

ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Regulatory roles of three miRNAs on allergen mRNA expression in *Tyrophagus putrescentiae*

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Funding information

National Natural Sciences Foundation of China, Grant/Award Number: NSFC31572319, NSFC31272369 and NSFC81971511; 333 project of Jiangsu Province in 2017, Grant/Award Number: BRA2017216; Primary Research & Development Plan of Jiangsu Province; Project of Wuxi Health Commission, Grant/Award Number: MS201949

Abstract

Background: *Tyrophagus putrescentiae* is an important mite species in rural and urban environments, causing sensitization and allergic disease. While evidence suggests that microRNAs (miRNAs) may regulate the expression of allergen-encoding genes, no study has directly investigated this possibility. Here, this gap was addressed by profiling miRNAs and elucidating their target allergen messenger RNAs (mRNAs) in this mite species.

Methods: Small RNA and transcriptome libraries were constructed for eggs, larvae, nymphs, and adults. After deep miRNA and whole-transcriptome sequencing were performed, the miRNA and allergen-encoding mRNA regulatory networks were explored.

Results: A total of 540 miRNAs were identified, including 155 with expression levels differing significantly across the four mite developmental stages ($p < .01$), 59 of which were novel. The mRNA expression for allergens was higher for Tyr p 1 in adults than in other developmental stages; Tyr p 2–5, 7, 10, 13, 33, and 34 in immature stages; and Tyr p 28, 35, and 36 in eggs and adults. A combined miRNA and transcriptome bioinformatics analysis showed that allergen Tyr p 3 was regulated by miRNA PC-5p-5698441_1, Tyr p 4 was regulated by PC-5p-7050653_1, and Tyr p 34 was regulated by PC-5p-5534223_1 and PC-5p-5698441_1. These three allergen mRNA and three miRNAs were identified using qRT-PCR, and their regulatory roles were confirmed by double-fluorescent reporter gene system and site-directed mutagenesis technology.

Conclusions: For the first time, allergen mRNA expression and miRNAs were profiled throughout the life cycle for an allergen-producing mite, and the results showed that miRNAs bind to target allergen mRNAs to regulate their expression.

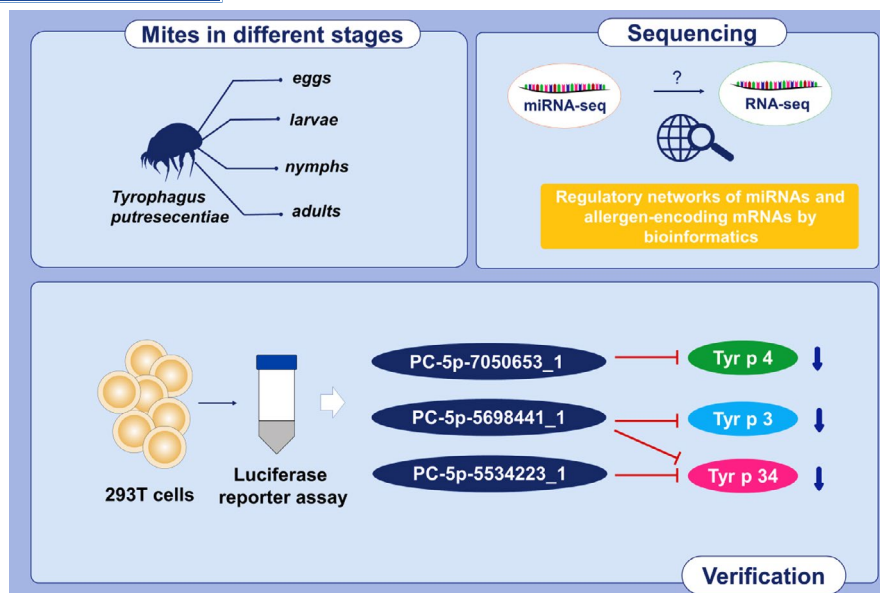
KEYWORDS

double-fluorescent reporter gene system, microRNA, qRT-PCR, transcriptome, *Tyrophagus putrescentiae*

Abbreviations: IUIS, International Union of Immunological Societies; KEGG, Kyoto Encyclopedia of Genes and Genomes; KOG, Eukaryotic Ortholog Groups; miRNA, microRNA; mRNA, messenger RNA; NR, non-redundant; OTUs, operational taxonomic units; Pfam, protein family; RLUC, Renilla luciferase; RPKM, reads per kilobase of exon model per million mapped reads; UTR, untranslated region.

Ying Zhou and Qingqing Li contributed equally to this article.

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GRAPHICAL ABSTRACT

We collected the eggs, larvae, nymphs, and adults of *Tyrophagus putrescentiae*. We verified mRNA (Tyr p 3, Tyr p 4, and Tyr p 34) and microRNA (PC-5p-5698441_1, PC-5p-7050653_1, PC-5p-5534223_1, and PC-5p-5698441_1) expression by RT-PCR. The luciferase reporter assay showed that Tyr p 3 was regulated by the miRNA PC-5p-5698441_1, Tyr p 4 was regulated by PC-5p-7050653_1, and Tyr p 34 was regulated by both PC-5p-5534223_1 and PC-5p-5698441_1.

1 | INTRODUCTION

The term “domestic mites” refers to mites inhabiting human dwellings, including house dust mites (HDM) and storage mites.¹ The ubiquitous and common mold mite, *Tyrophagus putrescentiae* (Schränk, 1781; family Acaridae) is one of the most important stored food pests, infesting a wide variety of products, such as grain, flour, peanuts, dried fruits, dried meats, and cheese. It is also the most clinically important allergen-producing storage mite species,^{2–4} causing allergic respiratory diseases and representing an occupational hazard for farmers, grain workers, and food industry workers.⁵ A survey on the prevalence of mite allergy in Taiwan showed increased sensitization with IgE responsiveness to *T. putrescentiae* extract (35.6%, 106/298), especially in elderly subjects (44.4%, 71/160), by ELISA.⁶ In Guangzhou (Southern China), the positive rates of skin prick test (SPT) and specific IgE (sIgE) to *T. putrescentiae* were 61.1% and 24.6%, respectively, in respiratory allergic individuals.⁷ A total of 39 (6.5%) of 600 serum samples selected from a 1-day submission to a clinical diagnostic laboratory in Southwest Ohio had serum IgE to the protein of *T. putrescentiae*, and 15 were sensitized to only *T. putrescentiae*.⁸ In northern Europe, the positive rate of SPT to *T. putrescentiae* was close to that of HDM (6% vs 8%), with 26% co-sensitization to HDM.⁹ These data suggested that this mite species plays a comparable role with HDM in allergen-induced IgE-mediated allergic diseases.

For this species, the International Union of Immunological Societies' (IUIS) Allergen Nomenclature Subcommittee lists 12 allergen groups: *T. putrescentiae* (Tyr p) 1–3, 7–8, 10, 13, 20, 28, and 34–36 (<http://www.allergen.org/>). The nucleotide sequences of Tyr p 4,

Tyr p 5, and Tyr p 33 are available in GenBank. In 106 sera from allergic subjects with ELISA-positive reactions to *T. putrescentiae*, the frequencies of IgE responsiveness to these recombinant allergens were as follows: Tyr p 1 (61.3%), Tyr p 2 (79.2%), Tyr p 3 (49.1%), Tyr p 7 (37.7%), Tyr p 8 (44.3%), Tyr p 10 (18.9%), and Tyr p 20 (48.1%). Therefore, the major IgE-binding components were Tyr p 1, Tyr p 2, and Tyr p 3.⁶ Novel allergens are actively being discovered in this species,¹⁰ and more allergens are likely to be identified, given that the two best-studied house dust mites (genus *Dermatophagoides*) have more than 30 allergen groups.^{11,12} However, the mechanisms underlying the regulation of the production of different mite allergens are not fully understood. While microRNA (miRNAs) have the potential to regulate the expression of allergen-encoding genes, this possibility has not yet been explored in any mites.

MiRNAs can regulate gene expression at the transcriptional and post-transcriptional levels by targeting sites within messenger RNAs (mRNAs). MiRNAs are also involved in almost every biological process, including cell differentiation, proliferation and apoptosis, embryogenesis, organogenesis, morphogenesis, physiological metabolism, environmental coordination, cognitive behavior, and immune defense.^{13,14} Interestingly, miRNAs can be transmitted from one species to another, inducing signal interference in distant species, even in cross-kingdom manner. This cross-species communication might mediate symbiotic and pathogenic relationships between organisms.¹⁵ The parasitic wasps *Cotesia vestalis* teratocytes express several miRNAs and its associated bracovirus (CvBV) encodes several miRNAs that are expressed in infected host, showing cross-species regulation by miRNAs in animal parasitism and their possible function in the alteration of host physiology during

parasitism.¹⁶ Therefore, identification of organisms' miRNAs and their cross-species regulation roles on host will be helpful to find modulator of human diseases.

MiRNAs have been identified in plant-feeding mites, including 594 known and 31 novel miRNAs in the developmental stages of the citrus red mite *Panonychus citri*.¹⁷ In the red spider mite *Tetranychus urticae*, the endosymbiotic bacterium *Wolbachia* regulates 91 miRNAs in females and 20 miRNAs in males, with an overall suppression of miRNAs in *Wolbachia*-infected mites.¹⁸ MiRNAs were differentially expressed in infected versus uninfected mites and regulated the expression of genes with various functions, including sphingolipid metabolism, lysosome function, apoptosis, and lipid transportation in both sexes and reproduction in females.¹⁸

Here, the potential regulatory activity of miRNAs and their target mRNAs in *T. putrescentiae* were investigated using deep miRNA and whole-transcriptome sequencing. Due to the clinical importance of this mite, this study focused particularly on allergen-encoding mRNAs and their related miRNAs. qRT-PCR was used to verify several mRNAs and miRNAs to validate the sequencing results, and their regulatory relationships were confirmed using luciferase reporter assay. The mite's eggs, larvae, nymphs, and adults were separately analyzed because endogenous allergen expression in mites is variable in different developmental stages.¹⁹ The present study provides a unique window on the role of miRNAs in allergen mRNA expression, which may be helpful to explore cross-species regulation by mite miRNAs and their possible function in allergic diseases caused by these allergen, leading to improved treatments for mite-induced allergic disorders.

