5). The amplified miRNA product ranges between 80 to 100 bp in length.

Table 1: Selected mature miRNA sequences predicted by the MatureBayes web tool and their apoptotic target genes

miRNA Candidates	Start	End	Sequence	MFE kcal/mol	Experimental Validation	Apoptotic target genes
MD942_p3	83	105	AAAAGUCAUACAUGUUCAAAUC	-80.2	Validated	APAF1
MR1402_p5	9	31	CGGCGAGUGCCGUGCGCGGGCA	-31.3	Validated	Caspase 5, APAF1, Fas1, Caspase DRONC
MR94_p3	114	136	UCGGGCGAUAGUUGAGCGGACG	-56.1	Validated	APAF1
MD946_p5	40	62	UUCGAAUCGAACAUGGCCGACU	-51.7	Validated	APAF1
MD638_p5	33	55	UGUCGUCGGUAAACUUGUUCGU	-34.3	Validated	Caspase 5, Caspase DRONC, Caspase 8
MD653_p3	66	88	AGUCGGCCAGGUUCGAUUCAAA	-44.1	Validated	Fas1, Fas2, Caspase DRONC
MD749_p3	55	77	AGGCGGCGGCCGUAGUGAUCAA	-46	Validated	Fas 1
MR31_p5	23	45	UGUCUUCCACUUCCUUCGAUUU	-36.9	No	
MR1372_p5	15	37	UUUACCAAUUGGGCGUCCGUGG	-25.8	No	
MD291_p3	85	107	UGAACAGUCGGCCACGUUCGAU	-52	No	
MD942_p5	46	68	AUCGAACGUGGCCGACUUUUCA	-47.4	No	
MR632_p5	51	73	UUGGUAUCAAACCUGUCUGACU	-60.8	No	

