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Insight into the mechanism of action of scoparone inhibiting egg development of *Tetranychus cinnabarinus* Boisduval

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ABSTRACT

Investigating the mechanisms of action of natural bioactive products against pests is a vital strategy to develop novel promising biopesticides. Scoparone, isolated from *Artemisia capillaris*, exhibited potent oviposition inhibition activity against *Tetranychus cinnabarinus* (a crop-threatening mite pests with strong fecundity). To explore the underlying mechanism, the vitellogenin (Vg) protein content, and Vg gene expression of mites from three consecutive generations of G0 individuals exposed to scoparone were determined, revealing marked inhibition. This study is the first to explore the egg development defect behaviour of mite pests induced by scoparone. The egg-laying inhibition of mites by scoparone was significantly increased by 47.43% compared with that of the control when *TcVg* was silenced by RNA interference (RNAi), suggesting that egg-development inhibition of female *T. cinnabarinus* by scoparone was mediated by low Vg gene expression. Furthermore, scoparone bound to the Vg protein *in vitro*, and its K_d value was 218.9 μM, implying its potential function in inhibiting the egg development of mites by directly targeting the Vg protein. This study will lay the foundation for the future applications of scoparone as an agrochemical for controlling the strong egg-laying capacity mite pests in agriculture.

1. Introduction

The carmine spider mite *Tetranychus cinnabarinus* Boisduval is a typical arthropod mite pest that is found globally in agriculture and feeds on more than 100 types of crops (*e.g.*, corn, wheat, sesame, cotton, beans, tomato, and citrus) (Feng et al., 2018; Zhou et al., 2021). These mites display strong fertility capacity, a small size, a short growth stage, and high resistance to pesticides, making them exceedingly difficult to prevent and control (Chen et al., 2020). To date, synthetic acaricides or insecticides, such as avermectin, pyridaben, etoxazole and spiromesifen, remain the primary means to control *T. cinnabarinus* (Dermauw et al., 2020; Sugimoto et al., 2020). However, the increasing and continued application of chemical acaricides has led to the development of resistance in mite populations, stimulation of egg-laying, and a negative impact on the sustainable development of human health and the environment (Fotoukkiaii et al., 2019; Gould et al., 2018; Mermans et al., 2017). Thus, environmentally friendly acaricidal compounds with novel modes of action should be developed to tackle these issues in agriculture (Li et al., 2019; Ma et al., 2019; Teong et al., 2014; Xu et al., 2019).

Botanical bioresources originating from plant secondary metabolites are “treasuries” of nature and have provided invaluable inspiration for discovering and developing novel acaricides (Gao et al., 2015; Ma et al., 2020; Xu et al., 2020). *Artemisia capillaris* (Yin-Chen), a herbal medicinal plant, is a naturally sustainable plant resource widely distributed in Japan, Korea, Europe, and northern China and belongs to the genus *Compositae* (Koo et al., 2002). The extracts of this plant are a traditional Chinese herbal medicine and exhibit a broad spectrum of pharmacological effects, such as insecticidal (Liu et al., 2010), anti-tumour (Kim et al., 2013), choleretic, liver-protecting, anti-asthmatic (Fang et al., 2016), anti-allergy (Choi and Yan, 2009), and anti-inflammatory (Jang et al., 2005) effects. Previous phytochemical studies on *A. capillaris* identified a naturally occurring bioactive component called scoparone (Zhao et al., 2014).