2 | METHODS

2.1 | Mite culture and species verification by amplification sequence analysis

Tyrophagus putrescentiae was cultured in the laboratory for many generations in cell culture flasks (75 cm, Corning, USA) in an artificial climate incubator (RXZ-280D, Jiangnan Instrument Factory, Ningbo, China) under 25°C ± 1°C and 85% ± 5% relative humidity.

Live mites were collected manually, washed with twice with phosphate-buffered saline (PBS, Ph 7.4), and placed into RNA sample preservation solution (TaKaRa Bio., Inc., Code No. 9750). The total genomic DNA was extracted with an Omega kit (Omega E.Z.N.A. Soil DNA Kit D5625). DNA quality was evaluated by electrophoresis on an EtBr-stained 0.8% agarose gel and visualized under an ultraviolet transilluminator.

Tyrophagus putrescentiae was identified by morphological characteristics.²⁰ In brief, the genus *Tyrophagus* has a prodorsal shield with a groove present, and seta *vi* (the internal vertical seta) is less than three times long as seta *ve* (the external vertical seta) (Figure S1). The purity of mite samples was assessed by PCR amplification and sequencing. The 18S ribosomal DNA V4 hypervariable domain (18S rDNA V4) was amplified with universal

primer pairs (547F 5'-CCAGCASCYGC GGTAATTCC-3' and V4R 5'-ACTTTCGTTCTTGATY RA-3') by using Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB). Reactions were generally run for 30 cycles. Amplicons were detected by electrophoresis on a 2% agarose gel, excised, and purified for target gene sequencing with the AxyPrep DNA Gel Extraction Kit (Axygen). Illumina libraries were prepared with the Illumina TruSeq Nano DNA LT Library Prep Kit and sequenced on an Illumina MiSeq sequencing instrument by using the 2 × 300 bp protocol yielding paired-end reads, which overlap almost completely. The amplicon high throughput sequencing reads were clustered into operational taxonomic units (OTUs) by using a 97% cutoff in QIIME (<http://qiime.sourceforge.net/>), and representative sequences of OTUs were used to identify species.

2.2 | Transcriptome sequencing

Mite eggs were collected on the same day after being deposited by females, larvae on the sixth day after egg incubation, nymphs on the 10th day after starting larval culture, and adults on the 20th day after starting nymph culture.

The total RNA of eggs, larva, nymphs, and adults was extracted separately using a Qiagen RNeasy Mini Kit 74104. Quality evaluation of RNA extractions was performed on a Bioanalyzer 2100 (Agilent Technologies), and RNA concentration, 28S/18S ratio, and RNA integrity number were recorded (Figure S1). Transcriptome next-generation sequencing libraries were constructed with the SMART cDNA Library Construction Kit (CLONTECH Company, Code No. 634901). In brief, mRNA was enriched and purified with Oligo (dT) magnetic beads, and fragments were then randomly sheared. These mRNA fragments were used as a template to produce cDNA. The fragments were purified; sticky ends were restored to flat ends, A-bases were added to the 3' ends, and the joints were added; and PCR amplification was conducted. The library was submitted for quality control on an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR system and then sequenced on an Illumina HiSeq TM 2000 instrument, producing 18 million paired-end 2 × 100 bp reads. After quality filtering of raw reads was conducted, 99.81% (adults and eggs) or 99.94% (larvae and nymphs) of reads were retained. These reads were assembled de novo in Trinity.²¹ The assembled transcripts and unigenes were functionally annotated by sequence similarity using BLAST (*E*-value ≤ 1.0E-5) and public databases SWISS-PROT, the NCBI non-redundant (NR) Protein Sequence Database, the Kyoto Encyclopedia of Genes and Genomes (KEGG), EuKaryotic Ortholog Groups (KOG), and the Pfam protein family database.¹⁰ The expression levels of unigenes were calculated and compared for different mite stages. The expression abundance in different samples was computed by sequence similarity comparison with the assembled and annotated genes as the library. Short read mapping was conducted on bowtie 0.12.8²² in single-end mode. Expression levels were computed as reads per kilobase of exon model per million mapped reads (RPKM) values by using the following formula: RPKM = total exon reads/[mapped

TABLE 1 Oligonucleotide primers used in qPCR to quantify four allergen-encoding mRNAs of *Tyrophagus putrescentiae*

Gene name	Gene function	GenBank accession number	Oligonucleotide primers (5'-3')	Amplicon length (bp)
Tyr p 4	Alpha-amylase	DQ983318.1	F: GAACCGCTGGCTCTCACTA R: CGCATTGTCTGTGTTGGAT	1557
Tyr p 3	Trypsin	EU302595.1	F: TGCTAAGCCACCTACAATTCTAG R: CGAGATGATAGAACCCACACAA	858
Tyr p 34	Troponin C	FJ534553.1	F: GGCTACATCCACACCAACAT R: AGTTCACCGCTGCCATCA	462
aTub	Alpha-tubulin	KF964489	F: CACTCTGGAGCACTCTGACT R: AGACGGTTCAAGTTGGTGTATG	124

reads (millions) $\times$ exon length (kb)]. The assembled transcripts were searched for homology to allergen and miRNA sequences in the NCBI databases by BlastN (E -value $< 1E-5$).

2.3 | MiRNAs in different mite stages

For RNA library preparation and sequencing, the eggs, larvae, nymphs, and adults of *T. putrescentiae* were collected manually as detailed above. Total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA quantity and purity were analyzed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent), yielding a RIN > 7.0 . Approximately 1 μ g of total RNA was used to prepare a small RNA library by using a TruSeq Small RNA Sample Prep Kit (Illumina). Single-end short read sequencing (1×36 bp) was performed using an Illumina HiSeq 2500 instrument at LC-BIO. Raw reads were processed using an Illumina pipeline filter (Solexa 0.3) and ACGT101-miR v4.2 (LC Sciences) to remove adapter dimers, low-quality and low-complexity regions, common RNA families (rRNA, tRNA, snRNA, snoRNA), and repeats.

Unique sequences of 18–26 nucleotides in length were mapped to specific species precursors in miRBase 22.0 (<http://www.mirbase.org/>) by using a BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. The mapped sequences were identified as known miRNAs. The remaining sequences were mapped to other arms of known hairpin precursors of selected species with close kinship; they were defined as conserved miRNAs. The unmapped sequences were analyzed by mapping to the specific genomes (<https://www.ncbi.nlm.nih.gov/genome/?term=Dermatophagoides%20farinae>) to establish their genome locations. These were considered as candidate novel miRNAs when the flank 80-nt sequences (from genome locations) were successfully predicated to contain hairpin RNA structures by using RNAfold software (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>).^{23–26}

The expression levels of miRNA for each mite stage and sample were calculated. The miRNA differential expression levels (as represented by normalized deep-sequencing counts) were compared across samples/groups by chi-squared test, with a significance threshold of $p < .01$.

2.4 | Regulatory networks of microRNA and their potential targets in allergen-encoding mRNAs

MiRNA target genes were predicted from the transcriptome data by using three computational algorithms: TargetScan 5.1 (<http://www.targetscan.org>), miRanda (<http://www.microrna.org>), and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). By using the whole-transcriptome sequence data, miRNA targets were predicted in allergen-encoding transcripts. TargetScan was used to search for miRNA seed matches (2–8 nucleotides at the 5'-end of miRNAs). miRanda was used to match the entire miRNA sequences with the following parameters: free energy < -20 kcal mol⁻¹ and context score > 50 . Finally, the data predicted by the three algorithms were combined, and a consensus was calculated.

2.5 | Validation of results

2.5.1 | Verification of allergen-encoding mRNAs by qRT-PCR

cDNA was synthesized from the total RNA with the PrimeScript RT Reagent Kit and gDNA Erase (TaKaRa No. RR047A) by following the manufacturer's protocol and using RNase-free reagents/supplies. Each reaction had 2 μ l of 5 $\times$ g of DNA, 1 μ l of gDNA Eraser, 1 μ g of total RNA, and DEPC H₂O to make a total reaction volume of 10 μ l. The reaction mixtures were incubated in a 42°C hot water bath for 2 min and then placed on ice. Subsequently, 1 μ l of PrimeScript RT Enzyme Mix I, 1 μ l of RT Prime Mix, 4 μ l of 5 $\times$ PrimeScript Buffer 2 (for real time), and 4 μ l of DEPC H₂O were added, and the RT reaction product was then incubated in a 42°C water bath for 15 min, transferred to a 85°C water bath for 5 s, and then immediately placed on ice. The cDNA product was diluted by adding 80 μ l of DEPC H₂O and then stored at -80°C for future use as a template for qPCR.

Two-step real-time qPCR was used to quantify allergen-encoding mRNAs. Each qPCR reaction contained 10 μ l of SYBR Premix Ex Taq, 0.6 μ l of a forward primer, 0.6 μ l of a reverse primer (Table 1), 5 μ l of cDNA product, and 3.8 μ l of DEPC H₂O. qPCR was performed in a LightCycler96 Real-Time PCR System as follows: 95°C for 30 min; 40 cycles of 95°C for 5 min and 60°C for 34 min; and then 95°C for

10 min (1 cycle), 65°C for 1 h (1 cycle), and 97°C for 1 (81 cycles) minute for melting curve preparation.