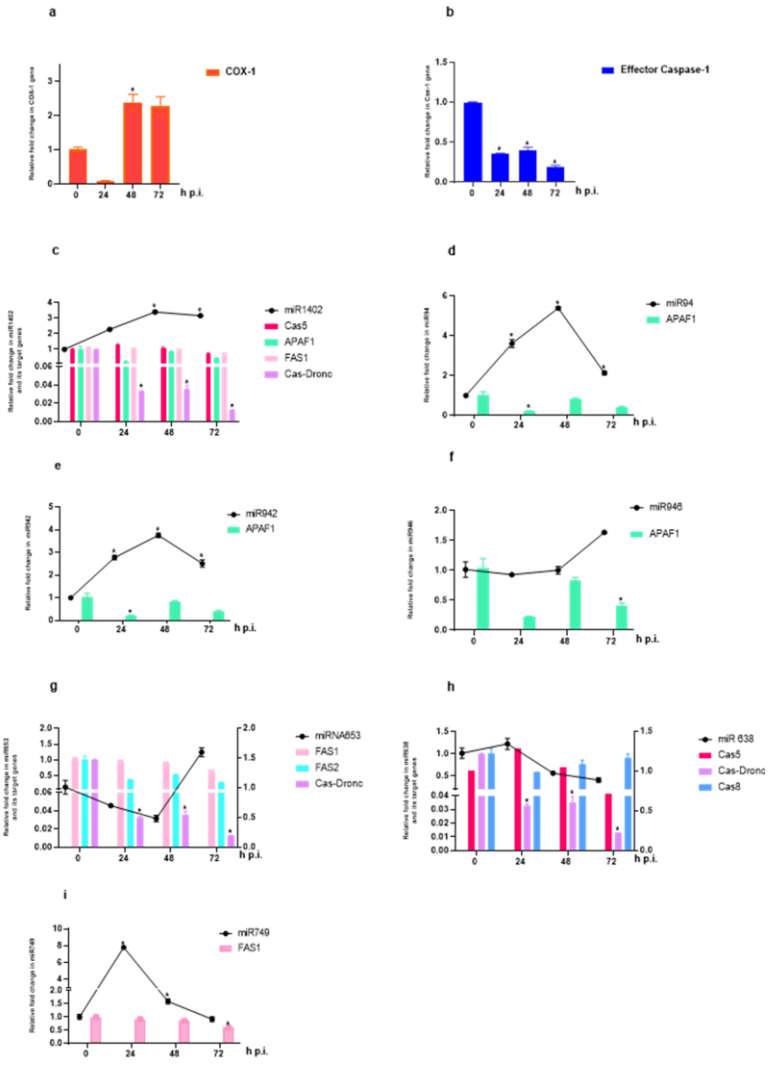

Fig. 4: Graphs represent the relative expression of predicted miRNAs and their apoptotic target genes determined by qRT-PCR. The Black lines show fold variations in miRNA expression relative to U6 rRNA expression. The colored columns represent fold changes of apoptotic target genes each with its candidate miRNA normalized to EF1 (translation elongation factor 1-alpha) expression. (**a**) COX-1. (**b**) Effector Caspase-1. (**c**) miR1402 and its target genes; Cas-Dronc, Fas1, Apaf1 and Cas-5. (**d**), (**e**) and (**f**) miR94, miR942, miR946 and Apaf1 target gene respectively. (**g**) miR653 and its candidate genes Cas-Dronc, Fas1 and Fas2. (**h**) miR638 and its target genes Cas-Dronc, Cas-8 and Cas-5. (**i**) miR749 and Fas1 target gene. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's multiple comparisons post hoc test, to compare the regulation of each miRNA and the expression of its apoptotic target genes at different time intervals after infection, with (*) $P < 0.05$ indicating statistical significance. The standard deviation is represented by the error bars

