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Development of reverse genetics tools in the two-spotted spider mite Tetranychus urticae

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

Tetranychus urticae, commonly known as the two-spotted spider mite, poses a significant threat to agriculture due to its ability to feed on a diverse range of plant hosts and its strong detoxification abilities in overcoming xenobiotic response. With global warming projected to increase spider mite infestations, it is vital to study the detoxification genes that enable the mite to adapt and survive. The spider mite genome sequence reveals a unique set of detoxification genes that can be studied using RNAi as a promising reverse genetic tool. However, the current genetic toolkit requires improvement. This study examined the effectiveness of three precursor molecules (dsRNA, shRNA, and amiRNA) in inducing RNAi response in spider mites. While shRNA and amiRNA demonstrated variable responses, dsRNA significantly reduced transcript levels of all three target genes. The establishment of primary cell cultures from *T. urticae* embryos provides a valuable tool for investigating the uptake mechanism of different precursor RNAi molecules through 'RNAi of RNAi' experiments. Ultimately, this research could contribute to the development of RNAi-based pesticides that selectively target spider mites detoxification pathways, leading to more effective pest control in agriculture. Future research should aim to improve our understanding of the spider mite RNAi machinery, including the uptake mechanism and precursor molecule stability, to facilitate functional gene analysis and the development of new pest control strategies.

Keywords: *Tetranychus urticae*, polyphagous pest, RNAi, genetic toolbox, primary cell culture, small RNA, reverse genetics, actin.

Summary for Lay Audience

The two-spotted spider mite, *Tetranychus urticae*, is a pest that can feed many different types of plants, causing significant economic losses in agriculture. One of the main challenges in controlling this pest is its ability to develop resistance to pesticides, making it crucial to explore alternative approaches for pest management. The two-spotted spider mite has developed high resistance to pesticides due to its ability to detoxify harmful compounds, which is controlled by a large number of genes. To understand the function of these genes, a tool called RNAi can be used to turn off their expression. The RNAi process begins by introducing precursor molecules into the system. These precursors are designed to interfere with the normal activity of the target gene, resulting in what is referred to as "knockdown" or suppression of its expression. However, the most commonly used precursor molecule, dsRNA, can have off-target effects, which means other genes may also be affected. To overcome this problem, my thesis investigated the use of alternative precursor molecules like shRNA and amiRNA for RNAi in spider mites. Additionally, primary cell cultures from spider mites were established, which are cells grown in a laboratory setting and are capable of mimicking the behavior of whole mites. This allowed us to study RNAi responses at the cellular level. The findings from this study suggest that dsRNA-induced RNAi is the most highly effective method, and that spider mite primary cell cultures are suitable for performing cellular assays such as RNAi experiments. Overall, this study establishes primary cell cultures as a valuable tool for performing RNAi, which can be used to further investigate the reasons for the lower efficiency of other precursor molecules compared to dsRNA in the future, by studying the enzymes involved in the RNAi machinery at the cellular level.

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List of abbreviations

Ago	Argonaute
amiRNA	artificial micro ribonucleic acid
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base pair
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
Ct	cycle threshold
CycA	cyclin A
CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
GFP	green fluorescent protein
L15	leibovitz 15
Mbp	mega base pair
MEGA	molecular evolutionary genetics analysis
MID domain	middle domain
miRNA	micro ribonucleic acid
MNNG	N methyl N' nitro N nitrosoguanidine
mRNA	messenger ribo nucleic acid
nt	nucleotide

NTO	non target organism
NRQ	normalized relative quantity
PAZ	piwi/Argonaute/zwille
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pcRNA	paperclip RNA
piRNA	piwi interacting ribonucleic acid
Piwi	P element induced wimpy testis
Pre-miRNA	precursor micro ribonucleic acid
qPCR	quantitative polymerase chain reaction
RdRP	RNA dependent RNA polymerase
RISC	ribonucleic acid induced silencing complex
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
RNase H	ribonuclease H
RP49	ribosomal protein 49
RT-qPCR	quantitative reverse transcription pcr
SE	standard error
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
T. urticae	*Tetranychus urticae*
TSSM	two spotted spider mite

UTR	untranslated region
VATPase	vacuolar H+ ATPase
WAGO	worm specific Argonaute
μL	microliter
μm	micrometer

1. Introduction

1.1 RNA Interference

RNA interference (RNAi) is a highly conserved, post-transcriptional gene silencing mechanism that involves the use of small RNA molecules to inhibit the expression of target genes. The discovery of RNAi in 1998 by Fire and Mello was a major breakthrough in the field of molecular biology, leading to a better understanding of gene regulation and the development of RNAi-based therapeutics.

The RNAi pathway can be divided into two major steps: (i) initiation and (ii) effector. In the initiation step, trigger RNA is recognized and processed by Dicer into siRNAs, which are then loaded into the ribonucleic acid induced silencing complex (RISC). In the effector step, the RISC-siRNA complex binds to complementary target mRNAs and cleaves them, leading to mRNA degradation or inhibition of protein synthesis (Figure 1.1). The Argonaute protein is responsible for the slicing activity of the RISC-siRNA complex, which is guided by the sequence complementarity between the siRNA and target mRNA**.**

RNAi can be classified into two types based on the extent of its effects: cell-autonomous and cell-non-autonomous RNAi . In cell-autonomous RNAi, the gene silencing effect is limited to the cell in which the siRNAs are generated (Whangbo & Hunter, 2008; Zibaee et al., 2016). The siRNAs are produced and act within the same cell, leading to the suppression of the target gene's expression only in that specific cell. On the other hand, in cell-non-autonomous RNAi, the siRNAs produced in one cell can be transported to other cells within an organism, leading to gene silencing in those recipient cells (Figure 1.2). This phenomenon is facilitated by a protein called systemic RNA interference deficient-1 (SID-1), which is an integral membrane protein encoded by the *sid-1* gene (Berry et al., 2002) SID-1 allows for the uptake and transport of siRNAs across cell membranes, allowing the spread of RNAi effects to distant cells and tissues. This cell-to-cell or systemic transport of siRNAs enables gene silencing to occur in multiple cells, even those that did not produce the siRNAs originally (Voinnet, 2005).

The ability of RNAi to exert cell-non-autonomous effects has been observed in various organisms, including plants and animals. In plants, cell-non-autonomous RNAi has been shown to play a role in systemic defense against pathogens, where siRNAs produced in response to infection in one part of the plant can move to other parts and induce gene silencing to limit the spread of the pathogen (Sohn et al., 2014). In animals, cell-non-autonomous RNAi has been implicated in processes such as neuronal development and regulation of gene expression in response to environmental cues (Cohen & Xiong, 2011).

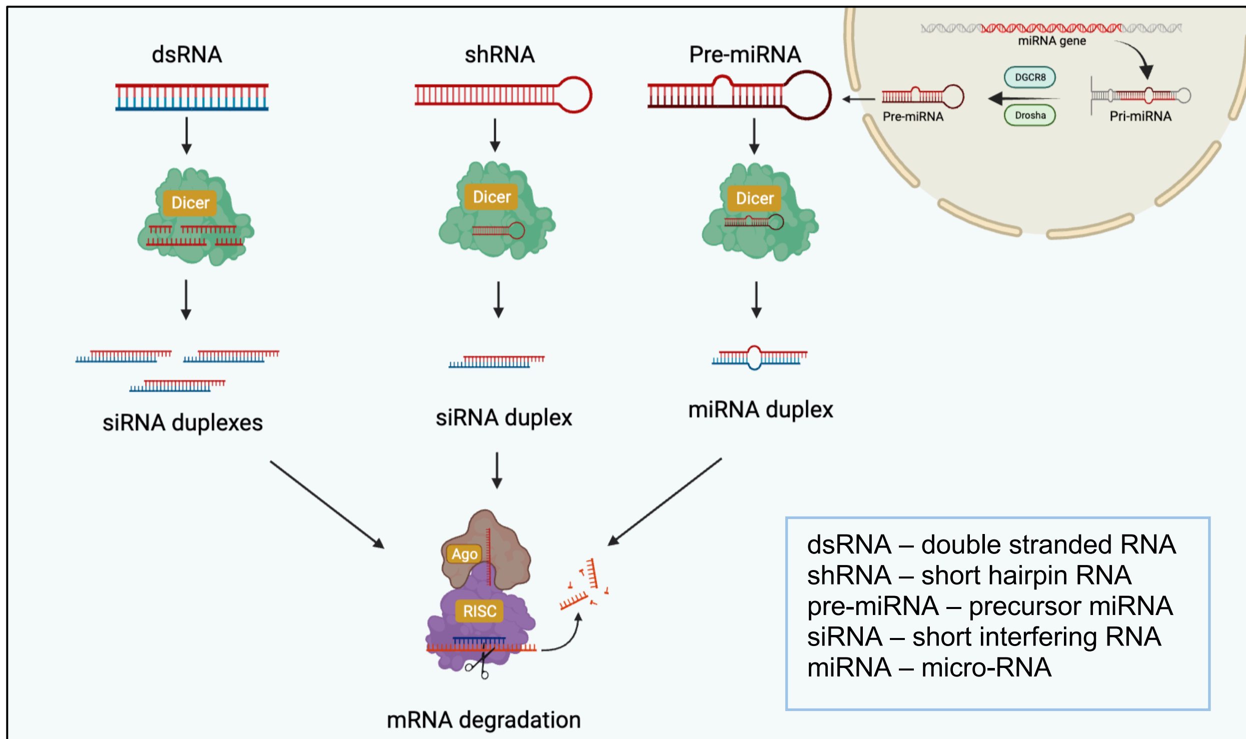

Figure 1.1. General RNAi pathway. Dicer converts dsRNA, shRNA, and pre-miRNAs (introduced artificially or transcribed) into siRNA/ miRNA that is loaded into the RISC. The passenger strand is cleaved by AGO, which is part of RISC. Active RISCs are guided to target mRNAs by the guide strand. As a result of the full complementary binding between the guide strand of siRNA/ miRNA and the target mRNA, the mRNA is cleaved. Image created using BioRender.

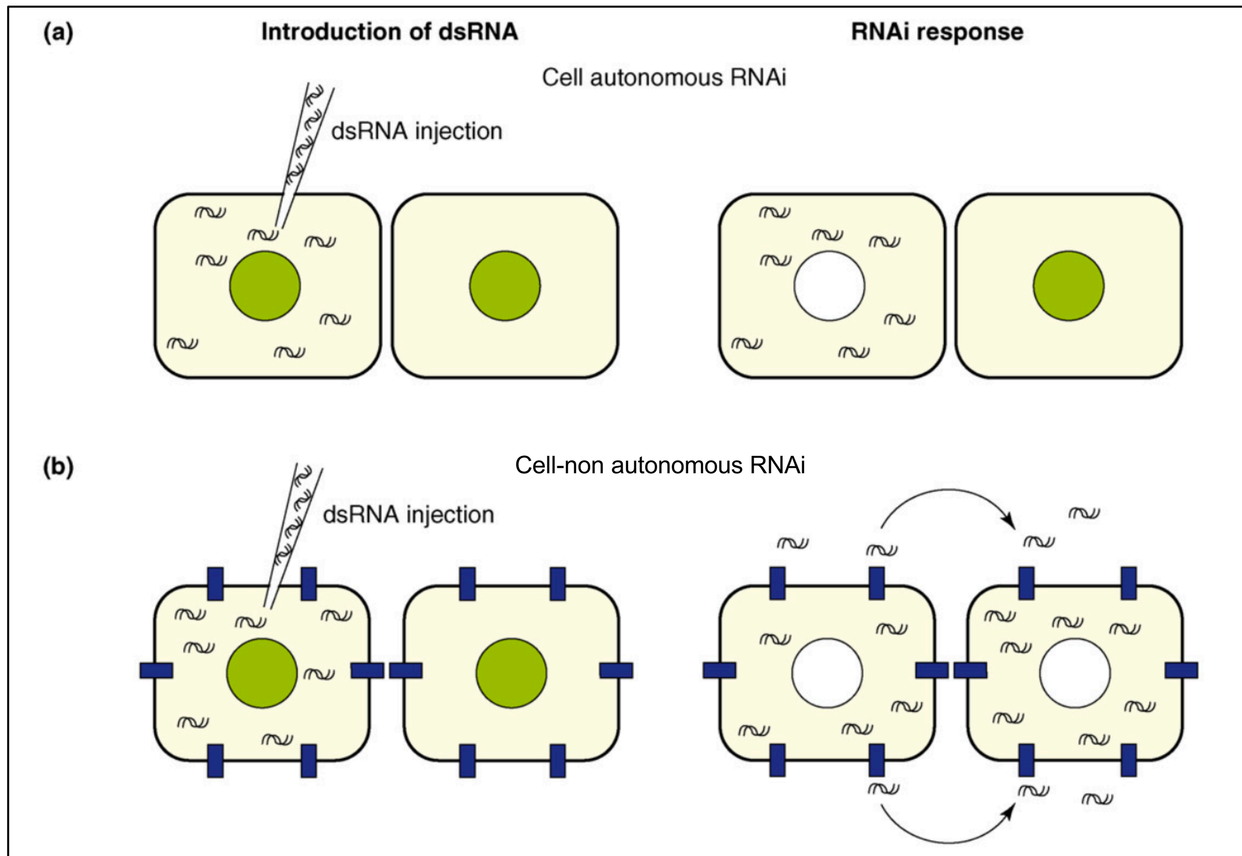

Figure 1.2. Schematic of cell autonomous and cell-non-autonomous RNAi. The left column depicts cells expressing green fluorescent protein (GFP). As illustrated in the left column, the cells are targeted by double-stranded RNA (dsRNA) to target GFP expression. A RNAi response is shown in cells in the right column in response to dsRNA that targets GFP expression. **(a)** Cell autonomous RNAi. There is a knockdown of GFP only in the cell that has dsRNA injected, while the other cell still expresses GFP. **(b)** Cell non autonomous RNAi. GFP is knocked down in the cell that has injected dsRNA, and the signal spreads to neighbouring cells, knocking down GFP in them. Image modified from Whangbo & Hunter, 2008.

1.2 COMPONENTS OF RNAi

1.2.1 Precursor molecules

Small RNA molecules play critical roles in regulating gene expression, and they can be classified into three main categories: siRNA, miRNA, and piRNA. These small RNAs are typically 20-25 nucleotides in length and trigger RNAi through association with the

Argonaute (Ago) family for siRNA/miRNA or the Piwi protein for piRNA. RNAi can be initiated by different precursor molecules, such as double-stranded RNA (dsRNA), short hairpin RNA (shRNA), and precursor microRNA (pre-miRNA) (Carthew & Sontheimer, 2009). The enzyme Dicer processes these precursors into small interfering RNAs (siRNAs) that are typically 21-23 nucleotides in length (Figure 1.1). The siRNAs are then loaded into RNA-induced silencing complexes (RISCs), which consist of the Argonaute protein and other accessory proteins (Carthew & Sontheimer, 2009). Exogenous long dsRNA or shRNAs can be introduced to trigger siRNA biogenesis, which results in the production of numerous siRNAs of differing sequence that target specific genes. Short hairpin RNAs contain only the 20-30 nucleotide sequence from the target gene's stem region, leading to the production of a single siRNA that specifically targets the gene of interest. On the other hand, miRNA biogenesis is endogenous, with non-coding miRNA genes transcribed to produce a pri-miRNA sequence that undergoes two rounds of processing to give rise to a mature miRNA duplex of ~22bp in size. Dicer then excises the miRNA, which acts as a guide for RISC complex to initiate post-transcriptional gene silencing (Shukla et al., 2011). Recently, artificial microRNAs have been developed by altering the mature sequence with the sequence of the gene of interest while maintaining the backbone of the miRNA duplex (Tiwari et al., 2014). This altered mature microRNA is then supplied exogenously and processed by Dicer, following the same pathway as the endogenous miRNA. In summary, small RNA molecules and their associated pathways have crucial roles in regulating gene expression, and their diverse classes offer different mechanisms for achieving gene silencing.

1.2.2 Dicer

Dicer is a class of endoribonuclease enzymes that typically weigh around 200 kDa and are composed of several crucial domains, such as the N-terminal helicase domain, the PAZ domain, the platform domain, two RNase III domains, a domain of unknown function (DUF283), and a dsRNA-binding domain (dsRBD) (Figure 1.3) (Carthew & Sontheimer, 2009; Zhang et al., 2004). The combination of domain components may vary among different species. During the cleavage of dsRNAs with a blunt end or a 5’ overhang, the N-terminal helicase domain plays a crucial role (Welker et al., 2011). The PAZ domain

binds to the 3' end of precursor molecules, while the platform domain interacts with the substrate to ensure proper positioning for cleavage (Song & Rossi, 2017). The RNase III domains, which are highly conserved catalytic domains, lead to the cleavage of precursor molecules into siRNA molecules.

Dicer forms a homodimer with each RNase III monomer, and this dimerization plays a critical role in cleaving both strands of the precursor molecule. The activity of the Dicer enzyme is regulated by a complex network of protein-protein interactions and post-translational modifications (Rajyaguru et al., 2021).