2.5.2 | Real-time PCR detection of miRNAs

Total RNA was extracted from samples by using TRIzol following the manufacturer's protocol to generate cDNA for real-time PCR. Reverse transcription was conducted using the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia) in accordance with the manufacturer's protocol. Each reaction (total volume of 25 μ l) had 1 μ l of Poly A Polymerase (2.5 U/ μ l), 1 μ l of RTase Mix, 5 μ l of 5 $\times$ PAP/RT Buffer, 2.0 μ g of total RNA, and 18 μ l of RNase-free H₂O. The reaction cocktail was mixed, centrifuged, and incubated at 37°C in a water bath for 60 min and then at 85°C for 10 min. The cDNA from qRT-PCR was kept in -80°C for future use as template for qPCR.

Oligonucleotide primers (Table 2) were synthesized by Shanghai Sonny Biotechnology Co., Ltd. Each reaction mix (total volume of 20 μ l) included 10 μ l of 2 $\times$ All-in-One qPCR Mix, 2 μ l of All-in-One miRNA qPCR Primer (2 μ M), 2 μ l of Universal Adaptor PCR Primer (2 μ M), 2 μ l of the first-strand cDNA, and 4 μ l of DEPC H₂O. qPCR was conducted as follows: 95°C for 10 min (initial denaturation step); 40 cycles of 95°C for 10 s (denaturation), 60°C for 20 s (annealing), and 72°C for 30 s (extension); and 95°C for 10 s, 65°C for 1 min, and 97.0°C for 1 s (melting). The primers All-in-One miRNA qPCR Primer (2 mM) and Universal Adaptor PCR Primer (2 mM) were provided in the kit. The 18S ribosomal gene was used as internal reference in qPCR.

Experiments were performed in three technical replicates and three biological replicates. The relative levels of miRNAs were detected using the Ct method. For each locus, the Ct value was directly compared and transformed into a fold-change difference with the following formula: $\Delta Ct = (Ct_A - Ct_B) = X$, where Ct_A represents the target gene, and Ct_B represents the internal reference gene in the sample.²² The sample was verified, that is, the expression of the target gene was 2^{-X} the expression of the internal reference. The relative expression of a given miRNA across mites was calculated using the following formula: relative expression = $AVG(2^{-\Delta Ct}) \pm STDEV(2^{-\Delta Ct})$.²⁷

2.6 | Dual-luciferase reporter assay

HEK 293T cells were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured under standard conditions²⁸ in Dulbecco's modified Eagle's medium

(Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific) and a 1% penicillin/streptomycin solution (Sigma-Aldrich). The effect of miRNAs on three allergen-encoding mRNAs (PC-5P-5698441_1 on Tyr p 3, PC-5P-7050653_1 on Tyr p 4, and PC-5P-5534223_1 and PC-5p-5698441_1 on Tyr p 34) were validated via dual-luciferase reporter assay (E1910, Promega). The HEK 293T cells were seeded on a 96-well culture plate 24 h before transfection. They were subjected to transfection at 60%–80% confluency.

The 3'-untranslated regions (UTRs) of Tyr p 3 mRNA (containing a PC-5p-5698441_1 binding site), Tyr p 4 mRNA (containing a PC-5p-7050653_1 binding site), and Tyr p 34 mRNA (containing miRNA PC-5p-5534223_1 and PC-5p-5698441_1 binding sites) were PCR amplified and cloned into the *Mlu* I/*Hind* III site of the pMIR-REPORT Luciferase (H306; OBIO) to construct three wild-type plasmids: luc-Tyr p 3, luc-Tyr p 4, and luc-Tyr p 34. The 3' UTR of Tyr p 3 mRNA was mutated from AACACAAT to TTGTGTTA. The 3' UTR of Tyr p 4 mRNA was mutated from ATGAAAAA to TACTTTTT. The mutant constructs containing the 3' UTR of Tyr p 34 mRNA were mutated from GGCCAACA to CCGGTTGT, from AGCCAAC to TCGGTTGA, and from AACACAAACACACAAA to TTGTGTTTGTGTTT.

The cells were transfected with a luciferase construct containing Tyr p 3, Tyr p 4, and Tyr p 34, with the wild-type or a mutated version of the binding site, and co-transfected with PC-5p-5698441_1, PC-5P-7050653_1, or PC-5P-5534223_1 mimic or with empty vector to identify the binding sites of PC-5P-5698441_1 in Tyr p 3 (GenBank No. EU302595.1), PC-5P-7050653_1 in Tyr p 4 (GenBank No. DQ983318.1), and PC-5P-5534223_1 and PC-5p-5698441_1 in Tyr p 34 (GenBank No. FJ534553.1). Luciferase vectors were constructed by Obio Technology (Shanghai) Corp., Ltd. to determine the luciferase activities of Tyr p 3, Tyr p 4, and Tyr p 34. After 48 h of transfection, luciferase activity was measured using dual-luciferase reporter assay (Promega) on the microplate reader of a multi-wavelength measurement system (Tecan). The firefly luciferase (LUC) measures were divided by Renilla luciferase (RLUC) measures, and the normalized mean of LUC/RLUC was used to draw charts in GraphPad PRISM (version 5.0, GraphPad Software, Inc.).

2.7 | Nomenclature of Tyr p 24 and Tyr p 34

Tyr p 24 was originally named in 2010 and identified as troponin C.²⁹ However, this designation was later changed from Tyr p 24 to Tyr p

TABLE 2 Oligonucleotide primers used to quantify select miRNAs of *Tyrophagus putrescentiae* by qPCR

miRNA name	miR_seq	Primer sequence
PC-5p-7050653_1	TTTTTCATTGGCCACAAA	CGGCGTTTTTCATTGGCCACAAA
PC-5p-5698441_1	TTTGTGTTTGTGTTTGTGTTT	CCGCGTTTGTGTTTGTGTTTGTGTTT
PC-5p-5534223_1	TGTTGGCTTGAATGGATGCT	CGCTGTTGGCTTGAATGGATGCT
18S ribosomal gene (internal reference)		TGTGAGGATTGACAGGTTGAAGAGGCTAGAGTCTCGTTCGTTAT

34 to provide a consistent allergen numbering for troponin C across mites (<http://www.allergen.org>).

3 | RESULTS

3.1 | 18S V4 amplicon sequence analysis

18S V4 sequence data (Figure S1) were used to identify OTUs and their abundances. A total of three phyla, three classes, five orders, six families, and nine genera were found. The sample had high purity, being dominated by the phylum Arthropoda (96.78%) and largely represented by the genus *Tyrophagus* (96.77%) (Figure 1). The next most abundant phylum, Streptophyta (2.89%), was largely represented by the genus *Triticum* (2.68%) or wheat, which is a part of the mite's diet.

3.2 | mRNA expression in different mite stages

Separate transcriptome next-generation sequencing libraries were constructed from eggs, larvae, nymphs, and adults, and the data were deposited in GenBank (BioProject Accession No. PRJNA730378). After the reads were processed to remove 5' and 3' adaptor sequences, low-quality reads, and contaminants, 4.70 million (99.8%), 4.64 million (99.9%), 4.70 million (99.9%), and 4.06 million (99.8%) clean reads were obtained from eggs, larvae, nymphs, and adults, respectively. In total, 389,027 transcripts and 200,829 unigenes were obtained. Among them, 39,727 proteins were annotated using the SWISS-PROT database, 46,529 using the NCBI NR protein database, 41,390 using Pfam, 48,322 using KEGG, and 40,686 using KOG. The differential expression levels of mRNAs representing 12 allergen groups of *T. putrescentiae* (IUIS website) are shown in Figure 2. Tyr p 1 had greater expression levels in adults than in other stages; Tyr p 2, Tyr p 3, Tyr p 4, Tyr p 5, Tyr p 7, Tyr p 10, Tyr p 13, Tyr p 33, and Tyr p 34 had greater expression levels in immature stages (eggs, larvae, and nymphs) than in other stages; and Tyr p 28, Tyr p 35, and Tyr p 36 had greater expression levels in eggs and adults than in other stages.

3.3 | MiRNA expression in different mite stages

Four miRNA libraries were constructed and deep sequenced using the Illumina method and deposited at NCBI's Sequence Read Archive (BioProject ID: PRJNA730637). After the reads were processed to remove 5' and 3' adaptor sequences, low-quality reads, and contaminants, 10.34 million (70.02%), 7.44 million (80.89%), 8.52 million (83.29%), and 6.84 million (73.15%) clean reads were obtained from eggs, larvae, nymphs, and adults, respectively. In total, 540 miRNAs were identified from all four development stages, including 81, 71, 71, and 82 from the eggs, larvae, nymphs, and adults, respectively. Among them, 155 miRNAs showed significant differences in terms of expression levels between the

developmental stages ($p < .01$, Table 3), including 59 miRNAs that were identified as novel. The other 96 miRNAs were identified as being conserved with a number of Arthropod species, including *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Acyrtosiphon pisum*, *Bombyx mori*, *Drosophila melanogaster*, *Drosophila persimilis*, *Daphnia pulex*, *Drosophila simulans*, *Drosophila willistoni*, *Heliconius melpomene*, *Ixodes scapularis*, *Nasonia vitripennis*, *Tribolium castaneum*, and *Tetranychus urticae*. A total of 36 miRNAs were only present in eggs, two (PC-3p-2363168_2 and PC-5p-1613595_3) only in larva, and four (dwi-miR-6_R-2_1ss18TC, isc-miR-5312_R+1_1ss10GA, isc-miR-3931_R+2, and tur-miR-87-3p) only in adults; these may be stage-specific miRNAs (Table 3).²⁹

3.4 | Regulatory networks of miRNAs and allergen-encoding mRNAs

Based on the miRNA and transcriptome bioinformatics analysis, only three allergen mRNAs were regulated by miRNAs; Tyr p 3, Tyr p 4, and Tyr p 34 expression were regulated by 18, 14, and 36 miRNAs, respectively (Figure 3). Accordingly, the mRNA expression of Tyr p 3 was regulated by miRNA PC-5p-5698441_1, Tyr p 4 was regulated by PC-5p-7050653_1, and Tyr p 34 was regulated by PC-5p-5534223_1 and PC-5p-5698441_1.