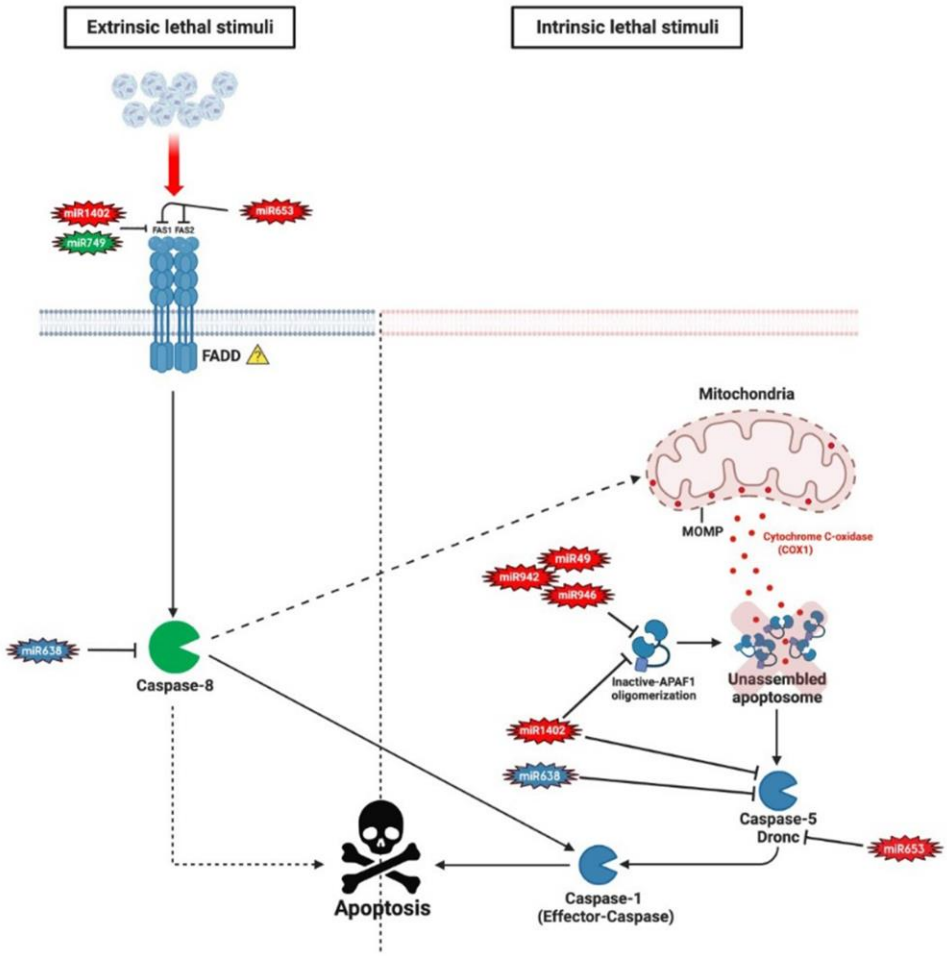

Fig. 5: *S. littoralis* proposed apoptotic pathway and SpliMNPV candidate miRNAs. The blue color signifies down-regulation, whereas the red color signifies up-regulation and the green color means normal expression. The diagram was created with BioRender.com, with respect to the model organism's apoptotic pathway identified in *C. elegance*, drosophila and mammals (Courtiade *et al.* 2011; Fuchs and Steller 2011; McIlwain *et al.* 2013; Bansal *et al.* 2021; Jang and Lee 2021; Umargamwala *et al.* 2024)

As shown in Fig. 4, Fas1 showed to be targeted by miR1402 and miR749. In addition, Fas1 and Fas2 showed also to be targeted by miR653, where both genes showed a mild down-regulation. Whereas, Caspase-8 which is detected to be regulated by miR638 showed a slight down-regulation at 48 and 72 hpi by 0.56 and 0.48, respectively. In contrast, COX1 gene expression revealed the most up-regulated gene in the apoptotic pathway at 48 and 72 hpi by 2.38 and 2.48 - fold, respectively. However, no miRNAs were detected for COX1 and Effector Caspase-1 genes. On the same context, the miR1402 is showed to target Fas1, Apaf1, Caspase 5 and Dronc genes, revealed the most down-regulation for the Dronc gene at 72 h pi by 0.01-fold. Whilst, miR1402 was up-regulated at 24 h pi by 2.28- fold, thereafter, increased up-regulation of its expression level at 48 h by 3.4- fold. Sequentially, its expression level was slightly down-regulated at 72 h pi, comparable with 48 h pi which was down-regulated by 3.16- fold. The Dronc gene is also detected to be targeted by miR653 and miR638, in which miR653 expression was slightly up-regulated at 72 h pi by 1.6-fold, whilst miR638 showed a mild down-regulation as mentioned above. Moreover, Apaf1 were detected to be regulated by 11 miRNAs, nevertheless; four miRNAs (miR1402, miR942, miR94 and miR 946) were selected and validated using qPCR. Apaf1 gene expression displayed a down-regulation especially at 24 hpi by 0.21-fold.

Proposed apoptotic pathway in *S. littoralis*

Schematic model summarizing the main effects of validated miRNAs and their target apoptotic genes (Fig. 5). Upon Baculovirus infection, the extrinsic pathway is initiated when a death ligand (SpliMNPV) binds to FAS1 and FAS2 (cell surface death receptors). This leads to the involvement of the adaptor molecule, Fas-associated death domain protein (FADD); which activates Caspase-8, that triggers the mitochondrial pathway, also referred as the intrinsic pathway for the mitochondrial outer membrane