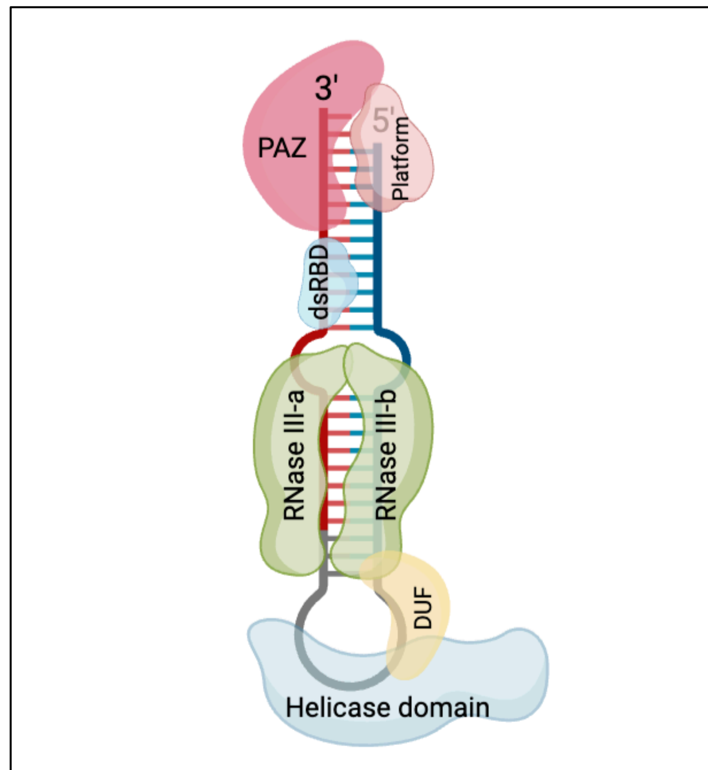

Figure 1.3. A schematic depicting the different dicer domains that process pre-miRNA. Image created using BioRender.

1.2.3 Effector complex

Upon processing of the precursor molecule by Dicer, the RNAi induced silencing complex (RISC) is formed, which is a multiprotein complex consisting of small RNA molecules (siRNA/miRNA), the Argonaute family of proteins and GW182 (Hameed et al., 2018).

These Argonaute proteins are categorized into three major groups: Ago, Piwi, and WAGO (Wang et al., 2013). While the Ago group specifically interacts with siRNA and miRNA, the Piwi group mainly works with piRNA, and the unique WAGO group is observed only in nematodes (Meister, 2013). The presence of numerous Argonaute genes is evident in all eukaryotic species. The fundamental domains present in Argonaute protein are the PAZ domain, MID domain, and PIWI domain. The Argonaute protein comprises a bilobed structure, where the N-PAZ domain binds to the 3' end of the small RNA molecule in one lobe and the MID-PIWI domains in the other lobe (Figure 1.4). The two lobes are connected by linker molecules L1 and L2, which undergo various structural rearrangements when binding small RNA molecules. The PIWI domain bears structural similarity to ribonuclease H (RNase H) and plays a crucial role in providing the splicing activity that cleaves the target mRNA sequence. However, not all Argonaute proteins possess this catalytic slicing activity. Recently, certain Argonaute proteins were found to exhibit splicing activity for selective small RNA bearing different flanking regions at the 5' and 3' ends, indicating that multiple Argonaute proteins may be present for different small RNA that they bind to (Wu et al., 2020).

The loading of small RNA molecules onto the Argonaute protein is an ATP-dependent process. Following unwinding of the double-stranded RNA molecule, one strand is selected as the guide strand based on thermodynamic principles, while the other passenger strand is discarded. The protein GW182 functions as a scaffolding protein and helps to organize protein complexes around the Argonaute protein (Liu et al., 2018). Although the combination of GW182 and Argonaute is not always essential for RNA interference, it is often involved in this process. Once the guide strand is bound to Argonaute, it guides the complex to the target mRNA and binds to it via sequence complementarity. This results in mRNA cleavage through the splicer activity of Argonaute, ultimately leading effective downregulation of target mRNA (Meister, 2013).

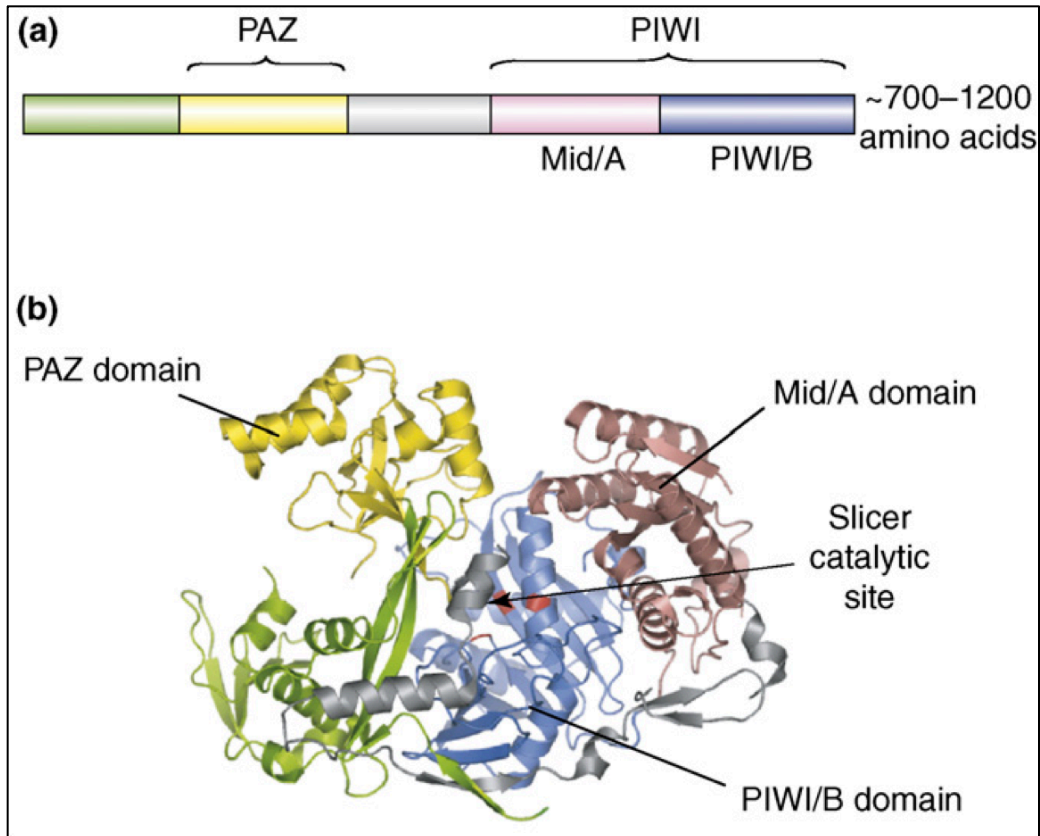

Figure 1.4. (a) Schematic showing different domains of Argonaute.
(b) A three-dimensional view of the Argonaute protein.

1.3 A pest control strategy based on RNAi

The development of resistance to pesticides in over 500 arthropod species over the past two decades has highlighted the urgent need for alternative pest management methods (Baum et al., 2014). One promising approach is the use of exogenously supplied double-stranded RNA (dsRNA) to regulate gene expression in insects, a phenomenon known as environmental RNAi (Figure 1.5). Environmental RNAi involves exposing insects to dsRNA through methods such as feeding or soaking, with the aim of disrupting genes crucial for their survival (Whangbo & Hunter, 2008). This approach has been attempted in various insect orders, including diptera, coleoptera, and dictyoptera, but has faced challenges due to variable sensitivity to RNAi among different insect species (Baum et al., 2014).

Several factors have been identified that affect the effectiveness of RNAi in insects. For example, the presence of dsRNA-degrading enzymes in the salivary secretions and haemolymph of aphids can reduce the stability of exogenously supplied dsRNA. In addition, inefficient uptake of dsRNA by midgut epithelial cells has been found to be a limiting factor in some insects (Baum et al., 2014). The exact mechanism of dsRNA uptake in insects is not well understood, but studies in the nematode *Caenorhabditis elegans* have proposed a model involving endocytosis mediated by the *sid-2* gene in intestinal cells, followed by spread of dsRNA/siRNA signals to distal tissues through the *sid-1* gene in epithelial cells. However, *sid-2* like genes have not been identified in any sequenced insect species so far (Saleh et al., 2006).

Despite these challenges, RNAi has shown promising efficacy in controlling certain insect species. For example, Western corn rootworm, fall armyworm, Colorado potato beetle, have been found to be highly susceptible to RNAi-based treatments. Recent studies have reported the effectiveness of Calantha, a dsRNA-based biopesticide, against Colorado potato beetle, resulting in significant reduction in fecundity even under field conditions (Pallis et al., 2023). However, the response to RNAi varies across different insect taxa, with Orthoptera and Coleoptera species generally being more responsive, while Lepidoptera and Diptera species exhibit lower sensitivity. Furthermore, the effectiveness of RNAi may also be influenced by factors such as dsRNA stability, mechanism of cellular uptake, and systemic RNAi response, which can vary among insect species (Cooper et al., 2019). In some cases, injection of dsRNA may be required for efficient RNAi induction, as demonstrated in the migratory locust (*Locusta migratoria*), rather than oral feeding.

In addition, recent findings highlight the potential of plastid-mediated RNAi (PM-RNAi) as an effective method for controlling spider mites, which are notorious for their resistance to traditional chemical pesticides (Wu et al., 2023). By introducing dsRNA into the plastids of plants, researchers were able to disrupt the essential genes of the spider mites, leading to their mortality and reduced population. This approach offers a promising alternative for pest control, expanding the application of RNAi-based technology beyond insect pests to include no insect pests like spider mites.

Further research is needed to optimize RNAi-based pest management strategies and identify more effective delivery methods for RNAi-based biopesticides. Understanding the mechanisms of dsRNA uptake and systemic spread in insects, as well as factors that influence RNAi efficacy, will be crucial for developing sustainable and effective RNAi-based pest control approaches in the future.

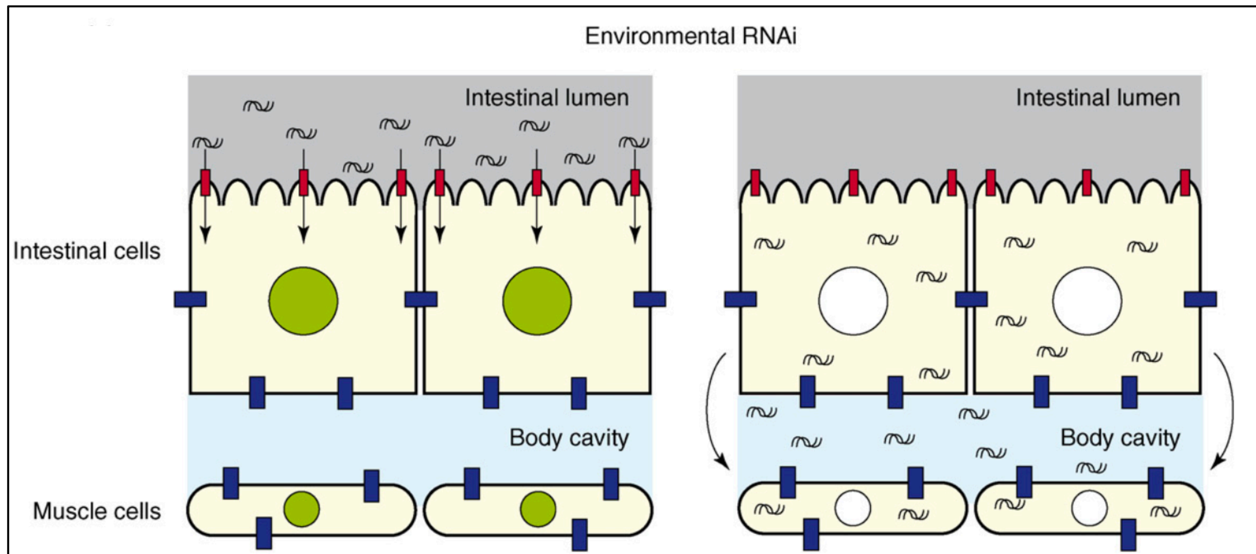

Figure 1.5. Environmental RNAi. The left column depicts cells expressing green fluorescent protein (GFP). A RNAi response is shown in cells in the right column in response to dsRNA that targets GFP expression. Intestinal cells are silenced by dsRNA molecules present in the lumen when they are imported by dsRNA importer proteins (red rectangles). GFP silencing in distant cells is caused by systemic RNAi spreading the dsRNA and dsRNA-derived silencing signals from the intestinal cells. Image modified from Whangbo & Hunter, 2008.

1.4 Two-Spotted Spider Mite: A Destructive Pest

Chelicerata is a significant group of land-dwelling animals that includes chelicerates, the fundamental lineage of arthropods. Pycnogonida, Xiphosura, and Arachnida are the most notable among the living chelicerates (Dunlop, 2010; Dunlop & Selden, 2009). Arachnida is an extensive collection of species that includes Acari, which encompasses more than 40,000 species with varying dietary habits, ranging from parasites to plant feeders and predators. *Tetranychus urticae* also known as the two-spotted spider mite, belongs to the

Tetranychidae family. The mite derives its name from the two conspicuous black spots on its body, which result from waste accumulation (Helle & Sabelis, 1985; Bensoussan et al., 2022). The two-spotted spider mite is a polyphagous pest that feeds on many plant species and is particularly destructive in greenhouses and fields, causing substantial damage to valuable crops such as cucumbers, strawberries, and tomatoes (Jeppson et al., 1975; Migeon et al., 2006). Therefore, effective management strategies are essential to mitigate the detrimental effects of this pest on agricultural productivity.

1.5 Life cycle of *Tetranychus urticae*

Tetranychus urticae, the two-spotted spider mite, has a short life cycle that typically lasts between 10-15 days, depending on various factors such as temperature, humidity, and leaf conditions (Cazaux et al., 2014). This pest species requires a minimum temperature of 12°C to develop, with an upper threshold of approximately 40°C. The life cycle begins with eggs, which take two to three days to hatch into larvae. The hatching of eggs is also influenced by environmental factors such as humidity and temperature (Ubara & Osakabe, 2015). Larvae can be differentiated from other developmental stages by the presence of only three pairs of legs, in contrast to four in all other nymphal and adult stages.

After hatching, the larva grows into a protonymph, with quiescent stages in between the nymphal stages. One such quiescent stage occurs before the protonymph develops into a deutonymph, and another occurs during the molting stage of the deutonymph. Finally, the adult mite emerges after molting. Female adult mites are larger in size compared to male mites (Figure 1.6), and the primary distinguishing feature is that the adult male mite has a tapered posterior, while female mites have a rounded posterior. The adult female mite can lay up to 100 eggs during her lifetime (Helle & Sabelis, 1985).

Interestingly, the two-spotted spider mite exhibits haplo-diploid sex determination, meaning that eggs from unmated females develop into male mites that inherit only a haploid set of chromosomes from their mother, while mated females can produce both male and female eggs (Carrière, 2003). These unique features, such as rapid

development time, high fecundity rates, and haplo-diploid sex determination, are essential factors that contribute to the high pesticide resistance observed in this species.

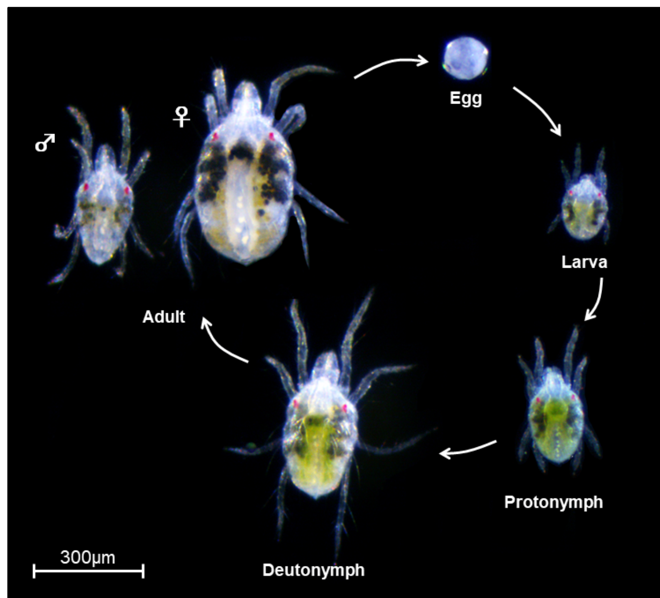

Figure 1.6. An overview of the life cycle of *T. urticae.* Image credit: Zoran Culo.

1.6 Genome of *Tetranychus urticae*

In 2011, the sequencing of the genome of *Tetranychus urticae*, the smallest known arthropod and chelicerate, was conducted, revealing a compact genome size of only 90 Mbp. Notably, 54% of this genome consists of protein-coding regions, which is in line with the polyphagous feeding behavior of *T. urticae*. Analysis of the genome also revealed an expansion of detoxification gene families, such as cytochrome P450s, Glutathione-s-transferases, and UDP-glucuronosyltransferases, explaining the ability of *T. urticae* to adapt to its diverse diet (Grbic et al., 2011).