Scoparone (1, Fig. S1), a well-known phenolic coumarin, is the principal component isolated from *A. capillaris* (containing 4.06 mg. g^{-1} in its buds) (Kim et al., 2013). Recently, we explored the acaricidal activity of a host of natural coumarin derivatives against *T. cinnabarinus* (Fig. S2) (Hou et al., 2017; Luo et al., 2018) and found, for the first time,

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that scoparone exhibited more potent acaricidal activity than a commercial acaricide, spirodiclofen, in greenhouse and field trials (Tables S1–S2; Fig. S1) (Hou et al., 2017). Most importantly, scoparone showed significant oviposition inhibition activity against *T. cinnabarinus* (Fig. 1). It is noteworthy that *T. cinnabarinus* displays strong egg-laying capacity, and each female adult produces an average of 100 eggs (Liu et al., 2016). Traditionally, numerous strategies are employed to control the mite pest population; however, using acaricides to inhibit egg-laying is an important approach (Ali et al., 2017). For example, Mansour et al. reported that 14 essential oils of Lamiaceae possessed a potent control effect on *T. cinnabarinus* by reducing the egg-laying of female adults (Mansour et al., 1986). Similar results have been reported in other pests, including *Apolygus lucorum* and *Nilaparvata lugens*, where sulfoxide exhibits an adverse effect on its transgenerational egg-laying (Liu et al., 2012; Zhen et al., 2017). These negative effects on the egg-laying capacity of females have also been reported in *Hippodamia variegate* (Rahmani and Bandani, 2013), *A. lucorum* (Tan et al., 2012), *Bradysia odouriphaga* (Zhang et al., 2014), and *Brevicoryne brassicae* (Lashkari et al., 2007) following exposure to thiamethoxam and imidacloprid. Additionally, the key to preventing and controlling agricultural pest mites is to effectively reduce their egg-laying or destroy the development of eggs (Ali et al., 2017; Attia et al., 2013; Kawakami et al., 2009). Thus, if the number of pest mites can be greatly decreased by reducing their egg-laying, the damage to crops will be reduced.

Additionally, we previously studied the transcriptome response of *T. cinnabarinus* to scoparone by RNA sequencing (RNA-Seq). Interestingly, a gene expressing vitellogenin (Vg), which may mediate the inhibition of egg-laying of female *T. cinnabarinus* by scoparone, was significantly downregulated by 2.94-fold (Zhou et al., 2019). Vg is a precursor of egg yolk protein, and plays a vital role in the egg development process of insects and numerous other oviparous species (Asad et al., 2020; Lin et al., 2020; Roy-Zokan et al., 2015). This protein is mainly synthesized by fat bodies in insects outside the ovary in a female-, tissue-, and stage-specific fashion, secreted into the haemolymph, and then absorbed by oocytes through endocytosis mediated by receptors (Ihara et al., 2015; Sappington and Raikhel, 1998; Tufail and Takeda, 2008). Vg molecules are rapidly cleaved by proteases and synergistically and post-translationally modified after synthesis in the fat body (Sappington and Raikhel, 1998). This post-transcription process allows Vg molecules to carry carbs, lipids and other nutrients into the ovary (Ihara et al., 2015). Vg is eventually stored in the form of vitellin (Vn) and becomes a component of the egg yolk, which is the main nutrient reserve for embryonic development (Tufail et al., 2014). Notably, evidence has shown that the Vg gene mediates the egg development behaviour of pests and could be regarded as a novel target of pesticides for pest control in crop protection. For example, silencing the Vg gene expression in *Panonychus citri* by RNAi significantly inhibited its oviposition and population (Ali et al., 2017), and the inhibition of egg development in *Chilo suppressalis* by chlorantraniliprole was mediated by downregulating the Vg gene (Huang et al., 2016). Thus, we intended to confirm whether a potential link exists between the egg development-related gene mediated by Vg and inhibition of the egg-laying capacity of female *T. cinnabarinus* treated with scoparone.