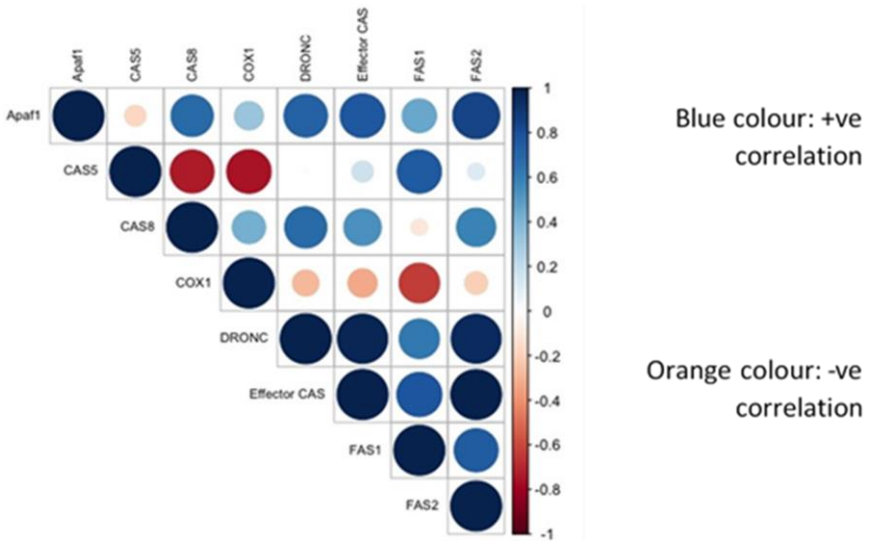

Fig. 6: Correlation matrix for Apoptotic target gene expression. The size and color of the circles indicate the strength of correlation. The blue color reveals a positive correlation, whilst orange color infers a negative correlation between apoptotic genes

permeabilization (MOMP), which stimulates the Cytochrome C release into the cytoplasm. COX1 gene develops a protein complex with Apoptotic protease activating factor-1 (Apaf-1), so called the apoptosome. Initiator caspases Dronc and Caspase-5 require the adaptor protein Apaf-1 to function. This, in turn activates the downstream effector caspase-1, which triggers apoptosis directly.

Correlation matrix for apoptotic target gene expression

Apaf1 gene expression shows a positive correlation with most apoptotic target genes, including Caspase-8, COX1, Dronc, Effector Caspase-1, Fas1 *and* Fas2. Additionally, the expression levels of Dronc, Effector Caspase-1, Fas1 and Fas2 exhibit a strong positive correlation with one another. However, *COX1* gene expression displays a negative correlation with Dronc, Effector Caspase-1, Fas1 and Fas2 expression. Furthermore, *Caspase-5* expression shows a strong negative correlation with Caspase-8 and COX1 (Fig. 6).

Evolutionary conservation analysis

Among the seven validated miRNAs, four miRNAs (miR1402, miR94, miR638 and miR749), were reported to be unique for SpliMNPV. In addition, the rest three validated SpliMNPV miRNAs SpliMNPV miRNAs showed an extensive similarity with SpltNPV miRNAs, in which miR942 and miR653 showed 100% similarity and miR946 showed 95% similarity (Table S6). However, other baculovirus genomes, including AcMNPV, *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) and *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), showed no similarirty to the seven validated SpliMNPV miRNAs.

Discussion

In this study, eight apoptotic target genes (Fas1, Fas2, Caspase-8, Caspase-5, COX1, Apaf1, Dronc and Effector Caspase-1) of S. littoralis were identified. By applying *in silico* prediction tools, the miRanda program and various filter screening methods based on several critical statistical parameters, a total of 43 miRNAs encoded by SpliMNPV were predicted. Since most viral target genes, host cell lysis and genes required for occlusion derived virus (ODV) assembly are late gene. This suggests that the majority of early-expressing miRNAs are responsible for late genes expression of late genes during infection (Hussain and Asgari 2014; Rohrmann 2019). Additionally, the majority of the identified SpltNPV encoded miRNAs had increased expression levels, at 72 hpi (Singh 2020). Therefore, this study planned to investigate the miRNAs encoded for apoptotic genes of SpliMNPV in the late phase of viral infection (12, 24 hpi) and in the very late phase (48, 72 hpi). Seven of the twelve selected SpliMNPV-encoded miRNAs targeting the apoptotic genes were validated. Interestingly, evolutionary relationship analysis revealed that of the seven validated SpliMNPV miRNAs, only three miRNAs (miR942, miR653 and miR946) showed a high degree of similarity to SpltNPV miRNAs. In contrast, the other four SpliMNPV miRNAs identified (miR1402, miR94, miR638 and miR749) were found to be remarkably unique to SpliMNPV. The miR1402, predicted to target the Fas1, Apaf1, Caspase-5 and Dronc genes, showed strong down-regulation of the Dronc gene, which in turn stimulates Effector Caspase-1, leading to stimulation of the apoptotic pathway (Fig. 5). It was hypothesized that Caspase-5 and Dronc play a comparable role in developmental apoptosis in Drosophila, as Dronc and Caspase-5 were found to be in the same phylogenetic tree. Nevertheless, Dronc shares a