Furthermore, the genome of *T. urticae* was found to contain 52 miRNAs, which were classified into 43 families (Grbic et al., 2011). Comparison of these miRNAs with those of 27 other species using the miRBase repository identified 26 conserved miRNA sequences, while 26 other miRNAs were found to be specific to *T. urticae* (Table 1.1). The RNA interference (RNAi) machinery in *T. urticae* genome has all essential

components for working RNAi machinery, with two dicer enzymes containing the PAZ and RNAse III domains, which play important roles in the cleavage of precursor molecules to produce siRNAs, seven Argonaute homologs, which mainly associate with siRNA and miRNA in eukaryotes. Drosophila Pasha and Drosha enzyme homologs were also identified (Grbic et al., 2011).

A notable finding in the *T. urticae* genome was the presence of five RNA-dependent RNA polymerase (RdRP) proteins, which are absent in other arthropods (Table 1.2). The RNAi machinery in *T. urticae* is relatively conserved, except for the expansion of the Argonaute gene family and the presence of five RdRP proteins, which warrant further study. However, the impact of this expansion of the Argonaute gene family on the working principle of RNAi in mites remains unclear and requires further investigation.

T. urticae miRNA	Number of matches	*Ixodes scapularis*	*Daphnia pulex*	*Aedes aegypti*	*Anopheles gambiae*	*Apis mellifera*	*Acyrthosiphon pisum*	*Bombyx mori*	*Culex quinquefasciatus*	*Drosophila ananassae*	*D. erecta*	*D. grimshawi*	*D. melanogaster*	*D. mojavensis*	*D. persimilis*	*D. pseudoobscura*	*D. sechellia*	*D. simulans*	*D. virilis*	*D. willistoni*	*D. yakuba*	*Locusta migratoria*	*Nasonia giraulti*	*N. longicornis*	*N. vitripennis*	*Tribolium castaneum*	*Strigamia maritima*
tur-mir-276	25	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
tur-mir-10	24	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	
tur-mir-2-1	23	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	●	
tur-mir-133	23	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	●	
tur-mir-184	23	●		●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	●	
tur-mir-2-2	23	●	●	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	●	
tur-mir-7	23	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●			●	●	●	
tur-mir-1	23	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●		●	●	
tur-mir-281	22			●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	
tur-mir-124-2	22	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●				●	●	
tur-mir-124-1	22	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●				●	●	
tur-mir-34	21		●	●	●	●		●		●	●	●	●	●	●	●	●	●	●	●	●		●	●	●	●	
tur-mir-305	21	●	●	●	●	●		●	●	●	●	●	●	●	●	●	●		●	●	●		●	●	●		
tur-mir-263a	21	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●					●	
tur-mir-12a	20	●	●	●	●	●			●	●	●	●	●	●	●	●	●	●	●	●				●	●	●	
tur-mir-87	18	●		●			●	●	●	●	●	●	●	●	●	●	●	●	●	●	●					●	
tur-mir-137	15	●	●	●	●	●	●	●	●				●			●		●					●	●	●	●	
tur-mir-190	11			●	●	●	●	●	●				●			●		●							●	●	
tur-mir-317	7	●	●		●	●																	●		●	●	
tur-mir-252	7						●	●	●				●			●		●							●		
tur-mir-263b	6		●			●	●																●	●		●	
tur-mir-279	4	●	●				●																			●	
tur-mir-745	1		●																								
tur-mir-3931	1																										●
tur-mir-92	1		●																								
tur-mir-278	1	●																									

● – denotes match between *T. urticae* miRNA and miRNA from other species.

Table 1.1 Conserved miRNA Genes in *Tetranychus urticae.* Image from Grbic et al., 2011.

Gene name	Gene function/pathway	*T. urticae*	*D. pulex*	*A. pisum*	*D. melanogaster*
ARGONAUTE	RNAi	7	2	3	3
Armi	RNAi	1	1	1	1
Aub/piwi/Ago3	RNAi	7	7	>4	2
Dicer	RNAi	2	3	2	3
R2D2	RNAi	0	0	0	1
vig	RNAi	1	1	0	1
RdRP	RNAi	5	0	0	0
Gw182	RNAi	2	1	1	1
Exportin 5	RNAi	2	1	1	1

Table 1.2. Genetic diversity across arthropods for core RNAi pathway genes. Image adapted from Grbic et al., 2011.

1.7 *Tetranychus urticae* – excellent model organism

Spider mites have a short life cycle and high fecundity, which make them easy to maintain in large numbers in laboratory settings (Cazaux et al., 2014). The completed genome sequence of *T. urticae* provides comprehensive genetic and molecular resources, making this species an outstanding experimental model. In situ and antibody staining experiments have been conducted to study the expression pattern of genes, and gene knockdown experiments have been successful in *T. urticae*, which is of significant importance (Bensoussan et al., 2022). By utilizing these experimental models, the molecular mechanisms underlying various biological processes such as development and pesticide resistance in spider mites can be studied. Moreover, understanding the gene expression pattern in spider mites can help in developing novel control strategies to mitigate the negative impact of spider mites on agricultural productivity.

1.8 RNAi experiments in *Tetranychus urticae*

In 2007, the first RNA interference (RNAi) experiment in spider mites showed that gene knockdown using dsRNA and siRNA could be achieved through microinjection (Khila & Grbić, 2007). However, using injection methods for screening lethal targets in biocontrol strategies via oral ingestion poses challenges. To overcome this, a leaf disc delivery method of dsRNA was developed, which effectively knocked down four candidate genes and increased mortality in spider mites (Kwon et al., 2013). Although this method was effective, it required large quantities of dissolved dsRNA, making it impractical for high-throughput RNAi screens.

To improve the efficiency of RNAi in spider mites and mitigate the requirement of large quantities of dissolved dsRNA, a comparative study of five different dsRNA delivery methods was performed (Suzuki et al., 2017). Leaf disc coating and soaking were found to be the most efficient methods for RNAi delivery and a Kim wipe-based approach was developed, which showed efficient uptake of dsRNA with minimal amounts of dsRNA required (Suzuki et al., 2017).

The efficiency of RNAi in spider mites is influenced by various factors, including the concentration and length of double-stranded RNA (dsRNA), the stability of external dsRNA in the mite body, and the cellular uptake of dsRNA. Through careful experimentation, it has been determined that a concentration of 160 ng/μL and a length of 400-600bp are optimal for inducing an effective RNAi response in spider mites (Bensoussan et al., 2020).

To better understand the spatial distribution of exogenously provided dsRNA in the mite body, fluorescently labeled dsRNA was used, revealing that the dsRNA ingested by the mites was primarily localized to the digestive cells and caeca lumen (Bensoussan et al., 2022). This finding suggests that the RNAi effect in mites is localized to the cells that are in direct contact with dsRNA, and that the effect is not transmitted to distal cells to elicit a systemic response.

As a result of efficient dsRNA delivery methods, high-throughput RNAi screens have been achieved in spider mites, making RNAi a feasible method for knocking down genes in

these pests. However, further research is needed to investigate the underlying mechanisms of RNAi in spider mites.

1.9 Cell culture – a tool to study mechanisms of RNAi

Cell culture techniques were first introduced in the early 20th century as a means of isolating cells from organisms and maintaining them in an artificial environment with all the necessary nutrients to sustain their growth (María, 2013). The resulting primary cells were able to divide and reach confluence before being continuously passaged to establish a cell line (figure 1.7). The use of cell cultures has proven valuable in mitigating systemic variations and environmental stresses associated with *in vivo* studies, as the culture conditions can be tightly controlled. While primary cell cultures better reflect *in vivo* conditions, the cells in a cell line have undergone physiological changes that confer an infinite lifespan. Over 600 cell lines have been established from approximately 100 insect species for various applications, including the study of cellular processes, screening for novel insecticidal activities, RNAi screening, and large-scale production of proteins and bio-insecticidal viruses (Smagghe et al., 2009). Primary cell cultures have been employed to perform RNAi screens by targeting genes such as myosin heavy chain, an important component of myofibril assembly, in *Drosophila melanogaster* gastrula embryos (Bai et al., 2009), and to enable gene function analysis using RNAi in marine bivalve cells (You et al., 2012). Furthermore, cultured cells have been utilized as a tool to study the mechanisms of RNAi response by targeting endocytic genes in *Aedes aegypti* (Abbasi et al., 2020). However, the finite lifespan of primary cell cultures poses a significant disadvantage, requiring laborious establishment of new cultures for each experiment.

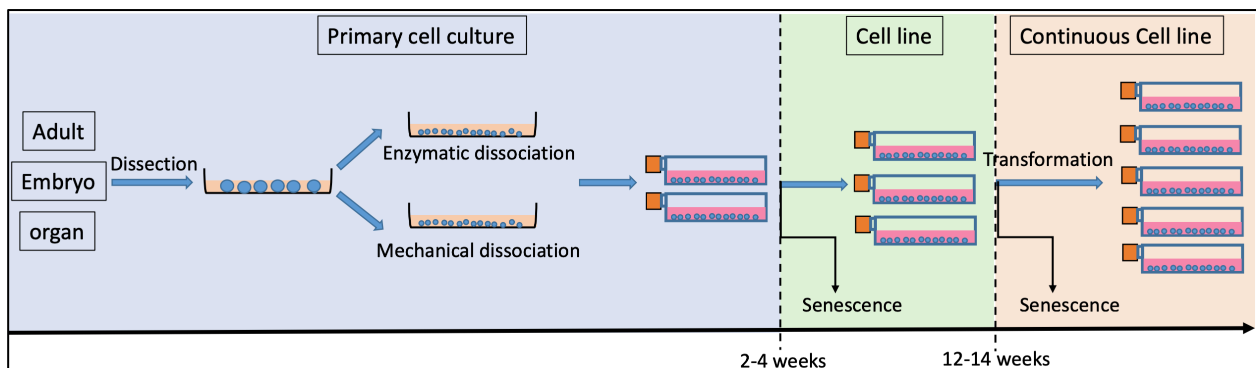

Figure 1.7. An overview of the process of establishing a primary cell culture and a cell line.

1.10 Rationale and objectives

Actin was selected as a target for RNA interference (RNAi) experiments due to its crucial role in various cellular processes, such as organelle movement and cell division. Actin proteins are broadly classified into three types, including α actin, β actin, and γ actin (Dominguez & Holmes, 2011). While α actin is primarily present in muscle cells and contributes to muscle contraction, β and γ actin are vital for maintaining cell structure and regulating fundamental cellular processes. Based on the selection criteria of producing phenotypic changes at both the cellular and whole mite levels, actin was chosen as the gene of interest for RNAi experiments. The hypothesis was that targeting actin using RNAi would result in significant phenotypic changes in mites and cells, given the abundance and importance of this protein in eukaryotic cells (as depicted in Figure 1.8).

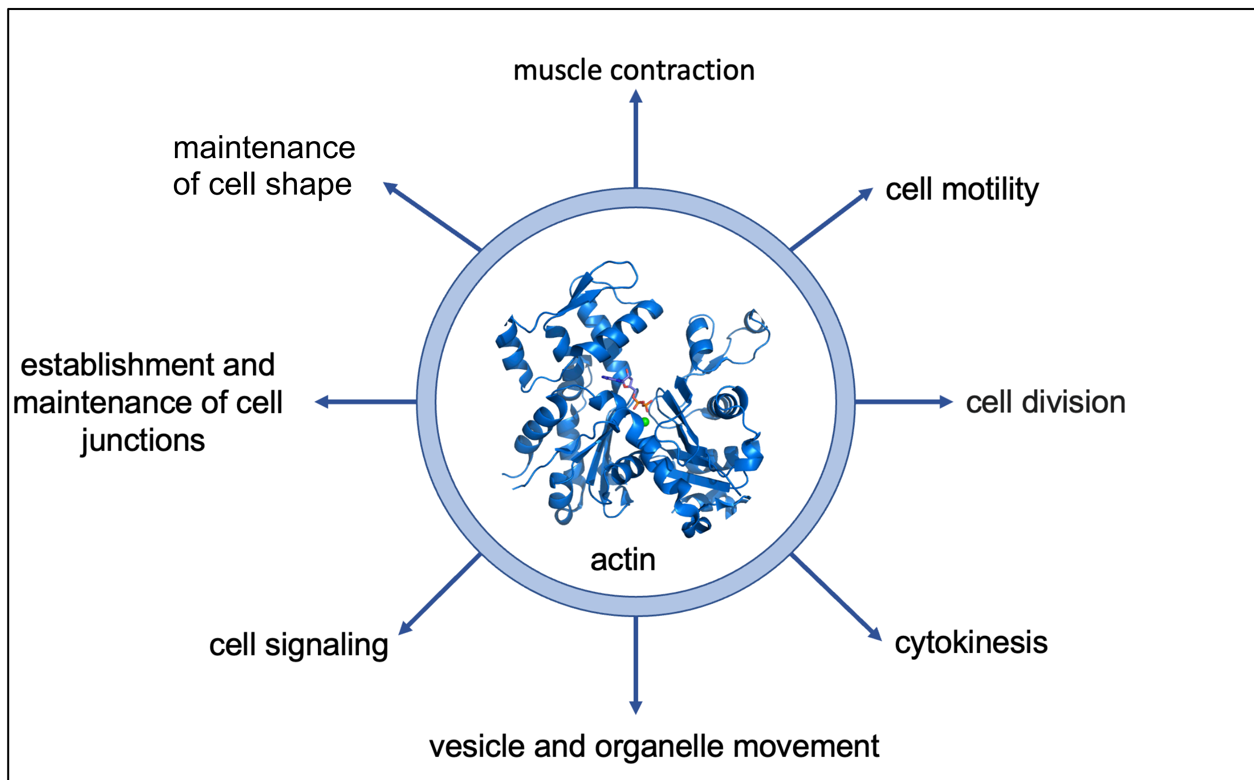

Figure 1.8. Roles of actin. Image created using BioRender.

Objectives

1. To examine the efficiency of various RNAi-inducing RNAs, such as dsRNA, shRNA, and amiRNA, on spider mite phenotype, gene expression and fecundity.
2. To determine the feasibility of establishing spider mite cell cultures and assess the efficacy of RNAi in primary cell cultures.

2. Materials and methods

2.1 Plant growth and mite rearing conditions

The California Red Kidney cultivar of Phaseolus vulgaris served as the host plant for *Tetranychus urticae.* The plants were grown in a Pro-MixR© BX Mycorrhizae TM mixture of peat and vermiculite under controlled conditions of 26°C temperature, 50% relative humidity, and a 16-hour light/8-hour dark photoperiod. Philips Fluorescent Plant Lightbulbs were used to provide a photosynthetic photon flux density of 150μmol $m^{-2}s^{-1}$. The two-spotted spider mite population was reared on bean plants under similarly controlled conditions of 26°C temperature and a 16-hour light/8-hour dark photoperiod.

2.2 Preparation of developmentally synchronized mites

To minimize the confounding effects of developmental variability in experiments involving spider mites, it is crucial to synchronize the mite population to obtain a uniform adult population of the same generation. Spider mite egg hatching and molting are regulated by environmental factors such as humidity and submergence in water (Ubara & Osakabe, 2015), which can be manipulated to generate a developmentally synchronized adult mite population. The protocol established by Suzuki et al. in 2017 was employed to prepare a synchronized mite population as depicted in figure 2.1.

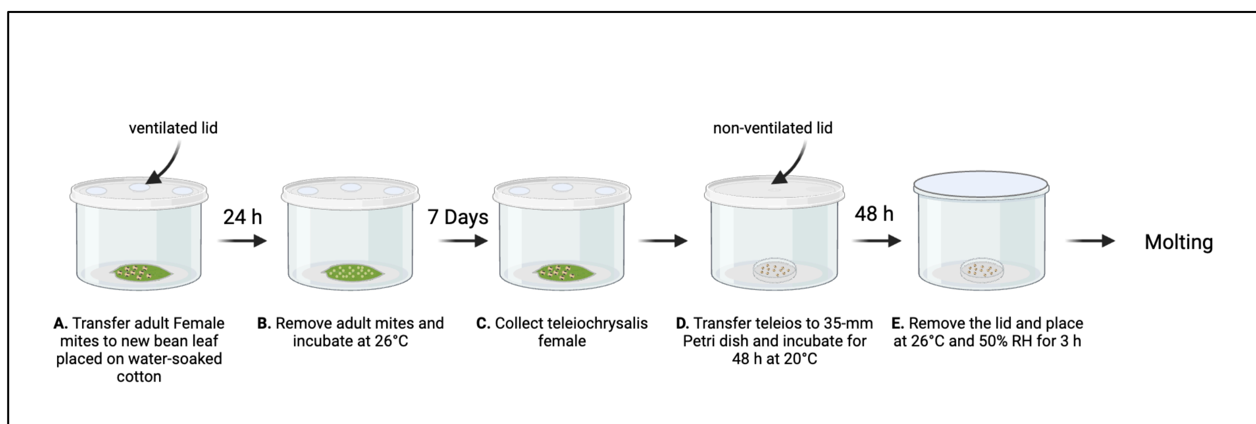

Figure 2.1. Synchronization of adult mites. The schematic was modified from Suzuki et al., 2017. Image created using BioRender.