3.5 | Verification of mRNA and microRNA expression by qRT-PCR

The expression levels of three allergen groups, Tyr p 3, Tyr p 4, and Tyr p 34, were quantified by qRT-PCR in three biological replicates and three technical replicates, with alpha-tubulin used as an internal reference (Figure 4).

Three miRNAs identified by TargetScan, miRanda, and PITA as being shared across these three allergen groups (PC-5p-5698441_1, PC-5p-7050653_1, and PC-5p-5698441_1) were then submitted for validation by qPCR in the same three replicates, with the 18S locus used as an internal reference (Figure 4). The melting curve for each category of amplicon is shown in Figure S2. All samples included a combination of eggs, larvae, nymphs, and adults because stage-specific validation of the miRNAs and mRNAs requires collecting a prohibitively large number of mites.

3.6 | Verification of microRNA regulating allergen-encoding mRNAs by luciferase reporter assay

A double-fluorescent reporter gene system and site-directed mutagenesis technology were used to explore the potential regulation of the three selected miRNAs of three major allergen-encoding mRNAs (Figure 4). The sequences and plasmids maps for mutant and wild-type versions of these three miRNAs are shown in Figure S3. The microscopic images of cells transfected with plasmids are shown in Figure S4.

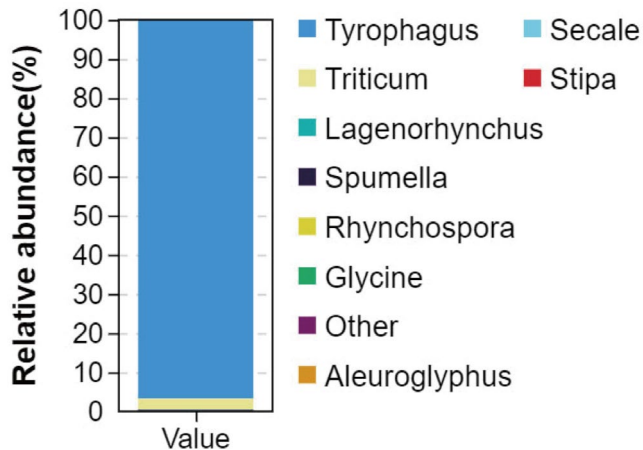


FIGURE 1 Genus-level composition of the mite culture sample based on 18S V4 amplicon sequence data

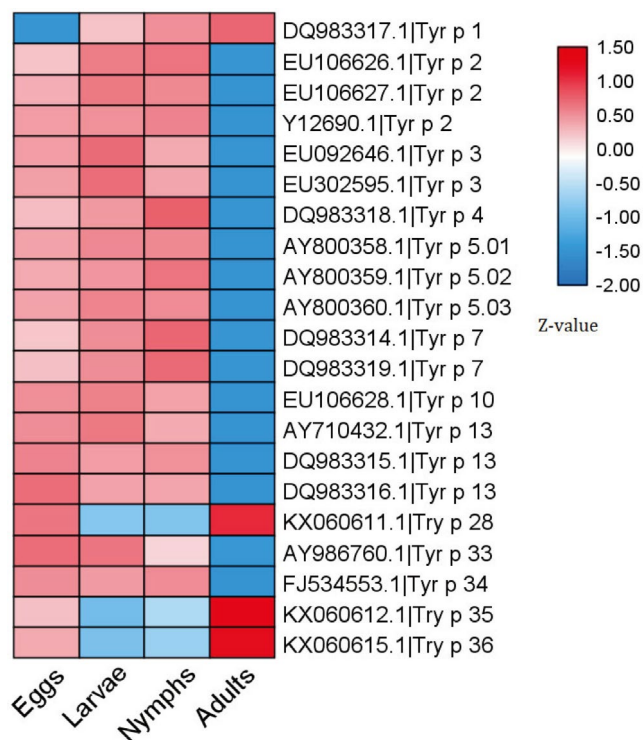


FIGURE 2 Differential expression of allergen-encoding mRNAs. RPKM values were estimated using Bowtie v.0.12.8 and then converted into z-values. Red represents upregulation and blue represents downregulation, compared with the mean value of a gene from all samples. Rows represent allergen-encoding mRNAs and columns represent developmental stages (eggs, larvae, nymphs, and adults). The expression values RPKM ($\log_{10}$ transformed) were scaled to the row mean. The color key relates the heatmap colors to the standard value (z-value), that is, the deviation from the row mean in units of standard deviations from the mean

The miRNA PC-5p-5698441_1 downregulated luciferase expression in Tyr p 3 by 43.22% ($p < .0001$). Mutating the 3' UTR binding sites increased luciferase expression by 15.53% ($p = .0001$, Figure 5A), suggesting that PC-5p-5698441_1 regulates luciferase expression by binding to this 3' UTR site.

The miRNA PC-5P-7050653_1 downregulated luciferase expression in Tyr p 4 by 23.68% ($p < .0001$, Figure 5B). Mutating the 3' UTR binding sites did not change luciferase expression ($p = .13$), suggesting that PC-5P-7050653_1 regulates Tyr p 4 mRNA expression while having affinity for several 3' UTR binding sites.

The miRNA PC-5P-5534223_1 downregulated luciferase expression in Tyr p 34 by 89.93% ($p < .0001$). Mutating the 3' UTR binding site increased luciferase expression by 22.45% ($p < .0001$, Figure 5C), suggesting that the miRNA binds to the 3' UTR as with PC-5P-5534223_1 and Tyr p 34 above.

The miRNA PC-5p-5698441_1 downregulated luciferase expression in Tyr p 34 by 43.73% ($p < .0001$). Mutating the 3' UTR binding sites increased luciferase expression by 18.31% ($p < .0001$, Figure 5D), indicating that PC-5p-5698441_1 regulates luciferase expression. Nevertheless, this miRNA also has a regulatory role on the mutant type 2 of Tyr p 34 mRNA. This observation could potentially be explained by (1) the presence of other binding sites outside of the mutated site and/or (2) the indirect effects of PC-5p-5698441_1 overexpression that leads to downregulated Tyr p 34 gene expression. Taken together, the results suggested that PC-5P-5534223_1 and PC-5p-5698441_1 regulate Tyr p 34 mRNA expression.

4 | DISCUSSION

The mold mite *T. putrescentiae* is a major source of allergens. However, the understanding of allergen production in this mite and other mite species is incomplete. Novel profiling of *T. putrescentiae* miRNAs revealed 155 differentially expressed miRNAs among four different developmental stages: eggs, larvae, nymphs, and adults. Some miRNAs were stage specific; for example, PC-3p-2363168_2 and PC-5p-1613595_3 were abundant in larvae; dwi-miR-6_R-2_1ss18TC, isc-miR-5312_R+1_1ss10GA, isc-miR-3931_R+2, and tur-miR-87-3p were abundant in adults; and 36 miRNAs were abundant in eggs. However, some abundant miRNAs were enriched in only two or three of the four developmental stages. These stage-specific miRNAs may be exploited in the future for molecular identification of mite developmental stage.

The transcriptomic data allowed the access of the expression levels of the 15 currently known allergen groups across the four developmental stages. Allergen Tyr p 1 had a greater expression level in adults than in other stages; Tyr p 2-5, 7, 10, 13, 33, 34 had higher expression in immature stages (eggs, larvae, and nymphs) than in other stages; and Tyr p 28, 35, and 36 had higher expression in eggs and adults than in larvae and nymphs. In recent years, accurate measurements of individual allergen concentrations have gained great importance in standardization and quality control in industrial allergen production.³⁰⁻³² For example, standardization is an important aspect for the major allergen Tyr p 2, which is found in sera from 80% of sensitized patients.³³⁻³⁶ Tyr p 3 also plays an important role in the pathogenesis of IgE-mediated allergic diseases caused by *T. putrescentiae*, being recognized by a high percentage (50%) of IgE-binding groups.³⁷ Thus, one of the main conclusions of the present study is that immature stages of mites should be also included as

TABLE 3 Differential expression of miRNAs in mite's eggs, larvae, nymphs, and adults ($p < .01$)