homologous activation process with mammalian Caspase-9 (Courtiade *et al.* 2011). In mammals, miR133 and miR24a repress Caspase-9 to control the cell fate (Kumar 2007; Xu *et al.* 2007; Walker and Harland 2009). In contrast, in Drosophila miR14 suppress Dronc gene expression (Kumar and Doumanis 2000). The results thus confirm previous findings that Dronc is an essential initiator caspase in the intrinsic signaling pathway of Lepidoptera (Liu *et al.* 2020), that cleaves and induces Effector Caspase known as Caspase-1, which in turn cleaves downstream proteins to cause apoptosis (Daish *et al.* 2004). The results of gene expression analysis showed that the expression of apoptotic target gene was mainly down regulated after SpliMNPV infection. According to a previous study, most AcMNPV-encoded miRNAs showed down-regulation of target gene expression (Wang *et al.* 2021). However, it has been found that the interaction between miRNA and their target genes does not always lead to a reduction in gene expression, but in certain cases can also promote gene expression (Ørom *et al.* 2008; Hussain and Asgari 2010). The expression level of Fas1 is slightly down regulated by miR1402, while the expression of Fas1 and Fas2 at 72 hpi shows a mild downregulation by miR653 miRNA, which triggers the activity of Caspase-8 regulated by miR638 to promote the mitochondrial pathway. In contrast, in mammals, Fas expression in osteosarcoma cells is down regulated by miR-20a, miR-146a and miR-196b (Huang *et al.* 2012; Li *et al.* 2012; Guo *et al.* 2013). In this process, the expression of caspase-8 is suppressed by miR-874 to stimulate the necrosis process (Wang *et al.* 2013). In addition, Cytochrome-c was shown to play an important role in the mitochondrial signaling pathway through the release of Cyto-c, which causes Apaf1 activation and thus the formation of apoptosomes (Liu *et al.* 2012; Ma *et al.* 2021). In our study, COX1 from *S. littoralis* showed the highest gene expression up-regulation. Accordingly, a recent study demonstrated that the overexpression of cytochrome c oxidase subunit 4 isoform 1 (COX4I1) in host cells promoted H5N1 influenza virus replication, while silencing or knocking out COX4I1 inhibited viral replication (He *et al.* 2022). In another study, it has been proven that the early phase of Human immunodeficiency virus type 1 (HIV-1) infection enhances the up-regulation of cytochrome c oxidase activity in host cells to meet energy demands and regulate apoptosis (Ogawa *et al.* 2015). This suggests a potential regulatory role of cytochrome c oxidase in viral infections. Therefore, the up-regulation of COX1 may contribute to the activation of the mitochondrial apoptotic pathway; however, further research is needed to elucidate the precise regulatory mechanisms of COX1 expression in Lepidoptera during Baculovirus infections. In addition, Apaf1 displayed mild downregulation of gene expression, leading to failure of apoptosome assembly and inhibition of the apoptotic pathway. Moreover, Apaf1 expression was shown to be regulated by the four validated miRNAs: miR1402, miR94, miR942 and miR947. Furthermore, correlation matrix analysis demonstrated that Apaf1 gene expression exhibited a strong positive correlation with most of the detected apoptotic target genes (Caspase-8, COX1, Dronc, Effector Cas-1, Fas1 and Fas2), confirming that Apaf1 plays a crucial role in the apoptotic signaling pathway of *S. littoralis*. In contrast, in mammals miR23a/b and miR27a/b repressed the Apaf1 expression (Chen *et al.* 2014). Furthermore, our results support the possibility that mature miRNAs from the 5′- and 3′-arms of the hairpin precursor function, with the validated *S. littoralis* miRNAs miR942, miR94, miR653 and miR749 being the 3'-arms of hairpins, while miR1402, miR638 and miR946 are 5'-arms of hairpins. As previous studies have shown that miRNA in insects binds to the 3′ UTR of the mRNA target sequence, it was later demonstrated that miRNA responsive elements (MREs) were also recognized in the 5’ UTR sequences of mRNA targets, moreover, previously identified Baculovirus miRNAs overlap with regions encoding proteins (Bartel 2009; Cerrudo *et al.* 2023). Furthermore, it is known that one miRNA can regulate the expression of multiple genes, while one gene can be controlled by multiple miRNAs (Bielska *et al.* 2021). Considering their established functions in regulating biological mechanisms in insects, as well as their heritability and environmental friendliness, therefore, scientists synthesized artificial miRNAs (amiRNAs) engineered to target specific mRNAs in insect pests that enter their bodies during feeding with transgenic plants or *via* food to control insects (Gualtieri *et al.* 2020). Pre-miRNA from amiRNA processing has been shown to consistently knock out genes involved in insect pathogenicity, development and metabolism during plant production, with less possibility of off-target effects (Bordoloi and Agarwala 2021). In previous research, expression of amiRNA-319a targeting the ecdysone receptor of *Helicoverpa armigera* leading to reduction in transcripts of target genes in insects feeding on leaves of transgenic tomato plants (*Solanum lycopersicum*), affecting insect survival and growth development (Yogindran and Rajam 2021). Another efficient method for pest control is the trans-kingdom RNAi technique, which is based on the transfer of miRNAs within the food to the recipient organisms through bacterially expressed precursors for miRNAs designed to knock out the desired insect gene. Other approaches for miRNA transfer are currently under investigation, *e.g.*, CRISPR-based genome editing, endogenous vesicles such as exosomes, nanoparticles, viral and non-viral vectors such as lipid- or polymer-based vectors (Zhang *et al.* 2021). Therefore, miRNAs are believed to be a promising approach for the development of pesticides. However, our understanding of the miRNAs role in the apoptotic pathway of lepidopteran needs to be enhanced by additional research, in addition to studying the molecular mechanisms underlying virus-insect interactions.