2.3 Comparitive phylogenetic analysis

A phylogenetic analysis was conducted between the target gene sequence and the Drosophila sequence using MEGA X software (Stecher et al., 2020). The ClustalW algorithm was used to align the sequences, and a maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model with 1000 bootstrap replicates.

2.4 Design and synthesis small molecules

2.4.1 dsRNA

The target gene sequences were obtained from *Tetranychus urticae* genome database available at BOGAS (http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Tetur). Subsequently, a 400-600 bp double-stranded RNA (dsRNA) fragment was designed to target the gene of interest as it was found to be the most optimal length for eliciting RNA interference (RNAi) responses in spider mite (Bensoussan et al., 2020). To ensure the specificity of the dsRNA sequences, a nucleotide BLAST was performed against the *T. urticae* genome. This step was crucial to ensure that the dsRNA only targeted the desired gene and not any other non-targeted genes in the genome. As a negative control, a 382 bp dsRNA fragment was designed to target a non-transcribing scaffold 12 region of the *T. urticae* genome, which was referred to as dsRNA-F3R3 (Suzuki et al., 2017). The use of this control was essential to distinguish the specific effects of the target gene knockdown from any non-specific effects caused by the dsRNA treatment.

Total RNA was extracted from spider mites using the RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA synthesis was performed using the SuperScript II cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). To prepare templates for dsRNA synthesis, specific primers were used to amplify the desired DNA sequences with a T7 promoter sequence located at the 5' end of primers. Following PCR, TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA) was used for *in vitro* transcription. DNAse was used to remove residual template DNA following *in vitro* transcription. Double-stranded RNA (dsRNA) was formed by denaturing RNA fragments at 95°C and slowly cooling them. Following phenol chloroform extraction, dsRNA was

purified using ethanol precipitation. To ensure the quality and quantity of the dsRNA, both Nano-Drop (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis analyses were conducted.

2.4.2 shRNA

To identify targets for siRNA treatment, siDirect 2.0 (https://sidirect2.rnai.jp/) was utilized (Naito et al., 2009), and off-targets were checked using blastn against a cDNA/transcript database. The seed region, which contains seven nucleotides at positions 2-8 from the 5′ end of the guide strand, was of particular importance. Oligonucleotides were designed to include a T7 promoter fragment, followed by the target (sense) sequence, TTCG (recommended for tetraloop), the reverse complement target sequence, and GC clamp. The folding efficiency of the RNA produced was checked using MC-Fold 2.32 (Parisien & Major, 2008). Synthetic structures were created by annealing oligonucleotides (Table 2.1), resulting in a T7 promoter at the 5′ end plus the structured RNA sequence. *In vitro* transcription was performed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA), and the product was purified through phenol chloroform extraction and ethanol precipitation.

2.4.3 amiRNA

The construction of an artificial microRNA (amiRNA) involved replacing the 24-nucleotide mature sequence of tur-mir-7 with homologous sequences for the target gene, while preserving the backbone of tur-mir-7. The synthetic structures were created by annealing oligonucleotides, resulting in an amiRNA construct that featured a T7 promoter at the 5' end (Table 2.1). *In vitro* transcription was performed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA), and the resulting product was purified via phenol-chloroform extraction and ethanol precipitation.

Primers	Oligonucleotide sequences
5_α_dsRNA	T7 promoter + cgagccgttttcccctcaattg
3_ α _dsRNA	T7 promoter + acaccatcaccagagtcgagg
5_ βγ_dsRNA	T7 promoter + ctggcatcacaccttctaca
3_ βγ_dsRNA	T7 promoter + catgattgaattgtaagtggttt
α_f1_shRNA	T7 promoter + agaaattgtccgagatatcaagttgccttgatatctcggacaatttctcccc
α_f2_shRNA	T7 promoter + atcactctttctacaacgaattgttgccaattcgttgtagaaagagtgatccc
βγ_f1_shRNA	T7 promoter + tggtagagatcttactgattattttcgaataatcagtaagatctctaccaccc
βγ_f2_shRNA	T7 promoter + caccggtattgttttggattctgttcgcagaatccaaaacaataccggtgccc
SL2	T7 promoter + gggggggggtcgaggacaataccactggtattgtcctcgaccccccccctatagtgagtcgtattaaatt
α_f1_amiRNA	T7 promoter + atctccttggagaaattgtccgagatatcaagtgtctgttagtaattaacttgatatctcggacaatttctctggagaa
α_f2_amiRNA	T7 promoter + atctccttgatcactctttctacaacgaattgtgtctgttagtaattaacaattcgttgtagaaagagtgattggagaa
βγ_f1_amiRNA	T7 promoter + atctccttgtggtagagatcttactgattatttgtctgttagtaattaaaataatcagtaagatctctaccatggagaa
βγ_f2_amiRNA	T7 promoter + atctccttgcaccggtattgttttggattctgtgtctgttagtaattaacagaatccaaaacaataccggtgtggagaa

T7 promoter – aatttaatacgactcactataggg

Table 2.1 The primers used in the experiment.

2.5 Delivery of small molecules in whole mite

In the study, 80 newly emerged adult female mites were treated with a solution containing 160ng/μL and 0.1%v/vTween20 for dsRNA treatment, and with a solution containing 500ng/μL and 0.1%v/vTween20 for shRNA and amiRNA treatment using the soaking protocol by Suzuki et al. (2017). The mites were then transferred to fresh bean leaves for recovery (Figure 2.2).

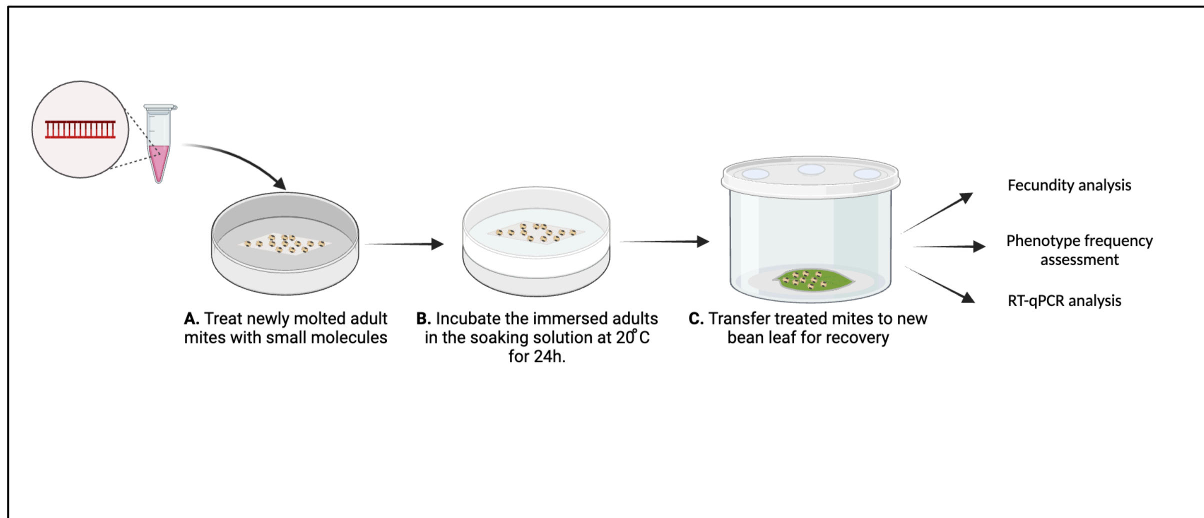

Figure 2.2. Delivery of small molecules in whole mite. Image created using BioRender.

2.6 Determination of RNAi effects

2.6.1 Mite fecundity assay and Phenotype frequency assessment

After 24 hours of treatment and subsequent 24 hours of recovery (Figure 2.2), the mites were transferred to bean leaf discs, with 5 mites per leaf, and their fecundity was observed after 72 hours. The experiment was performed in five trials, with each trial consisting of 5 biological replicates. The effect of treatment on fecundity was analyzed using one-way ANOVA followed by planned comparisons with a Dunnett's test using the "glht" function in the R package multcomp. Additionally, mite phenotype changes were observed, and the frequency of these changes was assessed using Fisher's test.

2.6.2 RT-qPCR analysis

Approximately 60 treated mites had their RNA isolated using the RNeasy Mini Kit (Qiagen), followed by reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). The reference genes used for normalization of target quantity were *TuRP49(tetur18g03590)* and *TuCycA (tetur01g12670)*, and the RT-qPCR primers are listed in Table 2.2, designed not to overlap with small RNA fragments used in treatment. Three technical replicates were averaged to obtain the cycle threshold (Ct) values of biological replicates. Normalized relative quantity (NRQ) for each target gene was calculated as described in Rieu &

Powers, 2009. For plotting purposes, NRQ values were calibrated to the dsRNA-F3R3 (control group) set at one. For statistical analysis NRQ values were log2 transformed and used in ANOVA to detect an effect of dsRNA treatment. Dunnett's test was applied using the "glht" function in the R package multcomp as a method of pairwise comparison following ANOVA. Statistical significance was set at $p<0.05$ for all analyses.

Primer names	Forward primer sequence	Reverse primer sequence	Primer efficiency
actin-α	TTGGAGGTTCCATCTTGGCTT	CCCGCAATTGTCCACAGAAAG	100.2%
actin-βγ	CCTCACCACTAGAGAAGGTC	TCAAACAAAACCGCGAAATCA	104%
actin-βγ’	CCTACCAGCCTGTTGAGCTTTTA	CACGCCTTTAGCCCGATAGA	104.1%
RP49	CTTCAAGCGGCATCAGAGC	CGCATCTGACCCTTGAACTTC	97.6%
CycA	GCTTCAAGGCGGTGACTTT	ACCTGGTCCAGTGTGTTTGAG	102.2%

Table 2.2. Primers used in RT-qPCR with amplification efficiency.

2.7 Establishment of primary cell culture

The primary cell culture of spider mites was established using a protocol previously established by that involved collecting embryos from bean plants that had been infested with spider mites (Shukla, in preparation). The embryos were first collected by washing the bean leaves in a 0.001% tween solution, followed by passing them through sieves of decreasing mesh sizes ranging from 500 μm - 160 μm. After collection, the embryos were sterilized using a solution of bleach and 1x PBS, which was repeated twice to ensure complete sterilization. Following this, the embryos were washed with L15 media, 1x antimycotic, and 50 μg/ml gentamycin. A suspension of the embryos was then created by adding 2 mL of serum-free media and mixing it gently with a pipette. The suspension was homogenized using a Tenbroek homogenizer by pushing the plunger all the way to the bottom and turning it while pushing down. The suspension was allowed to settle for 5 minutes before transferring it to a falcon tube. The suspension was then centrifuged at 40G for 2 minutes to remove the vitellin membrane, and the supernatant was transferred to a new tube and centrifuged at 40G for 4 minutes. The resulting pellet containing cells was suspended in 200 μL of complete media and mixed with a pipette. Cell counting was performed by taking 20 μL of the suspension and adding it to an 2 mL Eppendorf tube,

followed by mixing with 20 µL of 4.4% trypan blue. The sample was then placed on a counting chamber, and the number of cells present in the center grid was counted to calculate the number of cells per mL. After cell counting, 3µM MNNG was added to the cell suspension, and a 24-well plate was used to seed the cell suspension. Five hundred µL of cells was added to the bottom of each well, and the sample was checked under a microscope to ensure the absence of debris. The plate was then placed in an incubator at 27°C and left undisturbed for 2-3 days. After 2-3 days, the cells were viewed under a microscope to assess cell growth and morphology (Figure 2.3).

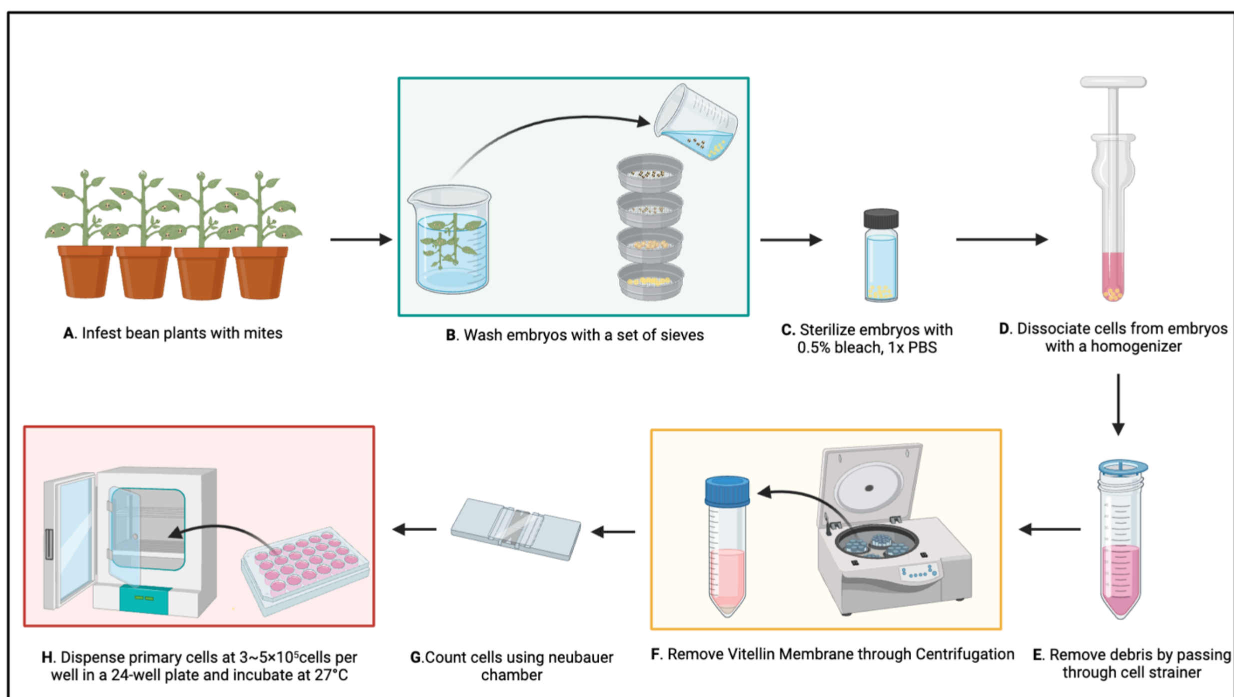

Figure 2.3. Establishment of spider mite primary cell culture. Image created using BioRender.

2.8 Delivery of dsRNA in primary cell culture

In mite primary cell culture, delivery of dsRNA was achieved through a simple cell bathing method. The cells were treated with 250µL of serum-free media containing 50 ng/µL dsRNA after five days of culture. Prior serum-free starvation of cells was necessary for optimal cellular uptake of dsRNA. This concentration has been previously validated for effective cellular uptake of dsRNA.

2.9 Determination of RNAi effects in primary cell culture

After 4 days of RNAi treatment, primary cells were harvested by adding lysis buffer directly into the 24-well plate. RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen), followed by reverse transcription using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). Statistical analysis and plotting of RT-qPCR data was performed as previously mentioned.