An in-depth investigation of the molecular mechanisms of natural product-based acaricidal candidates inhibiting the egg-laying of mites may help develop a novel strategy for controlling the *T. cinnabarinus* population by decreasing its fecundity. Here, we demonstrated that the egg-laying of mite pests were significantly inhibited in the G0, G1, and G2 generations by the G0 individuals exposed to LC_{40}, LC_{50}, and LC_{60} doses of scoparone. The Vg protein contents were determined, revealing

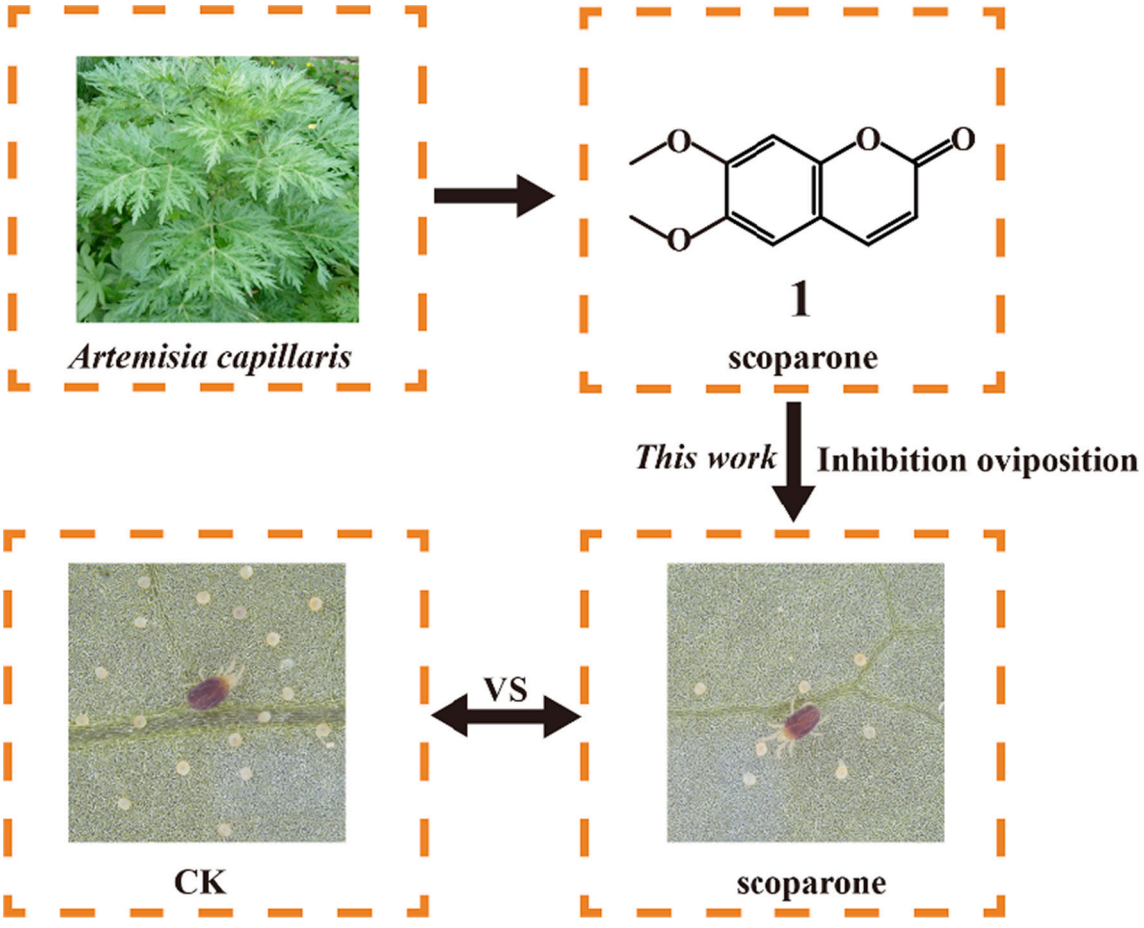

Fig. 1. Chemical structure of scoparone (1), and representative reproductive defect of *T. cinnabarinus* produced by scoparone for 3 days.

markedly lower levels in the treatment groups than in the control groups. To explore the molecular mechanism, a gene expressing Vg from *T. cinnabarinus* was sequenced, analysed, characterized, expressed, and purified. We also employed RNAi and ITC (isothermal titration calorimetry) technology to investigate the link between the Vg gene and inhibition of egg-laying of female mites by scoparone. This study is the first to demonstrate that scoparone is a green inhibitor of Vg gene-mediated egg-laying of mites and provides critical evidence for elucidating the mechanism of action underlying the inhibition of egg-laying of *T. cinnabarinus* by scoparone.