Index	miR_name ^b	TPM ^a			
		Egg	Larva	Nymph	Adult
1	aae-miR-305-5p_R+1_1ss1AT	343.54	309.91	120.27	43.73
2	aae-miR-276-3p_R+1	30.91	0.48	0.91	18.74
3	aae-miR-281-5p_R+4	11.49	0.00	0.00	0.42
4	aae-miR-92b-3p_1ss1AT	1.95	2.89	5.47	27.48
5	aae-miR-14	12.36	0.00	0.91	2.50
6	aga-miR-219_R+2	0.00	6.74	10.93	1.25
7	ame-miR-283_R+2	19.52	0.96	0.00	1.25
8	ame-miR-31a_L+1	4.55	0.00	0.91	0.00
9	ame-miR-316_R-3	3.25	0.00	0.00	0.00
10	api-miR-3050	4.55	0.00	0.00	0.00
11	bmo-miR-2b-3p_R+1_1ss18TG	2178.34	6.26	19.59	128.68
12	bmo-miR-6495-5p_L-4R-1_1ss5CA	96.95	0.00	0.00	14.99
13	bmo-miR-210_R+2	189.99	920.12	905.67	789.56
14	bmo-miR-275-3p_R-1	1.95	0.96	0.00	39.98
15	bmo-miR-6496-3p_L-4R-1	20.17	0.00	0.00	0.00
16	bmo-miR-281-5p_R+1	11.82	0.00	0.00	0.42
17	bmo-miR-2779_L-2_1ss14AC	27.33	5.77	2.73	18.74
18	bmo-miR-281-3p_L-2R+2	4.55	0.00	0.00	1.25
19	bmo-miR-1175-3p_R-2	2.93	0.00	0.00	0.00
20	bmo-miR-190-5p_R+3	21.47	28.87	7.29	7.50
21	bmo-miR-71-3p_L-1R-1_1ss20CG	51.73	48.60	28.70	38.10
22	bmo-miR-2766-3p_R+3	2.60	0.00	0.00	0.00
23	dme-miR-92a-3p_R-2_1ss1CT	1.63	5.77	7.29	27.48
24	dme-miR-993-5p_L-2R-1_1ss12TA	0.65	14.44	20.04	33.11
25	dpe-miR-133_R+2	0.65	34.65	30.07	14.99
26	dpu-miR-9-5p	1639.40	9795.97	11,085.69	11,795.63
27	dpu-miR-9-3p_R-1	134.03	1052.46	1199.05	1477.10
28	dpu-miR-96_R-1_1ss16AT	62.14	1734.36	1618.63	1897.07
29	dpu-miR-100_L-1R-1_1ss12CT	24.07	11,008.68	28,712.56	37,366.73
30	dpu-miR-252b_R+1_1ss22AG	251.80	1270.45	1373.99	1522.90
31	dpu-miR-263b_1ss11GA	0.00	4.81	11.84	13.74
32	dsi-miR-993-5p_L-3R-1_1ss13TA	0.00	14.44	20.04	31.86
33	dwi-miR-6_R-2_1ss18TC	0.00	0.00	0.00	8.75
34	hme-miR-263a_R+1	2143.86	3730.52	4089.17	3669.21
35	isc-miR-184_R+1	1.30	1909.53	1617.26	1008.19
36	isc-miR-263a_R+2	2857.61	13,770.47	16,926.13	13,143.32
37	isc-miR-279	22,537.21	19,586.17	14,655.13	13,691.14
38	isc-miR-315_1ss11TA	1944.11	959.58	713.42	484.73
39	isc-miR-96_R+1_1ss10AG	1151.31	35,555.39	30,013.20	26,214.81
40	isc-miR-279_1ss22AT	7598.83	5046.69	3160.72	3470.57
41	isc-miR-317_R-3_1ss19TA	3.90	817.13	622.30	1096.89
42	isc-miR-184_R+2	2.60	440.81	325.27	202.39

(Continues)

TABLE 3 (Continued)

Index	miR_name ^b	TPM ^a			
		Egg	Larva	Nymph	Adult
43	isc-miR-8	34.48	16.36	7.29	164.91
44	isc-miR-3931	0.00	0.00	0.91	73.71
45	isc-miR-315_L+1_1ss12TA	43.59	0.96	0.00	0.00
46	isc-miR-285	0.00	0.00	1.82	71.21
47	isc-miR-5309_1ss10TA	0.00	0.96	0.00	49.97
48	isc-let-7_R+1_1ss12GA	71.57	37.54	37.36	131.18
49	isc-miR-10_R-4	66.37	21.17	14.58	38.73
50	isc-miR-278_L+1R-2	0.00	102.98	79.27	126.18
51	isc-miR-5312_R+1_1ss10GA	0.00	0.00	0.00	24.99
52	isc-miR-750_R-2	7.16	0.00	0.00	0.00
53	isc-miR-305_L-1R+2	3.90	36.57	30.07	17.49
54	isc-miR-87_R+1	0.65	0.00	0.00	6.87
55	isc-miR-219_R+1	87.84	114.53	138.49	81.20
56	isc-miR-3931_R+2	0.00	0.00	0.00	5.00
57	nvi-miR-375_R-1	696.19	5417.72	4633.11	4382.56
58	PC-5p-7267_233	144.44	12.03	9.57	16.24
59	PC-3p-350_4057	6199.30	3108.76	3310.15	2264.99
60	PC-3p-39713_70	127.53	0.00	2.73	2.50
61	PC-3p-1050_1263	0.65	537.06	797.24	1151.86
62	PC-5p-14051_149	156.80	9.62	23.69	103.69
63	PC-3p-118984_25	55.96	0.00	0.00	0.00
64	PC-3p-13768_147	493.19	337.83	420.03	282.34
65	PC-5p-39897_69	46.85	0.00	0.00	0.00
66	PC-3p-82721_35	31.23	0.00	0.91	1.25
67	PC-3p-20227_110	64.09	21.66	25.06	13.12
68	PC-3p-184604_17	20.82	0.00	0.00	1.25
69	PC-3p-19054_118	282.38	269.97	323.91	197.39
70	PC-3p-124454_27	0.00	34.65	0.00	2.50
71	PC-5p-210005_15	15.62	0.00	0.00	0.00
72	PC-5p-180351_17	14.96	0.00	0.00	0.00
73	PC-5p-185191_17	14.31	0.00	0.00	0.00
74	PC-3p-17769_122	266.76	272.86	252.84	196.14
75	PC-3p-259160_12	12.36	0.00	0.00	0.00
76	PC-5p-249920_14	12.36	0.00	0.00	0.00
77	PC-3p-381437_9	11.71	0.00	0.00	0.00
78	PC-5p-19295_114	0.00	24.06	58.31	33.73
79	PC-5p-92268_33	0.65	5.77	6.38	29.98
80	PC-5p-872666_4	9.11	0.00	0.00	0.00
81	PC-5p-78509_36	0.00	7.70	9.11	31.23
82	PC-5p-5765_280	100.85	87.58	87.01	58.72
83	PC-3p-427230_10	9.76	0.00	0.00	1.25
84	PC-3p-527815_7	7.81	0.00	0.00	0.00
85	PC-3p-1353094_3	7.16	0.00	0.00	0.00
86	PC-3p-568651_9	0.00	2.89	3.64	18.74

(Continues)

TABLE 3 (Continued)

		TPM ^a			
Index	miR_name ^b	Egg	Larva	Nymph	Adult
87	PC-3p-298726_11	1.30	1.92	1.82	17.49
88	PC-5p-322783_10	7.81	0.00	0.00	1.25
89	PC-5p-20640_107	0.65	38.50	45.56	27.48
90	PC-3p-372302_9	5.86	0.00	0.00	0.00
91	PC-3p-674318_5	5.21	0.00	0.00	0.00
92	PC-3p-20892_106	217.31	274.30	345.32	247.36
93	PC-3p-270081_12	0.00	1.92	1.82	12.49
94	PC-3p-2363168_2	0.00	8.66	0.00	0.00
95	PC-3p-540603_6	4.55	0.00	0.00	0.00
96	PC-3p-150158_22	0.00	5.77	4.56	17.49
97	PC-3p-2552465_2	3.90	0.00	0.00	0.00
98	PC-5p-774361_5	3.90	0.00	0.00	0.00
99	PC-3p-945991_4	3.90	0.00	0.00	0.00
100	PC-5p-310651_11	5.86	0.00	0.91	1.25
101	PC-5p-223901_14	0.00	1.92	13.67	7.50
102	PC-3p-546579_6	0.00	10.59	4.56	0.00
103	PC-5p-2512763_2	3.25	0.00	0.00	0.00
104	PC-3p-8984562_1	3.25	0.00	0.00	0.00
105	PC-3p-225948_14	0.00	9.14	2.28	13.12
106	PC-5p-1613595_3	0.00	5.77	0.00	0.00
107	PC-5p-1187333_4	0.00	0.96	0.00	6.25
108	PC-3p-144234_23	0.00	19.25	15.49	19.99
109	PC-5p-1456851_3	2.60	0.00	0.00	0.00
110	PC-3p-980639_4	2.60	0.00	0.00	0.00
111	PC-3p-2547599_2	2.60	0.00	0.00	0.00
112	PC-5p-874316_4	2.60	0.00	0.00	0.00
113	PC-5p-2602083_2	2.60	0.00	0.00	0.00
114	PC-5p-4615102_1	2.60	0.00	0.00	0.00
115	PC-3p-9062_196	31.23	95.28	61.96	63.71
116	PC-3p-1367059_4	0.00	0.00	7.29	3.75
117	PC-3p-1729239_3	0.00	1.92	0.00	6.25
118	tca-miR-125-5p_R-1_1ss18TG	61.81	2344.57	2524.75	3755.41
119	tca-miR-750-3p_R-2	37.74	0.00	0.00	0.00
120	tca-miR-3869-3p_R-1	22.77	0.00	0.00	0.00
121	tca-miR-279c-3p_R-2	11.06	0.00	0.00	0.00
122	tca-miR-1175-3p_R-1	6.83	0.00	0.00	0.00
123	tca-miR-124-5p_L-1R+1_1ss20TA	0.00	40.42	27.33	37.48
124	tca-miR-190-5p_R+5	3.25	21.17	5.47	2.50
125	tca-miR-3869-5p_L-2_1ss24CT	2.60	0.00	0.00	0.00
126	tur-miR-1-3p_1ss3GT	3642.29	797.88	908.40	948.22
127	tur-miR-2-3p_R-2_1ss18AG	146.39	0.96	5.47	33.73
128	tur-miR-71-5p_R-1	2577.19	20,172.31	11,329.04	10,810.24
129	tur-miR-92-3p	95,940.83	10,179.99	10,163.70	8881.31
130	tur-miR-124-3p	616.16	2820.99	2723.83	2358.69

(Continues)

TABLE 3 (Continued)