3. RESULTS

3.1 Comparative phylogenetic analysis

The genome of *T. urticae* was found to contain three actin genes: *tetur09g05360*, *tetur09g05350*, and *tetur03g09480* (Grbic et al., 2011). In order to classify and annotate these actin genes, a comparative phylogenetic analysis was conducted using Drosophila as a model organism, which has two non-muscle actins and four muscle actins. Protein sequences from all six Drosophila actins and the three *T. urticae* actins were used to generate a multiple sequence alignment, taking advantage of the higher sequence diversity of protein sequences for more informative alignments (Figure 3.1). Subsequently, a phylogenetic tree was constructed using the maximum likelihood method, and the results, depicted in Figure 3.2, revealed that tetur09g05360 and tetur09g05350 clustered with the non-muscle actins of Drosophila, while tetur03g09480 was closely associated with the muscle actin of Drosophila. Based on these findings, tetur09g05360 and tetur09g05350 were annotated as actin βγ and actin βγ', respectively, while tetur03g09480 was identified as actin α, as listed in Table 3.1.

```
ACT2_DROME/1-376       1 MCDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNE 94
ACT4_DROME/1-376       1 MCDEEASALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDCYVGDEAQSKRGILSLKYPIEHGIITNWDDMEKVWHHTFYNE 94
ACT5_DROME/1-376       1 MCDDEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNE 94
ACT1_DROME/1-376       1 MCDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNE 94
ACT3_DROME/1-376       1 MCDDEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNE 94
ACT6_DROME/1-376       1 MCDDDAGALVIDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHTFYNE 94
tetur09g05360/1-376    1 MCDDEVAALVVDNGSSMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNE 94
tetur09g05350/1-376    1 MCDDEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNE 94
tetur03g09480/1-376    1 MCDDEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIITNWDDMEKIWHHSFYNE 94

ACT2_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
ACT4_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
ACT5_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNAPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
ACT1_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
ACT3_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
ACT6_DROME/1-376      95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNSPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGFALPHAILRLDLAGRDLTD 188
tetur09g05360/1-376   95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
tetur09g05350/1-376   95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188
tetur03g09480/1-376   95 LRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLDLAGRDLTD 188

ACT2_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSYELPDGQVITIGNERFRCPESLFQPSFLGMEACGIHETTYNS 282
ACT4_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKSYELPDGQVITIGNERFRTPEALFQPSFLGMESCGIHETVYQS 282
ACT5_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKSYELPDGQVITIGNERFRCPESLFQPSFLGMESCGIHETVYNS 282
ACT1_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMEACGIHETTYNS 282
ACT3_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKSYELPDGQVITIGNERFRCPESLFQPSFLGMESCGIHETVYNS 282
ACT6_DROME/1-376     189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAAASTSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETVYNS 282
tetur09g05360/1-376  189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSIEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTYNS 282
tetur09g05350/1-376  189 YLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSIEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESSGIHETTYNS 282
tetur03g09480/1-376  189 YLMKILTERGYSFVTTAEREIVRDIKEKLCYVALDFEQEMITAASSSSLEKSYELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTYNS 282

ACT2_DROME/1-376     283 IMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIVAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF 376
ACT4_DROME/1-376     283 IMKCDVDIRKDLYANNVLSGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPGIVHRKCF 376
ACT5_DROME/1-376     283 IMKCDVDIRKDLYANIVMSGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPGIVHRKCF 376
ACT1_DROME/1-376     283 IMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF 376
ACT3_DROME/1-376     283 IMKCDVDIRKDLYANIVMSGGTTMYPGIADRMQKEITSLAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKEEYDESGPGIVHRKCF 376
ACT6_DROME/1-376     283 IMKCDVDIRKDLYANSVLSGGTTMYPGIADRMQKEITALAPSTIKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPGIVHRKCF 376
tetur09g05360/1-376  283 IMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF 376
tetur09g05350/1-376  283 IMKCDVDIRKDLYANTVLSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF 376
tetur03g09480/1-376  283 IMKCDIDIRKDLYANIVLSGGTTMYPGIADRMQKEITALAPSTLKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPAIVHRKCF 376
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Figure 3.1. Multiple sequence alignment between actin protein sequences of *D. melanogaster* and *T. urticae.*

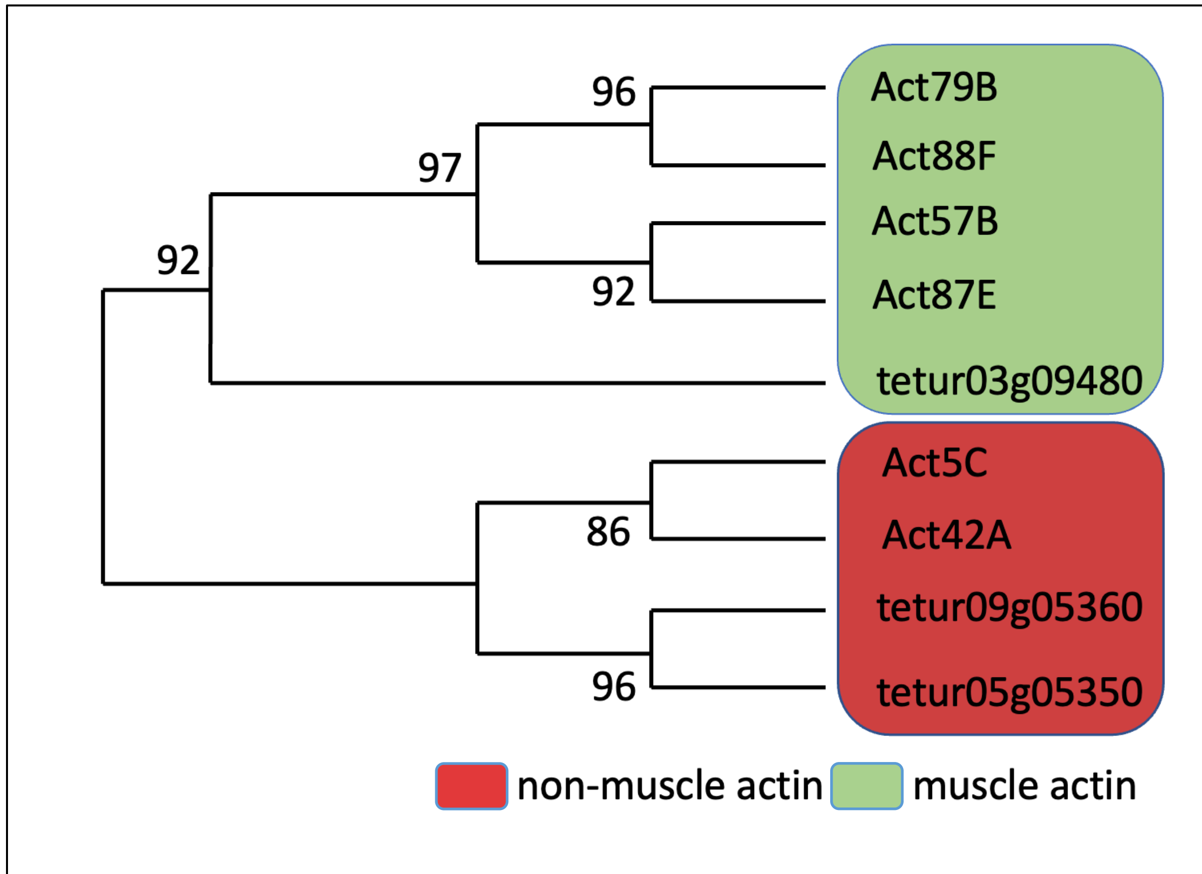

Figure 3.2. Maximum likelihood phylogeny of *D. melanogaster* and *T.urticae* actins.

Gene ID*	Functional description	Abbreviated name
tetur03g09480	Muscle contractile protein	actin α
tetur09g05350	Cytoskeletal protein	actin βγ'
tetur09g05360	Cytoskeletal protein	actin βγ

*Gene ID acquired from BOGAS: http://bioinformatics.psb.ugent.be/webtools/bogas/overview/Tetur

Table 3.1. Functional description of target gene.

3.2 Sequence similarity between *T. urticae actin* genes

Sequence alignments are a valuable tool for comparing nucleotide sequences, allowing for the identification of similarities between *actin* sequences. This information can be used to design specific dsRNA, shRNA, or amiRNA fragments that target individual actins without producing off-target effects.

In this study, the CDS regions of three actins, namely actin βγ, actin βγ', and actin α, were subjected to multiple DNA sequence alignment to determine their similarities. The results revealed that actin βγ and actin βγ' displayed a high degree of similarity, with a similarity rate of 98.5%. In contrast, actin βγ and actin α exhibited only an 85% similarity rate (Figure 3.3). Based on these findings, it can be inferred that the non-muscle actins, actin βγ and actin βγ', are more closely related to each other than they are to the muscle actin, actin α. This information can be applied to design specific dsRNA, shRNA, or amiRNA fragments that selectively target different types of actins.

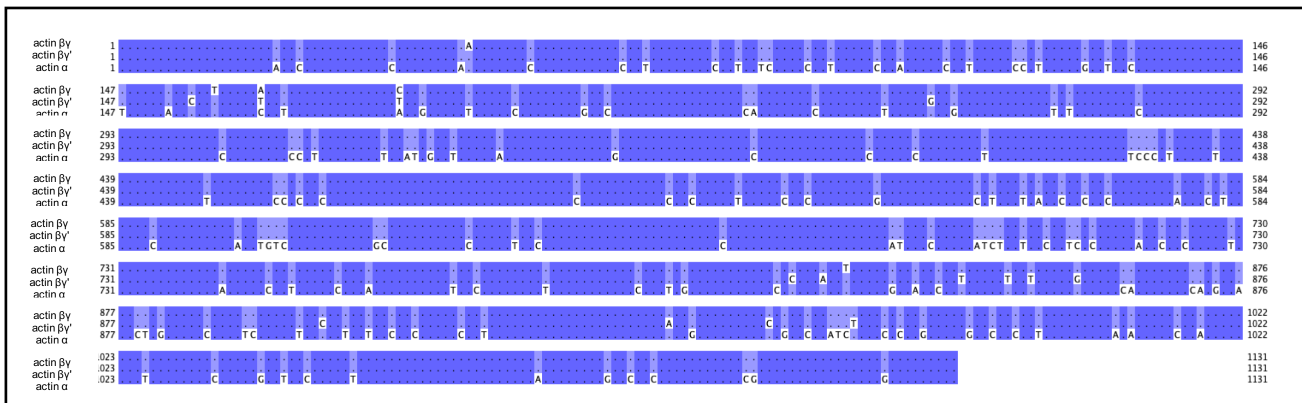

Figure 3.3. Multiple sequence alignment between CDS region of *T.urticae actin* genes.

3.3 Targeted gene knockdown using different RNAi precursors

3.3.1 dsRNA fragments

To ensure the effectiveness of RNA interference (RNAi) in *T. urticae*, the dsRNA fragments should fall within the length range of 400-600 bp (Bensoussan et al., 2020). Following this guideline, a 519 bp dsRNA fragment was designed to target both non-

muscle actins, *actin βγ* and *actin βγ'*, while another 470 bp dsRNA fragment was designed to target *actin α*, as depicted in Figure 3.3. As a negative control, a 382 bp dsRNA fragment complementary to a non-coding region in scaffold 12 of the spider mite genome (F3R3) was also used. Before use, the dsRNA sequences were used as queries for a BLAST search against the *T. urticae* genomic database to ensure specificity to the target genes and to minimize off-target effects. This step was taken to ensure the accuracy and specificity of the designed dsRNA fragments for effective RNAi in *T. urticae.*

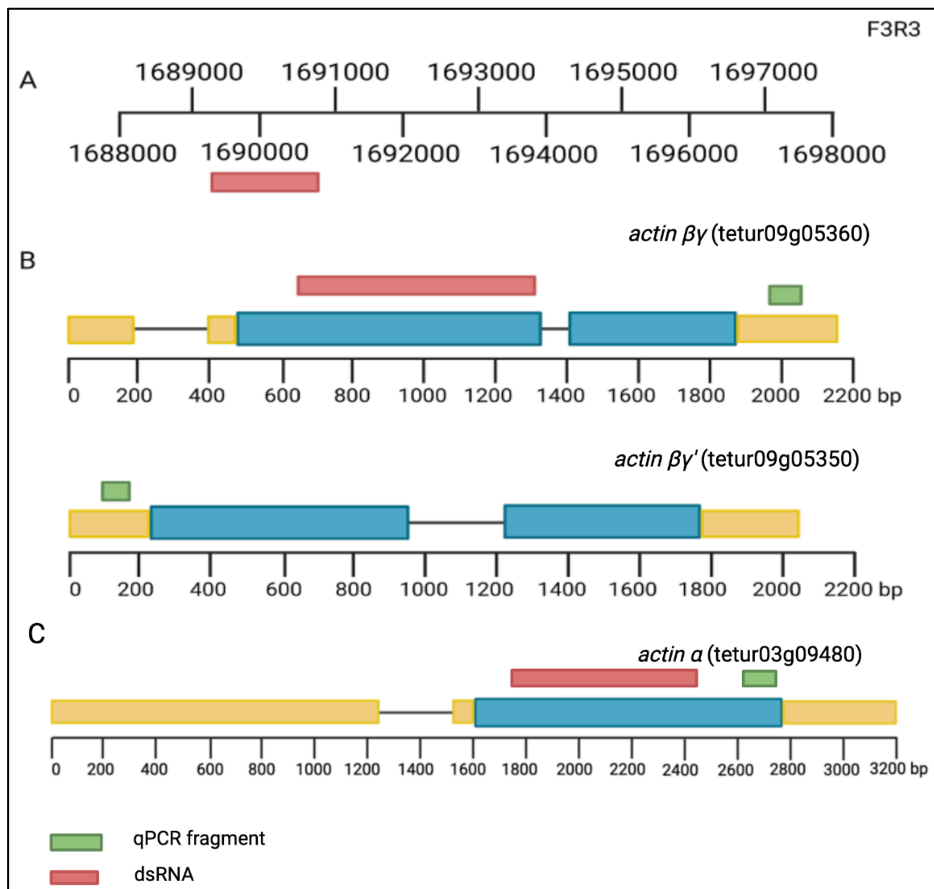

Figure 3.4. dsRNA fragments. (A) Schematic of the part of the scaffold 12 of *T. urticae* genome depicting the location of the 382 bp fragment that was used to synthesize dsRNA-F3R3. (B) Schematic of the *actin* βγ and *actin* βγ' locus. DNA sequence used for the generation of dsRNA-*actin* βγ (519 bp) is located in the 1st exon. (C) Schematic of *actin* α. DNA sequence used for the generation of dsRNA-*actin* α (470 bp) is located in the 1st exon. UTR, coding sequences and qPCR target site are shown in yellow, blue and green, respectively. Image created using BioRender.

3.3.2 shRNA fragments

To target non-muscle actins *actin βγ* and *actin βγ'*, two shRNA fragments named f1 and f2 were designed. Due to their high sequence similarity, these shRNA fragments are expected to target both *actin βγ* and *actin βγ'*. Additionally, two shRNA fragments f3 and f4 were designed to target *actin α* (Figure 3.5). The study also made use of the SL2 fragment previously reported by Mondal et al., 2021, which allowed for a direct comparison of the method's efficiency between the current study and the previous report.

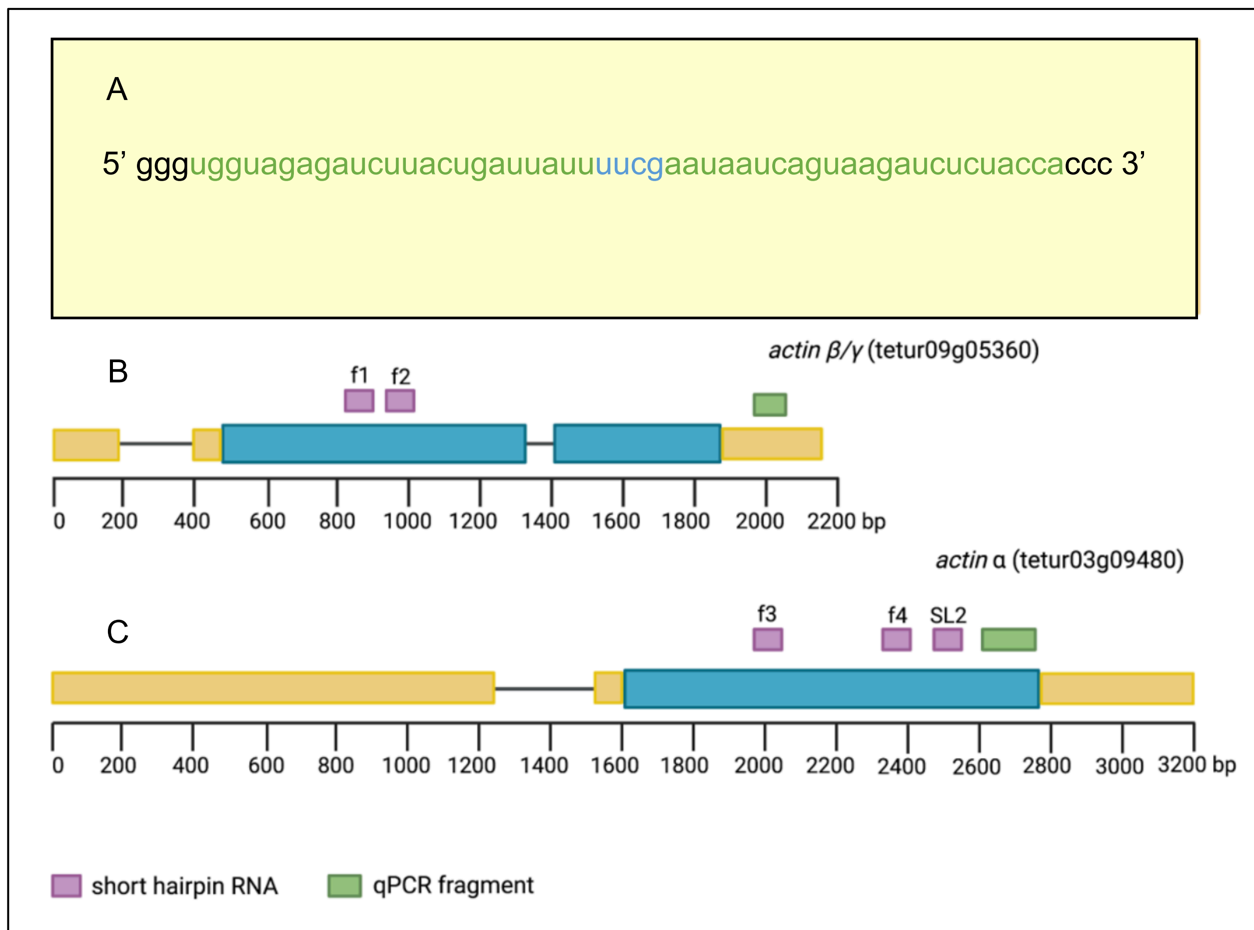

Figure 3.5. shRNA fragments. (A) Depicts the shRNA fragment, which includes the target sequence highlighted in green and the loop region highlighted in blue. (B) Schematic of the *actin* βγ locus. DNA sequence used for the generation of shRNA fragments 1 and 2 is located in the 1st exon. (C) Schematic of the *actin* α locus. DNA sequence used for the generation of shRNA fragments 3, 4 and SL2 is located in the 1st

exon. UTR, coding sequences and qPCR target site are shown in yellow, blue and green, respectively. Image created using BioRender.

3.3.3 amiRNA fragment

The highly conserved non-coding miRNA, tur-mir-7, was selected to construct an artificial miRNA fragment. The entire sequence of *T. urticae* mir7 was obtained from miRbase, a comprehensive database of published miRNA sequences. The mature sequence of tur-mir-7, which is 24 nt long and highlighted in the Figure 3.6, was replaced with the target sequence, while the remaining backbone sequence was maintained. To ensure specificity to the target gene and avoid off-target effects, a BLAST search was conducted against the *T. urticae* database using the sequences intended to replace the mature sequences. Two amiRNA fragments, f1 and f2, were designed to target *actin βγ* and *actin βγ'*, both non-muscle actins, while two other fragments, f3 and f4, were designed to specifically target *actin α*.

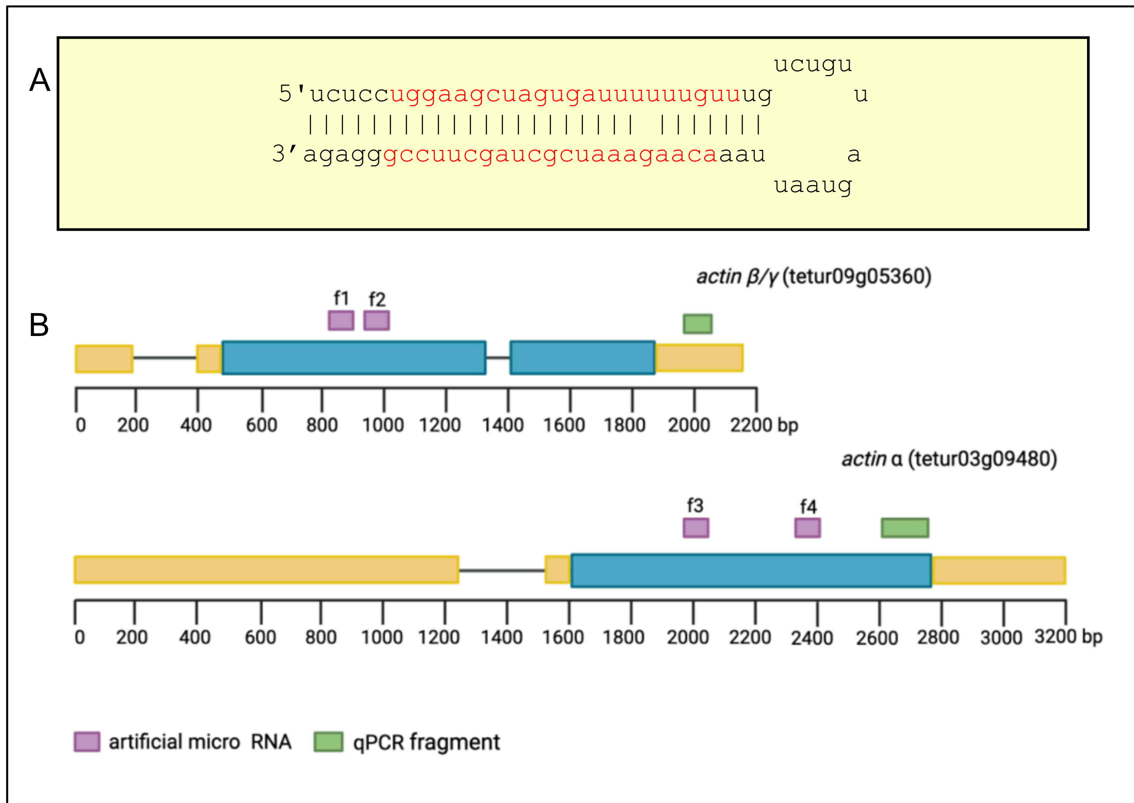

Figure 3.6. amiRNA fragments. (A) Schematic of tur-mir-7 sequence with the mature sequence region highlighted in red. (B) Schematic of the *actin* βγ and *actin α* locus. UTR,

coding sequences and qPCR target site are shown in yellow, blue and green, respectively. Image created using BioRender.

3.4 Effects of different small molecules induced RNAi

3.4.1 Effect of dsRNA induced RNAi