Conclusion

This study provides insightful knowledge to understand the apoptotic pathway in Lepidoptera insects after baculovirus

infection. Eight apoptotic genes (Fas1, Fas2, Caspase-8, Caspase-5, COX1, Apaf1, Dronc and Effector Caspase-1) were detected in *S. littoralis*. In addition, four miRNAs miR1402, miR94, miR638 and miR749 were identified as unique to SpliMNPV. Gene expression analysis showed that most apoptotic target genes were down regulated by viral miRNAs after SpliMNPV infection. The miR1402 showed the most acute down-regulation for the Dronc gene at 72 h pi. In addition, COX1, Apaf1 and Dronc may play an important role in the apoptotic pathway of *S. littoralis*. Baculovirus miRNAs could be suitable targets for the development of novel pest control techniques as they have significant effects on gene regulation of the target insect pest on the pathway to overcome pesticide resistance. Elucidating the role of miRNAs in the control of insect pests is therefore a vital and challenging task for future research.

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Author Contributions

All authors contributed to the study conception and design. LG, WE, MM and MA: Material preparation, data collection and analysis; LG and WE: wrote the first draft of the manuscript, NA and AA: commented on previous versions of the manuscript; LG, WE, MM and MA: carried out the experiments, data collection and analysis; MM conducted the *in silico* prediction analysis; LG and MA: performed statistical analysis of data; LG, WE, AA and NA wrote the article and all authors reviewed and approved the final manuscript.

Conflicts of Interest

No conflict of interest.

Data Availability

Data could be requested with a reasonable scientific purpose to corresponding author.

Ethics Approval

Not applicable to this study.

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