Index	miR_name ^b	TPM ^a			
		Egg	Larva	Nymph	Adult
131	tur-miR-133-3p	3704.75	23,437.95	26,580.50	23,509.43
132	tur-miR-184-3p	13.66	23,597.72	24,558.70	20,093.83
133	tur-miR-190-5p	8989.25	32,906.68	20,625.33	18,433.50
134	tur-miR-252-5p_R-1	0.00	773.82	745.31	717.10
135	tur-miR-263b-5p_R-1_1ss11GA	634.70	7195.39	10,784.63	12,973.41
136	tur-miR-276-3p_1ss12TA	1.30	7.70	1.82	258.61
137	tur-miR-278-3p_R+1	2375.49	24,955.76	20,916.90	33,706.27
138	tur-miR-305-5p_R+1	985.72	2472.57	1612.71	1179.34
139	tur-miR-317-3p_R-3	491.88	163.62	118.45	514.71
140	tur-miR-745-3p_R+1_1ss1CA	6613.76	2101.06	1450.52	907.00
141	tur-miR-993a-3p	884.22	669.72	554.88	571.14
142	tur-miR-993b-3p_R-2_1ss10TC	2192.66	39.30	41.46	118.27
143	tur-miR-5735-3p	9920.96	44,761.37	35,020.34	23,793.03
144	tur-miR-34-5p_R-2_1ss18GT	6.51	157.84	93.85	273.60
145	tur-miR-184-5p_1ss13CA	0.00	212.70	115.71	46.22
146	tur-miR-307-3p_R-2	101.50	635.23	497.48	388.53
147	tur-miR-137-3p_R+1	52.05	456.21	402.72	311.08
148	tur-miR-12a-5p_R+3	49.45	1.92	2.73	49.97
149	tur-miR-10-5p_L-1	32.53	0.96	3.64	3.75
150	tur-miR-279-3p	15.62	179.02	137.58	162.41
151	tur-miR-7-5p_R-3_1ss21TG	72.22	34.65	25.51	44.98
152	tur-miR-87-3p	0.00	0.00	0.00	16.24
153	tur-miR-263a-5p_L-1R+2	37.41	95.28	94.30	144.29
154	tur-miR-71-3p_1ss20GA	18.87	15.40	5.47	3.75
155	tur-miR-190-3p_R-1_1ss20CA	15.62	1.92	5.47	14.99

^aThe miRNA read counts were normalized as transcript per million (TPM) based on the following formula: Normalized expression = mapped read count/Total reads $\times 10^6$.

^bThe miRNAs beginning with PC were annotated as predicted candidates, representing novel miRNAs. The others were species-specific or identified as conserved miRNAs. The miRNAs beginning with aae- (*Aedes aegypti*), aga- (*Anopheles gambiae*), ame- (*Apis mellifera*), api- (*Acyrtosiphon pisum*), bmo- (*Bombyx mori*), dme- (*Drosophila melanogaster*), dpe- (*Drosophila persimilis*), dpu- (*Daphnia pulex*), dsi- (*Drosophila simulans*), dwi- (*Drosophila willistoni*), hme- (*Heliconius melpomene*), isc- (*Ixodes scapularis*), nvi- (*Nasonia vitripennis*), tca- (*Tribolium castaneum*), tur- (*Tetranychus urticae*), were species-specific or identified as conserved miRNAs. L-n means the detected miRNA is n base less than the referenced miRNA (from miRBase) in the left side; R-n means the detected miRNA is n base less than the referenced miRNA (from miRBase) in the right side; L+n means the detected miRNA is n base more than the referenced miRNA (from miRBase) in the left side; R+n means the detected miRNA is n base more than the referenced miRNA (from miRBase) in the right side; 2ss5TC13TA means 2 substitution (ss), which are T > C at position 5 and T > A at position 13.

sources of allergen extract production. In addition, the allergenicity of recombinant Tyr p 4 and Tyr p 34 have been determined by skin prick test and/or ELISA.^{29,38}

MiRNAs and transcriptomic data were compared to elucidate the functional regulation of miRNAs on allergen-encoding target mRNAs. The primary function of miRNAs is the regulation of gene expression at a post-transcriptional level.³⁹ In particular, the binding of miRNAs to complementary regions (either partial or complete) of the target gene mRNA may lead to inhibition of gene expression by degradation of the mRNA or suppression of translation. However, the interaction of miRNA with target mRNA may not always result in the suppression of gene expression, instead in certain instances, may nevertheless lead to induction of gene expression.⁴⁰ Based on the

three computational algorithms (TargetScan, miRanda, and PITA), the mRNA expression of Tyr p 3, Tyr p 4, and Tyr p 34 was regulated by 18, 14, and 36 miRNAs, respectively. The other allergen groups were predicted by fewer miRNAs, which may be due to the limited tools currently available for microRNA genomics. Considering the bioinformatics analysis results of the regulatory networks of miRNAs and allergen-encoding mRNAs and the pattern of miRNA seed binding to mRNA 3' UTR (7–8 mers), the associations between miRNAs (PC-5p-5698441_1, PC-5p-7050653_1, and PC-5p-5698441_1) and the mRNAs of three allergens (Tyr p 3, Tyr p 4, and Tyr p 34) were selected for verification test in this study.

Unfortunately, the attempts to establish cell cultures of *T. putrescentiae* did not yield sustainable cultures; thus, the miRNA

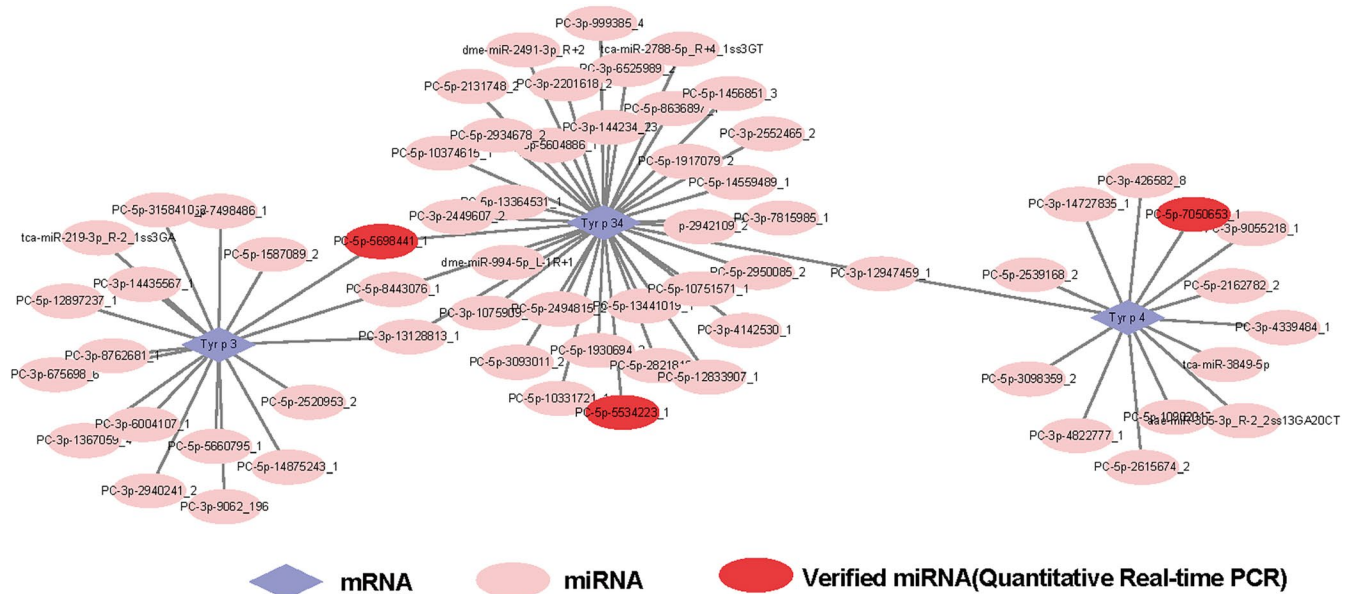


FIGURE 3 Regulatory networks of miRNAs and allergen mRNAs of Tyr p 3, Tyr p 4, and Tyr p 34. Connections suggest that microRNAs target allergen-coding genes

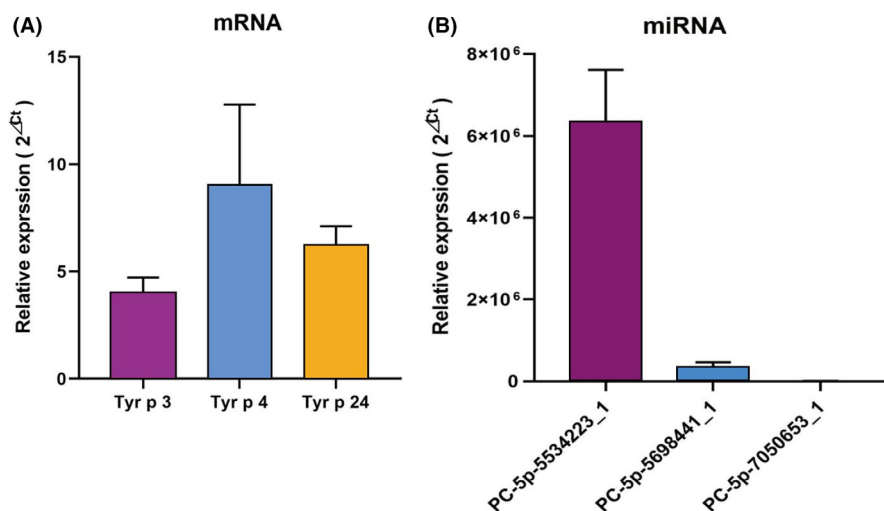


FIGURE 4 Expression levels of selected mRNAs (A) and microRNAs (B) in *Tyrophagus putrescentiae* by qRT-PCR. Experiments were performed in three technical replicates and three biological replicates. The relative levels of mRNA and miRNAs were detected using the Ct method (see text for details)

overexpression or silencing experiments could not be performed to further verify the results. Instead, 293T cells were used to verify the regulatory effects of mite miRNAs on allergen mRNA expression in a dual-luciferase reporter system,^{41–44} specifically miRNAs PC-5p-5698441_1 on Tyr p 3, PC-5p-7050653_1 on Tyr p 4, and PC-5p-5534223_1 and PC-5p-5698441_1 on Tyr p 34. The experiments confirmed the downregulating effects of these miRNAs on the expression of respective target mRNAs, indicating that they could be used for post-transcriptional regulation of major allergen-encoding genes in *T. putrescentiae*. To the authors' knowledge, this is the first study to explore the potential of miRNAs to regulate allergen expression levels by affecting target sites in allergen-encoding mRNAs in a mite. In recent years, antisense microRNA has been demonstrated in therapy of airway remodeling in house dust mite-sensitized mice,^{45,46} and heterologous miRNAs like plant-derived miRNAs have been suggested in therapy of human diseases.^{47,48} It is rational to identify specific mimics or inhibitors, or natural modulators of these