To assess the impact of dsRNA treatment on the entire mite, fecundity was measured over a 3-day period following treatment. It was observed that mites treated with dsRNA targeting *actin βγ* showed a significant reduction in fecundity, producing only 3 eggs/female/day as compared to control mites which laid 9 eggs/mite/day (Figure 3.7A). Furthermore, mites treated with dsRNA targeting *actin α* also exhibited a significant decrease in fecundity, producing only 6 eggs/female/day as compared to the F3R3 control (Figure 3.7A). This effect was also reflected in phenotypic changes observed in mites post RNAi treatment, where dsRNA targeting *actin βγ* resulted in a complete dark body phenotype, in contrast to F3R3 control which exhibited a regular two-spotted phenotype as depicted in Figure 3.7B. The proportion of dark mites produced after dsRNA treatment targeting *actin βγ* was found to be significantly higher, with approximately 90% of treated mites turning black. In contrast, targeting of muscle actin did not result in any phenotypic change post-treatment (Figure 3.7B).

To determine if there was a corresponding reduction in transcript levels of *actin* after RNAi treatment, RT-qPCR was conducted. From the data presented in Figure 3.8, it was evident that there was a significant reduction in the levels of actin transcripts for all three types of actins, namely *actin βγ*, *actin βγ'*, and *actin α* (ANOVA, ***P<0.001, n=3). The statistical analysis showed that the reduction in transcript levels was highly significant. Overall, the results suggest that the dsRNA induced RNAi treatment was successful in reducing the expression of actin genes in the treated samples, as compared to the control group.

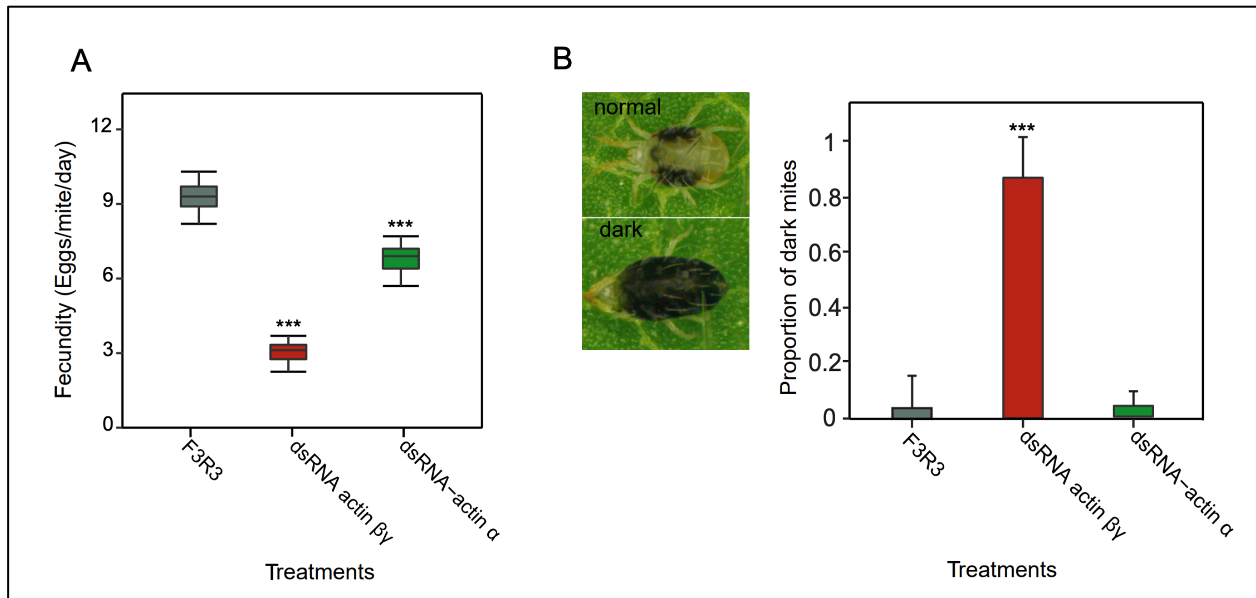

Figure 3.7. Effect dsRNA induced RNAi in whole mite. (A) Mite fecundity over a 3-day period upon mite transfer to detached bean leaves. (B) Mites showing the phenotypic changes after dsRNA *actin* treatment. The bar graph represents proportion of dark phenotype. Asterisk represents significant difference between treatment and control, ***P< 0.001, n=5)

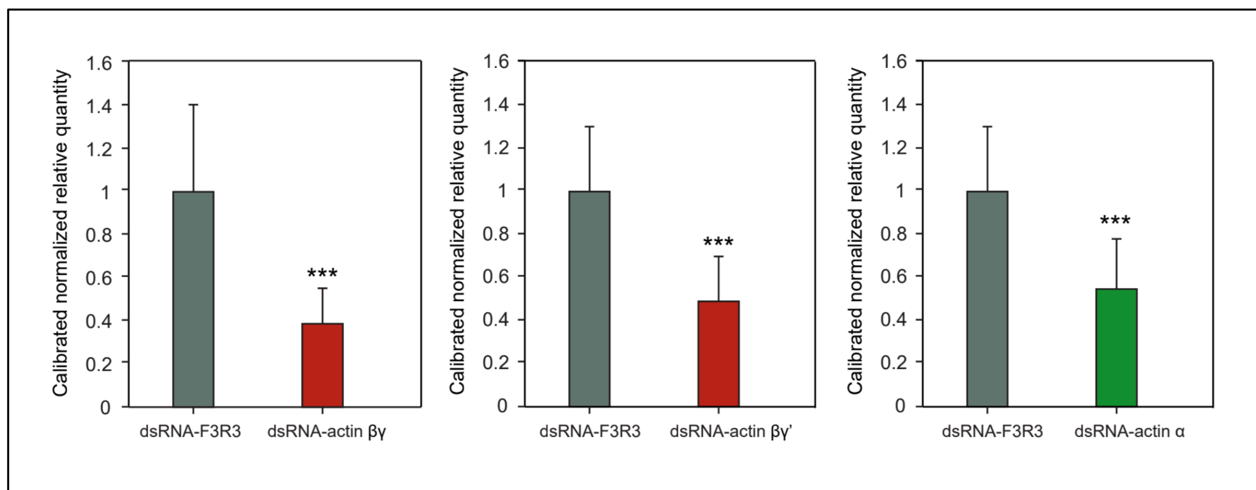

Figure 3.8. *actin* expression post dsRNA induced RNAi. *actin* β/γ, *actin* βγ’ and *actin* α expression level in whole mite. Data were represented as mean ±SE and analyzed using ANOVA relative to dsRNA F3R3 treatment (asterisk represents significant difference between treatment and control, ***P< 0.001, n = 5).

3.4.2 Effect of shRNA induced RNAi

In the conducted study, the response of mites to shRNA treatment was evaluated using the same methodology as that of dsRNA treatment. Specifically, the fecundity of treated mites was recorded after treatment with different shRNA fragments. The results showed that all shRNA fragments induced a significant reduction in mite fecundity, reducing it to approximately 6 eggs/female/day compared to the negative control (F3R3), which had a fecundity of around 9 eggs/female/day (ANOVA, ***$P<0.001$, $n=5$).

However, it was observed that the reduction in fecundity induced by dsRNA treatment included in the experiment was much higher compared to shRNA treatment. The dsRNA treatment resulted in the production of only around 2 eggs/female/day, which was significantly lower than that observed with all other shRNA fragments (Figure 3.9A). This difference was also reflected in the proportion of dark body phenotypes observed with dsRNA treatment, which was significantly higher than that observed with shRNA fragments (Figure 3.9 B). In contrast, the proportion of dark body mites produced by shRNA fragments was extremely low and not significantly different than the F3R3 control.

These findings suggest that while both dsRNA and shRNA treatments can significantly reduce mite fecundity, the dsRNA treatment is more effective in inducing a greater reduction.

Finally, RT-qPCR was performed to examine the impact of shRNA-induced RNAi on the levels of action transcripts. The results, as presented in Figure 3.10 A, indicated a significant reduction in the levels of *actin* β/γ transcripts following shRNA treatment compared to dsRNA treatment. This outcome was unexpected, given that the shRNA fragments did not achieve as high a reduction in fecundity levels as dsRNA did.

Furthermore, the levels of *actin* β/γ' transcripts were notably reduced by approximately 60% following dsRNA treatment, Although the treatment with shRNA resulted in a decrease in transcript levels, the observed reduction was not statistically significant (Figure 3.10 B). It is worth noting that there was also a higher degree of variability in the

results, which surpasses the statistical power available for further analysis. In addition, shRNA treatment led to a reduction in the level of *actin* α transcripts, although this reduction was not statistically significant (Figure 3.10 C). On the other hand, dsRNA treatment produced a significant reduction in the level of *actin* α transcripts by 60%. These results demonstrate that, although shRNA treatments can lead to a reduction in the levels of *actin* transcripts, the reduction is not statistically significant in most cases, thus making dsRNA treatments more effective.

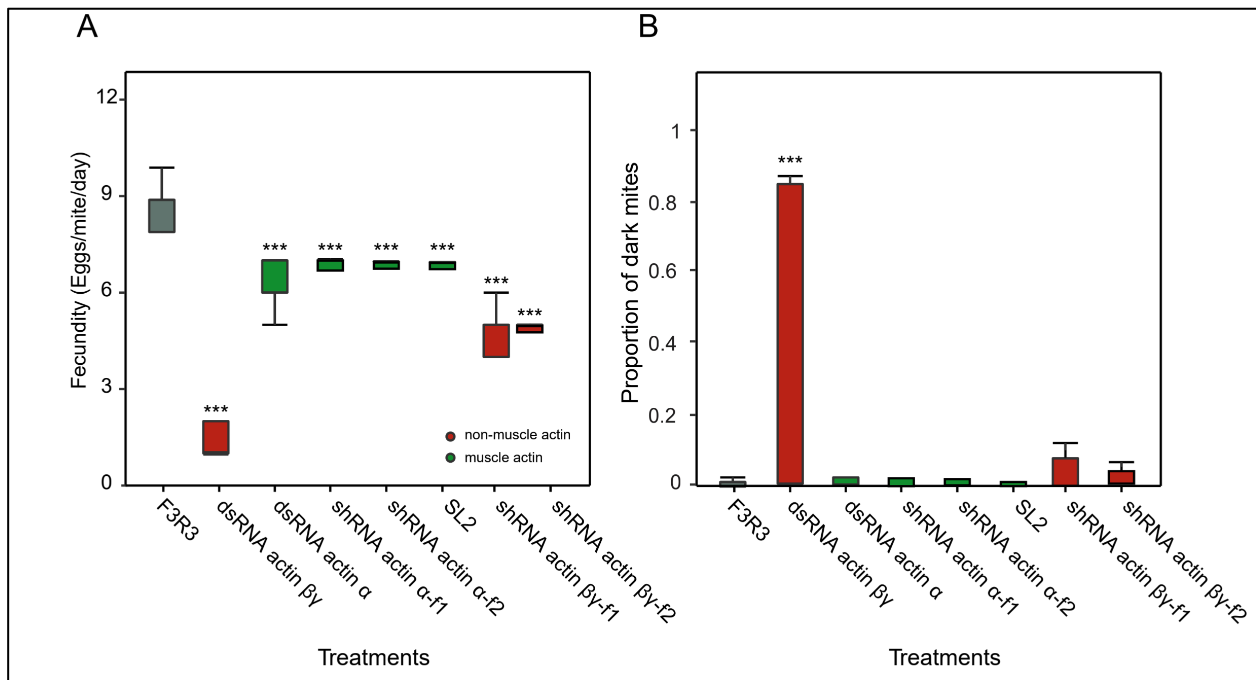

Figure 3.9. Effect shRNA induced RNAi in whole mite. (A) Mite fecundity over a 3-day period upon mite transfer to detached bean leaves. (B) Mites showing the phenotypic changes after shRNA *actin* treatment. The bar graph represents proportion of dark phenotype. Asterisk represents significant difference between treatment and control, ***P< 0.001, n=5)

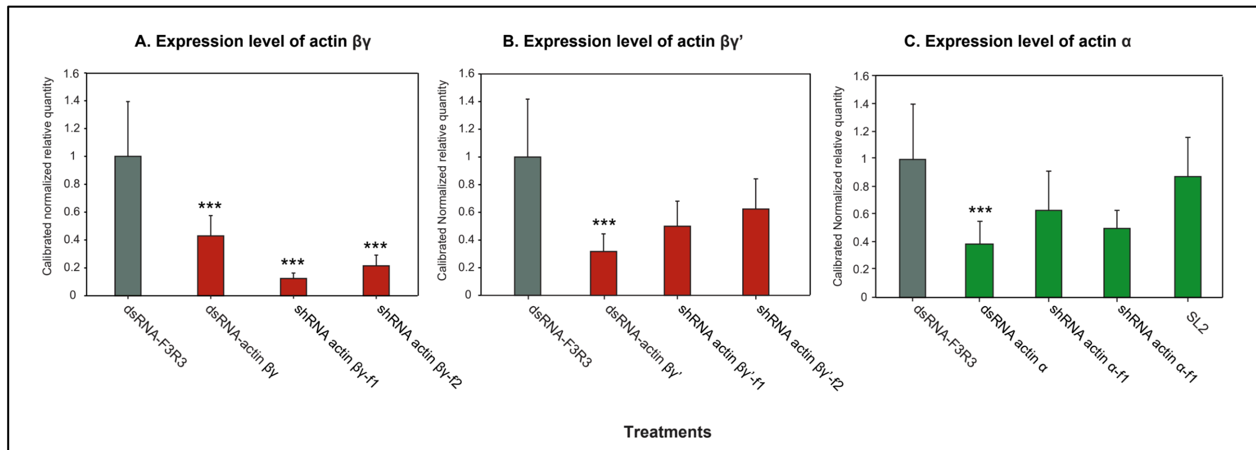

Figure 3.10. *actin* expression post shRNA induced RNAi. *actin* βγ, *actin* βγ', *actin* α expression level in whole mite. Data were represented as calibrated NRQ ± SE and analyzed using ANOVA followed by pairwise comparisons relative to dsRNA F3R3 treatment (asterisk represents significant difference between treatment and control, *** $P < 0.001$, $n = 5$).

3.4.3 Effect of amiRNA induced RNAi

Finally, the effect of amiRNA treatment on the fecundity of mites was assessed, the results of which are displayed in Figure 3.11. The data clearly showed that treatment with the dsRNA fragment had a significant impact on fecundity, reducing it by approximately 3 eggs per female per day. This reduction was consistent with the results of previous dsRNA trials, indicating the reliability of dsRNA in producing phenotypes.

Additionally, amiRNA fragments targeting *actin* βγ and βγ' also caused a significant reduction in mite fecundity, albeit not to the same extent as dsRNA. The ANOVA results indicated a statistically significant reduction in fecundity ($P<0.05$) for these amiRNA treatments.

Furthermore, the study found that amiRNA *actin* α-f2, which targeted *actin* α, was able to produce a significant reduction in mite fecundity compared to its counterpart amiRNA *actin* α-f1. The latter did not show a significant reduction in fecundity compared to control mites from the F3R3 treatment. Overall, these results suggest that dsRNA is far more effective in reducing mite fecundity compared to amiRNA.

In the final step of the study, RT-qPCR was conducted to assess the extent of reduction in actin transcript levels following amiRNA treatments. The results showed that all amiRNA fragments caused a slight reduction in actin transcript levels compared to the control group (F3R3), but these reductions were not statistically significant (Figure 3.12). In contrast, the dsRNA fragment produced a significant reduction in actin transcript levels. This finding is consistent with the previous results that showed dsRNA to be more effective in producing phenotypic effects compared to amiRNA.

Overall, these findings suggest that while all amiRNA fragments were able to produce some degree of reduction in actin transcript levels, they were not as effective as the dsRNA fragment. These results provide further support for the use of dsRNA in RNAi experiments.

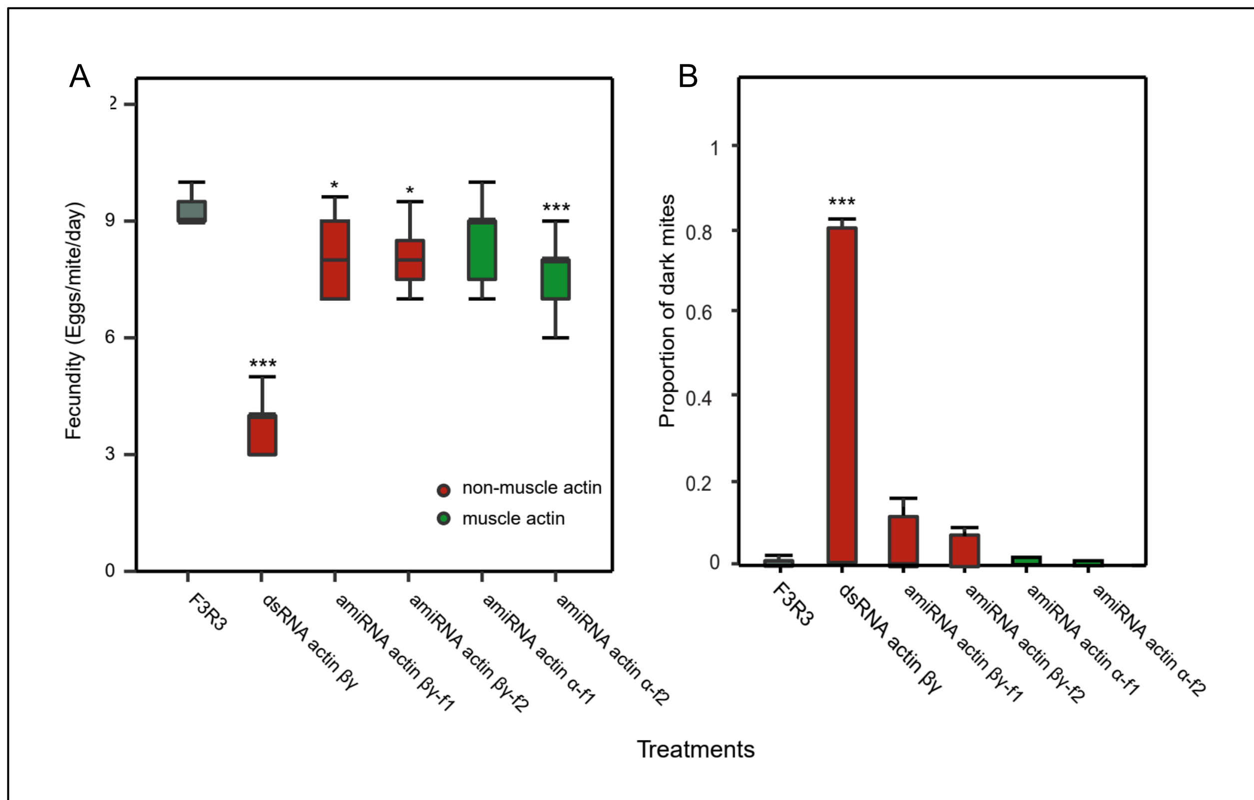

Figure 3.11. Effect amiRNA induced RNAi in whole mites. (A) Boxplots of mite fecundity over a 3-day period upon mite transfer to detached bean leaves. (B) Mites showing the phenotypic changes after amiRNA treatments. The bar graph

represents proportion of dark phenotype ± SE. Asterisk represents significant difference between treatment and control, *P< 0.05, ***P< 0.001, n=5)

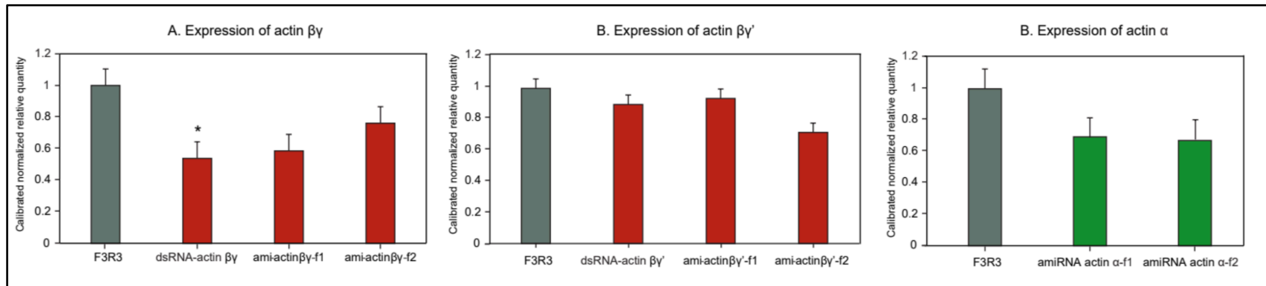

Figure 3.12. *actin* expression post amiRNA induced RNAi. *actin* β/γ, *actin* βγ', *actin* α expression level in whole mite. Data were represented as calibrated NRQ ± SE and analyzed using ANOVA followed by pairwise comparisons relative to dsRNA F3R3 treatment (asterisk represents significant difference between treatment and control, *P< 0.05, n = 5).

3.5 Establishment of mite primary cell culture

Primary cell cultures of *T. urticae* embryos were successfully established following various parameter optimizations, including media selection, cell supplement selection, and homogenization methods. Spherical cells, along with mite feces that were too small to be filtered out by the cell filter, were observed in the culture immediately after plating (as shown in Figure 3.13.A). Signs of attachment to the culture plate were observed in the cells after 48 hours of seeding, as they slightly elongated (Figure 3.13.B). Over the course of the following days, clear morphological features began to appear in the cells, and fibroblast-like cells were produced in the culture plate by day 6 (Figure 3.13.C).

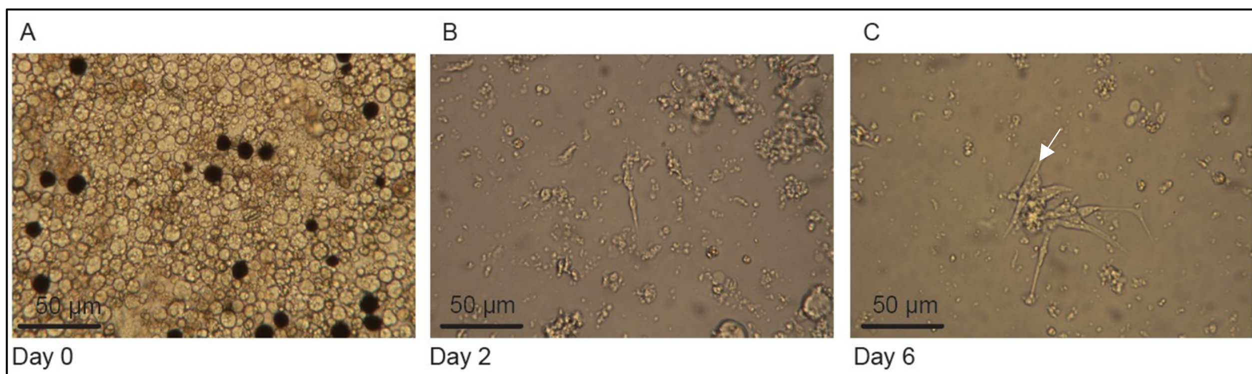

Figure 3.13. Images of cells in L15 medium. (A) Cells after seeding in 24 well plate. (B) Cells showing signs of attachment to the plate. (C) Cells begin to show clear morphology, fibroblast-like cells (white arrow) were observed in primary cell culture.

3.6 Cell density in primary cell culture

The total number of cells in the primary cell culture of *T. urticae* embryos was monitored for a period of 25 days using a cell counter. The results indicated that on the day of seeding, the number of cells was high, with approximately 3×10^5 cells/well. However, only about 25% of the cells attached to the culture plate, as shown in Table 3.2. Between days 3 and 15, the number of cells remained relatively constant, ranging from 7.5×10^4 to 3.5×10^4 cells/well. This time frame appears to be optimal for conducting experiments in primary cell culture. Towards day 21, the total number of cells decreased significantly, and the cells began to senesce. Thus, it is recommended to perform experiments within the time frame of day 3 to day 15 to obtain reliable results from the primary cell culture of *T. urticae* embryos.

Stage of cell culture	***Total number of cells***
Day 0	**~ 3×10^5**
Day 3	**~ 7.5×10^4**
Day 7	**~ 5×10^4**
Day 15	**~ 3.5×10^4**
Day 21	**~ 1.5×10^4**

Table 3.2. Total number of cells isolated and attached at different time periods of cell culture.

3.7 Effect of dsRNA induced RNAi in primary cell culture

In order to investigate the impact of dsRNA treatment on primary cells, the gene expression levels of treated cells were assessed by conducting RT-qPCR. The results indicated that *actin* βγ was significantly silenced with a reduction of approximately 35% (ANOVA, $p<0.05$, n=5), whereas the non-muscle *actin* βγ' showed a minor decrease in transcript level that did not reach statistical significance (Figure 3.14). These findings provide evidence that RNAi can be effective in primary cell culture.