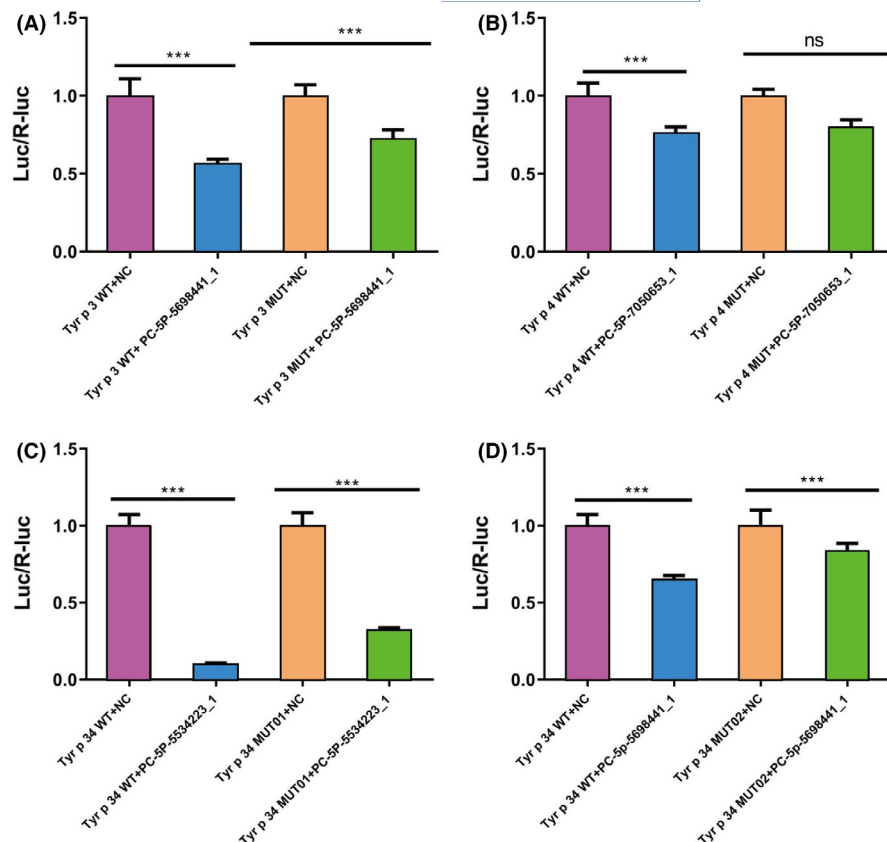
miRNAs, which target sites in allergen-encoding mRNAs, to develop strategies for the treatment of allergic disease caused by domestic mites.⁴⁹

In summary, miRNAs were profiled and their regulatory roles on allergen gene expression were explored for the first time in an allergy-inducing mite. Out of 12 known allergens of the mite *T. putrescentiae*, three (Tyr p 3, 4, and 34) were identified to be regulated by three miRNAs (PC-5p-5698441_1, PC-5p-7050653_1, and PC-5p-5534223_1).

ACKNOWLEDGMENTS

We thank Mrs. Zhenzhen Cai (HappyBio Co. LTD, Hangzhou, China) for assisting in bioinformatics analyses, and Dr. Pavel B. Klimov (University of Michigan, Department of Ecology and Evolutionary Biology, Museum of Zoology) for English language editing. This study was supported by the National Natural Sciences Foundation of China (Grant Nos. NSFC31572319, NSFC31272369, and NSFC81971511),

FIGURE 5 Validation of regulatory roles of three miRNAs in the expression of three major allergen-encoding mRNAs by luciferase reporter assay: PC-5p-5698441_1 on Tyr p 3 mRNA (A), PC-5p-7050653_1 on Tyr p 4 mRNAs (B), and PC-5p-5534223_1 and PC-5p-5698441_1 on Tyr p 34 mRNAs (C and D, respectively). Tyr p 3 wild-type (WT), Tyr p 4 WT, Tyr p 34 WT, Tyr p 3 MUT, Tyr p 4 MUT, Tyr p 34 MUT01, and Tyr p 34 MUT02 represent plasmids pMIR-REPORT-Tyr p 3 3' UTR-WT, pMIR-REPORT-Tyr p 4 3' UTR-WT, pMIR-REPORT-Tyr p 34 3' UTR-WT, pMIR-REPORT-Tyr p 3 3' UTR-MT, pMIR-REPORT-Tyr p 4 3' UTR-MT, pMIR-REPORT-Tyr p 34 3' UTR-MUT01, and pMIR-REPORT-Tyr p 34 3' UTR-MUT02, respectively. ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



the 333 project of Jiangsu Province in 2017 (BRA2017216), the Primary Research & Development Plan of Jiangsu Province (Grant No. BE2018627), and the Project of Wuxi Health Commission (Grant No. MS201949).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Y.C. designed the experiments. Y.Z., Q.L., R.P., Q.W., X.Z., C.Y., and F.C. conducted the experiments. Y.Z. and Y.C. analyzed the data. Y.G. and Y.C. drafted and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Zhou Y, Li Q, Pan R, et al. Regulatory roles of three miRNAs on allergen mRNA expression in *Tyrophagus putrescentiae*. *Allergy*. 2021;00:1-14. doi:[10.1111/all.15111](https://doi.org/10.1111/all.15111)

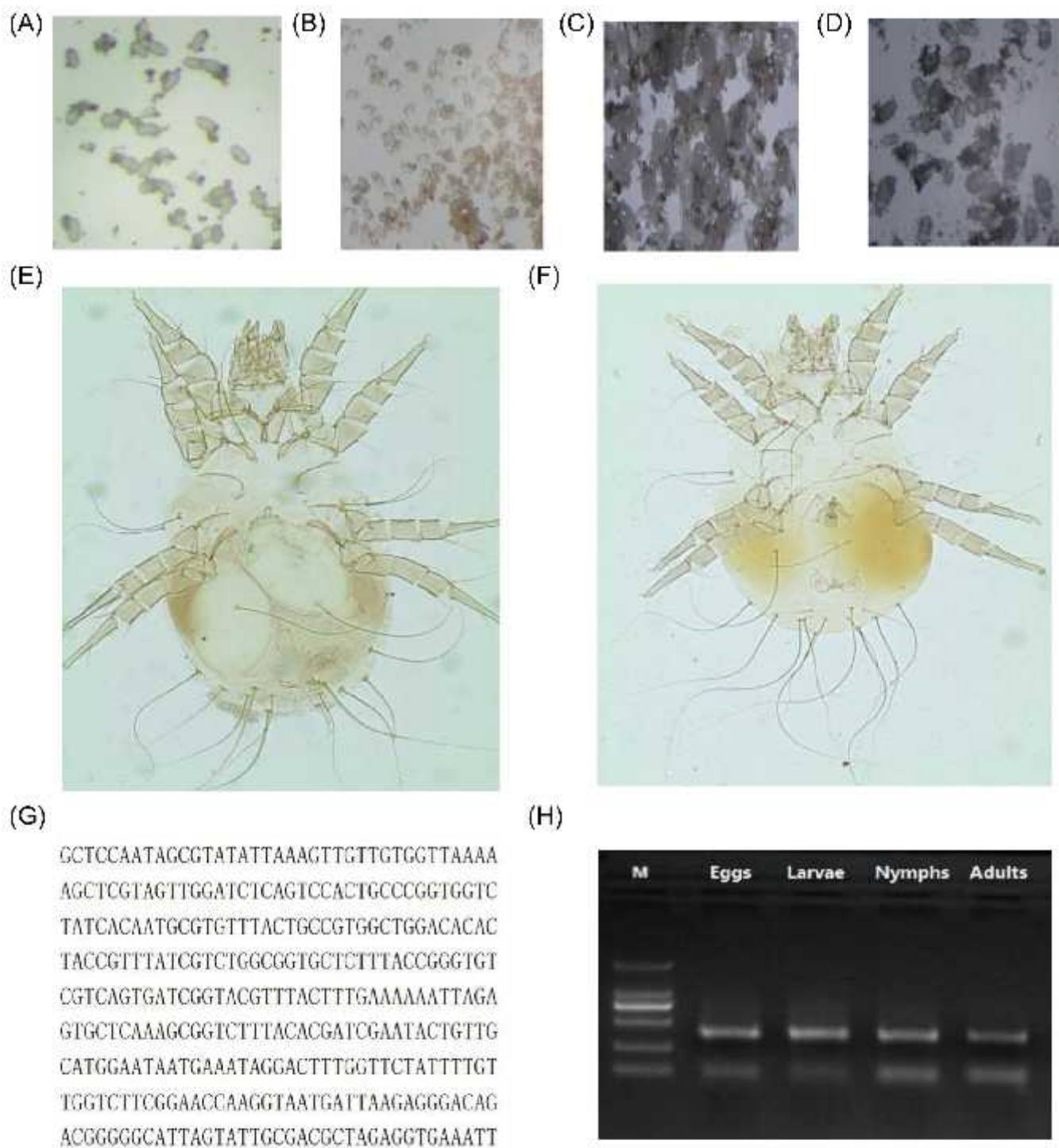


Figure S1_Ying Zhou et al.

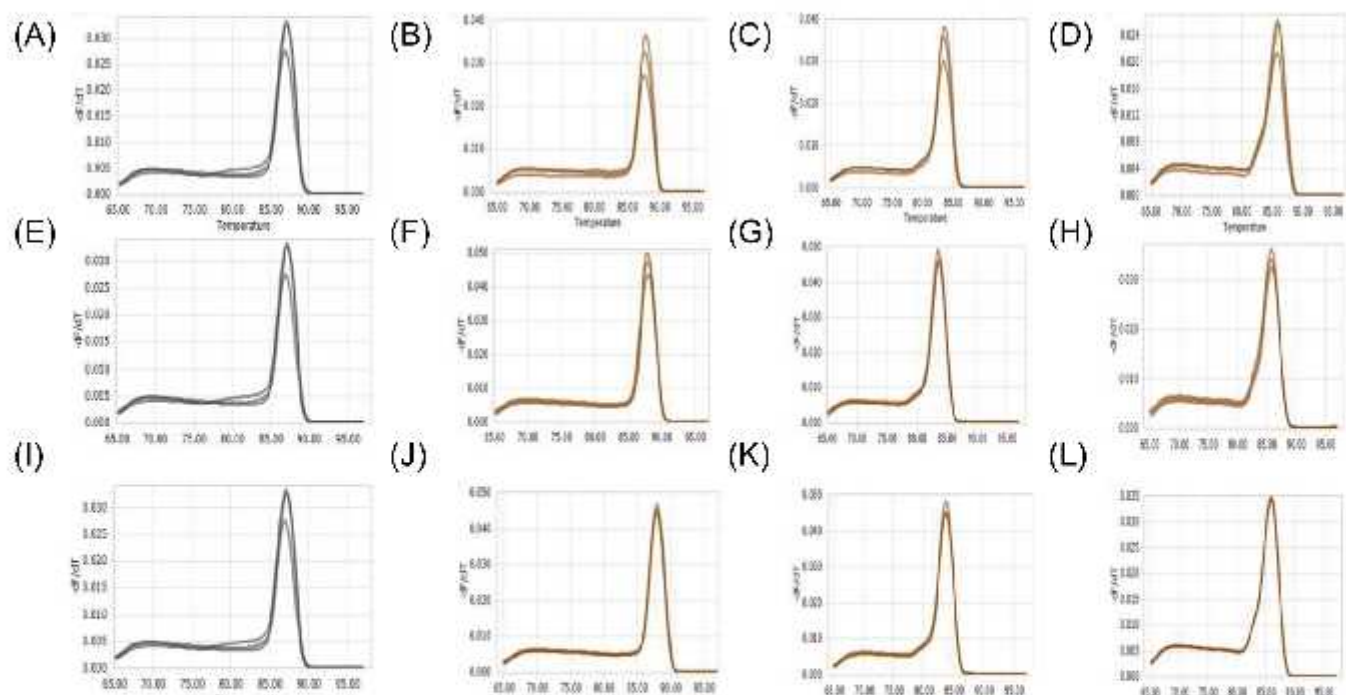


Figure S2A_Ying Zhou et al.

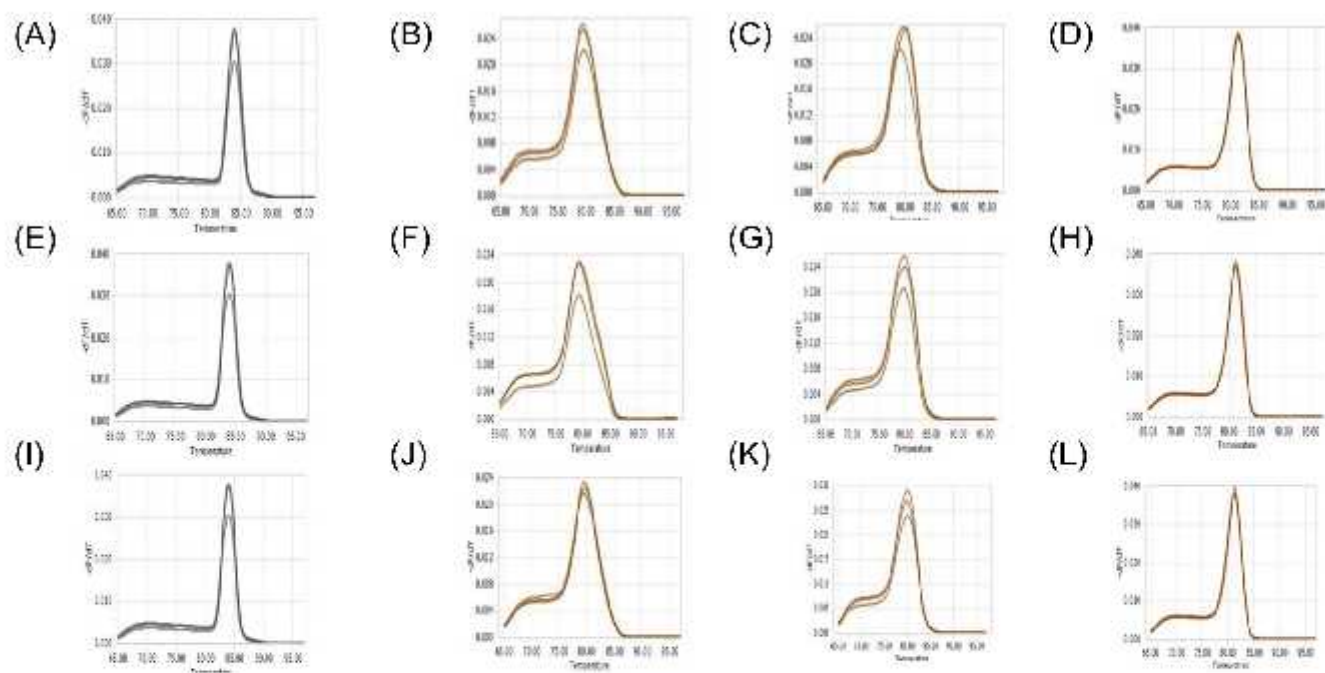


Figure S2B_Ying Zhou et al.

(A) **Tyr p 3** 3'UTR 137 bp

5'....TTGTACCTTTAAAAACAATGTCAAAATTATTTATTCCTGACT
CCTGAAAAATTATGTATTTTATAGACCACAGTTTATGTTTAAACA
ACAAGTGATACA**AACACAA**TTAAAAATACTTAAAAAAA
AAAAA...3'

PC-5p-5698441_1 3'...TTTGTGTTTGTGTTTGTGTTT...5'

WT 5'....AACACAA...3'

MT 5'....TTGTGTTA...3'

(B) **Tyr p 4** 3'UTR 144 bp

5'....CTTGCTTTTCTAATATAAAGGAAAAATACTGCAGTAATGTG
TCGTTTATATAACACAATGGGTAAATGTACCAAACTCAAATTT
ATAAACTAAATTTTAAATTTGTTTTAAAT**ATGAAAA**AAAAA
AAAAA...3'

PC-5p-7050653_1 3'...AAACACCGGTTTACTTTTT...5'

WT 5'....ATGAAAA...3'

MT 5'....TACTTTT...3'

(C) **Tyr p 34** 3'UTR 302 bp

5'...ACTGCTTACCTGAGCTGCTTTTCCCACACTGACT**GGCC**
AACATAAACACACAGCAAAATGGTAAAAACGGTGCCGAAAAAAC
TTGGCTGAATAATCAACCAACCATCCAAAGAAGTCAACCAAGCAA
GCACACACCAACACAAACACACAACTCCAGCCAATAACTCTCTG
TTACACTCTGATGATGTTGTTA**CTTACCA**ACCTGCTTACC**AGCCCA**
ACTACTCCTATTGGTACTATTTGGTTATTGAGATCATAAATTTTA
ATTAAAGCCAGACCACATCCCTGAAAAA...3'

PC-5p-5534223_1 3'...TCGTAGGTAAAGTTCGGTTGA...5'

WT01 5'....GGCCAACA...AGCCAAC...3'

MT01 5'....CCGGTTGT...TCGGTGA...3'

(D) **Tyr p 24** 3'UTR 302 bp

5'...ACTGCTTACCTGAGCTGCTTTTCCCACACTGACTGGCC
AACATAAACACACAGCAAAATGGTAAAAACGGTGCCGAAAAAAC
TTGGCTGAATAATCAACCAACCATCCAAAGAAGTCAACCAAGCAA
GCACACACC**AACACAA**ACACACAACTCCAGCCAATAACTCTCTG
TTACACTCTGATGATGTTGTTA**CTTACCA**ACCTGCTTACCAGCCA
ACTACTCCTATTGCTACTATTTCGTTATTGAGATCATAAATTTTA
ATTAAAGCCAGACCACATCCCTGAAAAA...3'

PC-5p-5698441_1 3'...TTTGTGTTTGTGTTTGTGTTT...5'

WT02 5'....AACACAAACACACAAA...3'

MT02 5'....TTGTGTTTGTGTTT...3'



Figure S4_Ying Zhou et al.

Supplementary Figures

Figure S1. Assessment of purity of *T. putrescentiae* samples. (A) Eggs; (B) larva; (C) nymphs; (D) adults; (E) females; (F) males; (G) 18S V4 sequence; and (H) gel electrophoresis images of total RNA of eggs, larvae, nymphs, and adults.

Figure S2. Melting curve of qRT-PCR for allergen-encoding mRNA (A) and the miRNAs targeting them (B).

Figure S3. Mature wild-type and mutated sequences of the three miRNAs.

Figure S4. Microscopic images of cells transfected with plasmids. **Note:** (A) Tyr p 3 MUT NC; (B) Tyr p 3 MUT+PC-5P-5698441_1; (C) Tyr p 3 WT NC; (D) Tyr p 3 WT+PC-5P-5698441_1; (E) Tyr p 4 MUT NC; (F) Tyr p 4 MUT + PC-5P-7050653_1; (G) Tyr p 4 WT NC; (H) Tyr p 4 WT+PC-5P-7050653_1; (I) Tyr p 34 MUT01 + NC; (J) Tyr p 34 MUT01 + PC-5P-5534223_1; (K) Tyr p 34 MUT02 + NC; (L) Tyr p 34 MUT02+PC-5p-5698441_1; (M) Tyr p 34 WT + NC; (N) Tyr p 34 WT + PC-5P-5534223_1; and (O) Tyr p 34 WT + PC-5p-5698441_1.