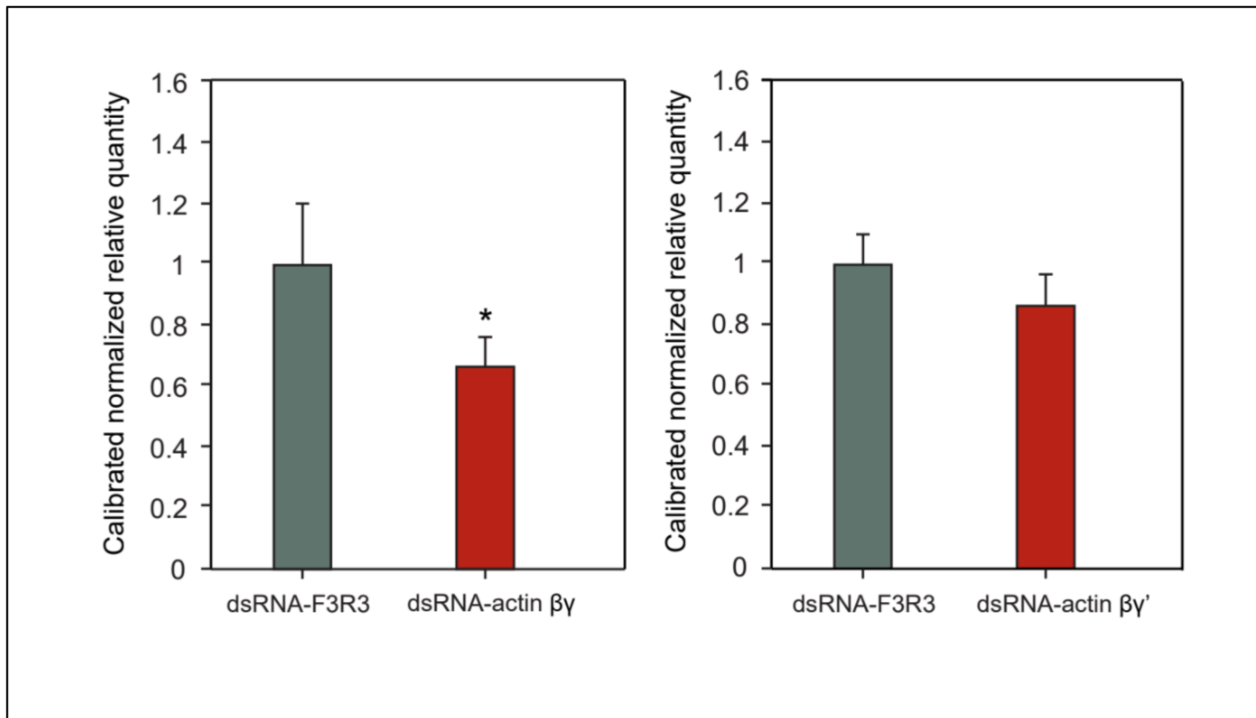

Figure 3.14. *actin* expression post dsRNA induced RNAi in cell culture. *actin* β/γ, *actin* βγ', *actin* α expression level in whole mites. Data were represented as calibrated NRQ ±SE and analyzed using ANOVA (asterisk represents significant difference between means *$P< 0.05$, n = 5).

4. DISCUSSION

4.1 RNA Interference: A promising reverse genetics tool for study of gene function and pest control

The advent of high-throughput sequencing has led to the availability of vast amounts of genomic data for various insect and pest species that are non-model organisms. For instance, the genomes of pests such as the red flour beetle *Tribolium castaneum*, the diamondback moth *Plutella xylostella*, and the corn earworm *Helicoverpa zea*, are now available (Pearce et al., 2017; Richards et al., 2010; You et al., 2013). This genomic information has opened up new avenues for studying the molecular basis of insect and pest biology and for developing novel pest management strategies. For decades, researchers have relied on traditional forward genetic tools such as mutagenesis and phenotypic screenings to understand the function of genes in model organisms. However, applying these tools to non-model organisms can be challenging due to limited genetic resources and necessity to maintain mutant stocks (Liu & Rand, 2019). Additionally, identifying and isolating mutants with specific phenotypes can be time-consuming and resource intensive. In contrast, reverse genetic tools such as RNA interference (RNAi) and CRISPR-Cas9-mediated gene editing offer a straightforward approach to studying a gene function in non-model organisms. RNAi is a powerful tool that can be used to knock down specific genes and study their function. For example, RNAi has been used to investigate the roles of genes involved in insect development, immunity, and behavior in non-model organisms such as the Colorado potato beetle *Leptinotarsa decemlineata* (Zhao et al., 2015) and the diamondback moth *Plutella xylostella* (Kumar et al., 2017). Another option for gene editing in non-model organisms is the CRISPR-Cas9 system. CRISPR-Cas9 enables precise genome editing and has been used successfully in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Kanca et al., 2022; Namiki et al., 2017), as well as in non-model organisms such as the red flour beetle *Tribolium castaneum* (Gilles et al., 2015) and the Asian citrus psyllid *Diaphorina citri* (L. Chen et al., 2016) However, establishing the CRISPR-Cas9 system in non-model organisms can be challenging, as it requires efficient transformation and expression of sgRNA and Cas9 in the target organism. This is not always feasible for some insects or pests due to their complex physiology and low transformation efficiency. For example,

CRISPR-Cas9 gene editing has been attempted but has proven difficult in other pests such as the tsetse fly *Glossina morsitans* (Geiger et al., 2018) Hence, RNAi remains a crucial and promising approach for studying gene functions in organisms where traditional genetic methods are not practical.

The genome sequence of the two-spotted spider mite, *Tetranychus urticae*, is available and provides a wealth of information about the genetic basis of its herbivorous adaptations and potential targets for pest control (Grbic et al., 2011). The genome sequence of *T. urticae* identified all components of RNAi machinery, which can be used for targeted gene knockdown to control pests. Multiple studies have confirmed the effectiveness of RNA interference (RNAi) in silencing target genes in *T. urticae* using dsRNA and siRNA ((Bensoussan et al., 2022; Khila & Grbić, 2007; Suzuki, Nunes, et al., 2017). In contrast, attempts to use CRISPR-Cas9-mediated gene knockout in spider mites were unsuccessful (Dermauw et al., 2020). The positive outcomes of RNAi-based knockdown experiments in *T. urticae* create new opportunities for developing alternative pesticide solutions to manage this harmful pest.

Environmental RNAi is a technique that involves delivery of double-stranded RNA (dsRNA) through methods such as feeding or soaking. The goal is to disrupt genes that are crucial for the survival of an organism. RNAi is a natural mechanism that regulates gene expression by degrading mRNA molecules, and it can be artificially induced by introducing dsRNA that matches the target gene's sequence. Studies have shown that environmental RNAi can significantly reduce transcript levels of target genes in predatory mites, ticks, and insects, with a reduction rate of 70-90% (Bolognesi et al., 2012; Rangasamy & Siegfried, 2012). RNAi-based pesticides can be applied in the field by spraying method or through transgenic crops produced by nuclear or chloroplast transformation. For instance, RNAi-based spray formulations have been developed to control Colorado Potato Beetle (Pallis et al., 2023). On the other hand, transgenic crops producing dsRNA can be effective in controlling various insect pests, such as the brown planthopper (Zha et al., 2011)and the cotton bollworm (Mao et al., 2016). However, the efficiency of RNAi varies across different insect orders and species. Coleopterans

generally show high efficiency in target gene knockdown and insect mortality, while lepidopterans exhibit minimal or no effects (Christiaens et al., 2020). Various barriers can influence the effectiveness of RNAi, such as the presence of dsRNA-degrading nucleases and inefficient uptake of dsRNA by midgut epithelial cells in certain insects. Furthermore, the life stage and tissue type where the target gene is expressed can impact the success of RNAi (Christiaens et al., 2020).

Comparing the barriers affecting RNAi in insects to spider mites, stability of dsRNA is not a problem, as ingested dsRNA labeled with fluorescent tag was detected in the caecal lumen while the breakdown product of dsRNA was not seen in the posterior midgut (Bensoussan et al., 2022). Furthermore, the dsRNA did not appear to be trapped in the endosomes, as no signal was observed in the endosomes using fluorescence microscopy (Bensoussan et al., 2022). Even though spider mites do not possess the *sid-1* gene, which plays a key role in the transport of precursor molecules and RNAi-induced signals to different cells, it is plausible that receptor-mediated endocytosis may be involved in the uptake of dsRNA molecules and can facilitate cell-to-cell movement (Saleh et al., 2006; Bensoussan et al., 2022). In fact, a gene in *T. urticae* that codes for a scavenger receptor shares high identity with the class B scavenger receptor (SRB) of the tick *Haemaphysalis longicornis,* where SRB plays a vital role in receptor-mediated endocytosis for systemic RNAi (Aung et al., 2011). the processivity of dsRNA by dicer has the greatest effect on long dsRNAs that are approximately 400-600 bp in length (Bensoussan et al., 2020). This presents a challenge in terms of the specificity of dsRNA when using it as RNAi-based pesticides, as long dsRNA can produce a large number of small interfering RNAs (siRNAs) that may cause off-target effects and unintended harm to non-target organisms.

4.2 Exploring Alternative RNAi Precursors for Effective Pest Management

The traditional approach of using dsRNA to trigger RNAi has some limitations, such as off-target effects, non-specific immune response, and limited ability to silence certain genes. For example, in the study by Kamath et al. (2003), it was observed that dsRNA-mediated RNAi in *C. elegans* triggered the activation of the Toll and Jak-STAT immune

pathways, leading to the expression of antimicrobial peptides and interferon-like genes. This immune response can be non-specific and may affect not only the target organism but also non-target organisms. In terms of the limited ability to silence certain genes, this can occur due to factors such as the position and structure of the target gene, as well as the presence of alternative splicing isoforms that may not be targeted by the dsRNA (Huvenne & Smagghe, 2010). Additionally, some genes may be resistant to RNAi due to factors such as secondary structures in the mRNA that prevent the efficient binding and cleavage by the RNAi machinery (Overhoff et al., 2005).

A viable alternative to using dsRNA to trigger RNA interference is the use of shRNA as a precursor molecule. shRNA forms a hairpin structure, which is processed by Dicer into a single siRNA that triggers RNAi. This method has demonstrated efficacy in reducing gene expression across a range of organisms and cell cultures. (Li et al., 2011; Shin et al., 2006; Ventura et al., 2004; Brummelkamp et al., 2002; Rossi, 2008). Another alternative is synthetic siRNA has been shown to be effective in knocking down genes in Drosophila and mammalian cell cultures (Doi et al., 2003; Elbashir et al., 2001). In addition to shRNA and synthetic siRNA, another RNAi precursor that has been explored is paperclip RNA (pcRNA). In pcRNA, two partially complementary RNA molecules form a hairpin loop structure similar to a paperclip (Abbasi et al., 2020). In addition, another type of small RNA molecule that can be used for gene silencing is artificial microRNA (amiRNA). This approach involves designing a short RNA molecule that mimics the structure of the endogenous microRNAs that are involved in post-transcriptional gene regulation in many eukaryotes. The amiRNA is then processed by the same RNAi machinery as endogenous miRNAs, resulting in the generation of a single siRNA molecule that is specific to the target gene. For example, Mao et al. (2016) successfully reduced the expression of a gene involved in chitin synthesis using an amiRNA approach in cotton bollworm (*Helicoverpa armigera*). While these RNAi precursors show promise in improving the efficiency and specificity of RNAi-mediated gene knockdown, they also have their own limitations. For instance, shRNA can cause toxicity in some cell types (Paddison et al., 2002). pcRNA has only been tested in cell cultures and its effectiveness *in vivo* remains to be seen (Abbasi et al., 2020).

Mondal et al. (2021) conducted a study investigating the effectiveness of various small RNA molecules, including shRNA, siRNA and piRNA in silencing genes in *T. urticae* through feeding experiments. The results showed that all tested small RNA molecules induced gene silencing in *T. urticae*, with shRNA exhibiting the highest knockdown efficiency. However, these data were not replicated, preventing statistical analysis. Using the same shRNA that should yield the same small RNA, I found that these RNAs were less efficient than dsRNA in inducing phenotypic effects in mites. While dsRNA, shRNA, and amiRNA were effective in reducing mite fecundity and actin transcript levels, the reductions achieved by shRNA and amiRNA were not statistically significant relative to the application of the control RNAi treatment in most cases. It is worth noting that the shRNA and amiRNA treatments exhibited a higher degree of variability in the results, surpassing the available statistical power to determine significant differences for further analysis. At present, the reasons for lower activity of these alternative RNAi-inducing molecules are not known. One possible explanation for the relative ineffectiveness of shRNA and amiRNA could be their lower stability and persistence compared to dsRNA. Differences in the uptake mechanism may also account for the variable RNAi response to different precursors. A fluorescent tag can be attached to small RNA molecules to visualize the uptake and distribution of the molecules over time within mite cells and tissues. This information can then be used to determine the stability of the molecules in the mite and to identify factors that may be impacting the molecules stability within the mite and their susceptibility to clearance by the mite's immune system.

4.3 *actin* as target gene for RNAi

Actin is an essential protein involved in various cellular functions, including muscle contraction and cell division (Dominguez & Holmes, 2011). Targeting both muscle and non-muscle actins using dsRNA treatment significantly reduced fecundity in mites. Moreover, dsRNA treatment targeting non-muscle actins resulted in a distinct dark body phenotype in mites. The dark body phenotype was caused by retention of chlorophyll pigment in the lumen, reflecting an altered digestive physiology, which may limit nutrient availability required for egg production (Bensoussan et al., 2022). The RT-qPCR analysis

also indicated a significant reduction in transcript levels of all actins following the treatment. This is in agreement with the results of Chen et al. (2021), who found that highly expressed genes are also highly susceptible to RNAi. Although *actin* may not be a feasible target for mite control because of its conservation across species, the study's findings demonstrate that RNAi technology can effectively decrease gene expression and elicit phenotypic alterations in mites, consistent with previous experiments that targeted constitutively expressed genes such as *VATPase* and *COPB2*. (Bensoussan et al., 2020, 2022; Kwon et al., 2013; Suzuki, Nunes, et al., 2017). Due to its constitutive expression, *actin* can serve as an ideal model target to study RNAi machinery in both whole mites and cell cultures, providing valuable insights into the broader applications of this technology for gene regulation and pest management.

4.4 Significance of primary cell cultures in mite research

Primary cell cultures are an essential research tool for insect and mite research, as they allow the study of cells under physiological conditions that closely mimic *in vivo* environments. Unlike immortalized cell lines, primary cells retain their properties of differentiated cells, including morphology, gene expression, and physiology (Ulvila et al., 2006). This makes primary cell cultures more useful in studying various cellular processes, including signaling pathways, immune responses, and developmental biology.

One significant advantage of primary cell cultures is their ability to be used in studies of the effects of RNAi on cellular processes. For example, in the study by Abbasi et al. (2020), cell culture was utilized to investigate the RNAi machinery in the mosquito Aedes aegypti. Based on their findings, cells took up pcRNA independently of clathrin-mediated uptake, suggesting an alternative mechanism of the uptake to dsRNAs. The study also identified several key components of the RNAi machinery in the mosquito cells, including Dicer-2 and Argonaute-2. Overall, the use of cell culture allowed for a detailed investigation of the RNAi pathway in *Aedes aegypti*, providing insights into the mechanisms of RNAi uptake and processing in this important mosquito species.

The establishment of primary cell cultures of *Tetranychus urticae* is a notable achievement in mite research (Shukla, in preparation). The results of monitoring the total number of cells in the primary cell culture of *T. urticae* for 21 days revealed that the number of cells remained relatively constant between days 3 and 15, suggesting that this time frame is ideal for conducting experiments in the primary cell culture. I performed an RT-qPCR assay to evaluate the impact of dsRNA treatment on primary cells, revealing a statistically significant reduction of around 35% in the transcript level of *actin βγ*. In contrast, non-muscle *actin βγ′* showed only a minor decrease in transcript level that did not reach statistical significance. These findings suggest that RNAi can effectively silence gene expression in the primary cell culture of *T. urticae*. Overall, my data demonstrate the suitability of mite primary cell culture for conducting experiments and highlight the efficacy of RNAi technology in silencing gene expression in this system.

Furthermore, primary cell cultures can be used to investigate the mechanism of small RNA uptake in mites. Subsequent investigations could focus on the function of the gene *Scv7* (tetur13g00340*)*, which encodes a scavenger receptor involved in the receptor-mediated endocytosis of small RNAs, based on previous findings indicating that RNAi targeting of *actin* genes reduced actin transcript levels in primary cells of *T. urticae*. To perform RNAi of RNAi experiments, RNAi knockdown of *Scv7* can be followed by RNAi knockdown of *actin* to observe whether the silencing of the receptor-mediated endocytosis gene affects the uptake of dsRNA/shRNA/amiRNA and thus impacts the efficiency of RNAi knockdown of *actin*. There are several other genes within the RNAi machinery of mites that have yet to be fully characterized and could be investigated using primary cell cultures. For example, other genes involved in small RNA processing and transport, such as Argonaute and Piwi genes. Additionally, the contribution of other cellular pathways to small RNA uptake and processing, such as exosome-mediated transport, could be investigated using this system. These experiments can provide valuable insights into the molecular mechanisms of small RNA uptake in mites and the factors affecting the efficiency of RNAi knockdown, which can be beneficial for developing RNAi-based strategies for mite control. The use of primary cell cultures in these experiments can provide a controlled and reproducible system to study these processes

in a cell-based environment, which can complement *in vivo* experiments and facilitate the discovery of key molecular players in the RNAi pathway.

4.5 Conclusions and future directions

In summary, advances in genomic data and high-throughput sequencing have created new avenues for investigating insect and pest biology. The reverse genetic tool RNA interference has shown promise for studying gene functions in non-model organisms where traditional genetic methods are impractical. RNAi-based pesticides have the potential to control insect pests by targeting specific genes that play a vital role in essential physiological processes. However, for spider mites, the requirement of long dsRNAs (approximately 400-600 bp) for efficient RNAi may results in RNAi off-target effects and effects on the non-target organism. To address this challenge, chimeric dsRNA molecules can be developed, consisting of short (~100 bp) of the target gene sequence and non-coding "stuffer" sequences or multiple target sequences (Bensoussan et al., 2020). The incorporation of stuffer sequences enhances dicer processing, increasing the likelihood of a specific RNAi response while minimizing off-target effects. Alternative RNAi precursors such as shRNA and amiRNA have been explored to improve efficiency and specificity, but have not been as effective as dsRNA, possibly due to instability or varied cellular uptake mechanisms. Primary cell cultures are crucial for insect and mite research, as they provide an opportunity to study cells under physiological conditions that closely resemble *in vivo* environments. Finally, RNAi knockdown of *actin* in primary cell cultures offers new possibilities for "RNAi of RNAi" experiments and investigating the uptake mechanism of dsRNA and other RNAi-inducing molecules. In conclusion, while developing RNAi-based pesticides for spider mites is challenging, the continued exploration of RNAi and alternative RNAi precursors, as well as the use of primary cell cultures, offer exciting prospects.

APPENDICES

R Studio information

platform aarch64-apple-darwin20

arch aarch64

os darwin20

system aarch64, darwin20

status

major 4

minor 2.2

year 2022

month 10

day 31

svn rev 83211

language R

version.string R version 4.2.2 (2022-10-31)

nickname Innocent and Trusting

Packages used:

ggplot2 - for general plotting

dplyr - for data frame manipulations

ggpubr - for creating plots

rstatix - for outlier detection

car - for Anova function (analysis of deviance in this case)

emmeans - for pairwise comparisons

multcomp - for pairwise comparisons readout with letters

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Curriculum vitae

Nivitha Bhaskar

Education

The University of Western Ontario London, Ontario

Master of Science, *Biology (Cell and Molecular biology)* May 2021 – April 2023

Relevant coursework: Applied Bioinformatics, Analytical methods, and study design

SRM Institute of Science and Technology Chennai, Tamil Nadu

Bachelor of Technology, *Genetic Engineering* July 2016 – May 2020

Experience

The University of Western Ontario London, Ontario

Graduate Student Researcher May 2021 – Present

- Conducted statistical analysis on the collected data using R to enhance efficiency
- Assisted and designed protocols to establish world's first spider mite cell culture with technical expertise and attention to detail
- Tested different precursor molecules of RNAi and identified their efficiency at whole mite level

Teaching Assistant September 2022 – Present

- Explained and helped students master scientific concepts through one-on-one and small group tutoring
- Demonstrated biology laboratories for groups of up to 40 students by creating age-appropriate enrichment activities to drive intellectual development

Visiting Undergraduate Researcher January 2020 – May 2020

- Collaborated with postdoctoral researchers and conducted research on topic "RNA interference in Two Spotted Spider Mite", which played an important role in identifying lethal genes for model organism
- Presented experimental findings in presentations with graphs, charts and other visual aids

Scholarships and awards

- OE3C (AGA) travel award 2023
- Western Graduate Research Scholarship 2021 – 2023
- MITACS scholarship 2021 – 2022
- Graduated with gold medal for academic excellence 2020
- Performance-based scholarship 2016 - 2019