2. Materials and methods

2.1. Mite rearing

The *T. cinnabarinus* strain was initially collected from *Vigna unguiculata* in Beibei, Chongqing, China and reared at 25 °C ~ 26 °C and 70 ± 5% relative humidity, with a 14-h/10-h (light: dark) photoperiod in a greenhouse for over 18 years without exposure to any compounds.

2.2. Bioassay

The slip-dip method was employed to determine scoparone toxicity against *T. cinnabarinus* (Zhou et al., 2021). Briefly, female adult mites of *T. cinnabarinus* on pea seedlings were transferred to double-sided glued slides, on which 30 adult females were placed. The slides placed with mites were dipped in prepared solutions containing scoparone for 5 s with the same volume of distilled water without any insecticide, and the treatment method was similar to that of the negative control. The acaricidal activity of spirodiclofen (a registered commercial acaricide for controlling mite pests globally) was employed for comparative purposes (positive control). Finally, each immersed slide was placed in a culture dish with a damp sponge to prevent the slide from drying, followed by preservation at the above culture conditions. After storage for 4 and 24 h, the death of each group was observed under a microscope. The mites were considered dead if they were motionless when touching their appendages with a brush head.

We then used log-probit analysis of bioassay data to measure the sublethal (LC_{40}) and acute toxicity concentration (LC_{50} and LC_{60}) concentrations of scoparone against mites. Each separate assay included 3 biological replicates. Traditionally, the assessment of acaricide efficacy in controlling mite pest populations has relied primarily on the determination of typical acute toxicity (LC_{50} or LC_{60}) represented by median lethal dose or concentration (LC_{50}) (Huang et al., 2016). However, under field conditions, owing to acaricide degradation by processes such as photolysis and hydrolysis, mite pests are exposed to sublethal concentrations (LC_{40}) of acaricides for longer periods than to acute toxicity concentrations (LC_{50} or LC_{60}) (Chen et al., 2016; He et al., 2013). Thus, in this bioassay, the LC_{40}, LC_{50}, and LC_{60} values of scoparone were 0.137, 0.279, and 0.569 mg/mL, respectively, which served as exposure concentrations in subsequent experiments, as shown in Table S1. A leaf-disc dip method (Chen et al., 2016) was employed to measure the egg-laying capacity of mites, the details of which were based on the description of Liu *et al* (Liu et al., 2016).

2.3. Determination of the Vg content

Three hundred female mites (3 days old) were collected from each sample and homogenized with Tris-HCl buffer (0.5 mM, pH 7.5). Next, the samples were centrifuged at 12,000 rpm and 4 °C for 10 min. Finally, the supernatants were collected to determine the Vg content, and three replicates were used for analysis.

The total protein content was measured using Bradford's method (Bradford, 1976), and the Vg protein content was measured using an ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's protocol. Briefly, a standard curve of the Vg protein content was produced according to the standards supplied by the kit to calculate the content in the sample. Next, 60 μL of enzyme extract was added to the 96-well microplate, followed by incubation for 30 min at 37 °C, and then the wells were rinsed with washing solution 5 times. Enzyme conjugate buffer (60 μL) was added to the wells, and then rinsed as described above. The absorbance was measured at 450 nm after incubation at 37 °C for 10 min, and Vg protein content was calculated based on a standard curve. We employed SPSS 17.0 software to analyse the ratio of the Vg content to the total protein.

2.4. General procedure for TcVg cloning

The Tiangen RNeasy® Plus Micro Kit (Beijing, China) was employed to extract total RNA from 3-day-old female adult mites following the manufacturer's procedure. A GE Healthcare Nanovue UV–Vis spectrophotometer (Fairfield, CT, USA) at $OD_{260/280}$ and 1% agarose gel electrophoresis were then used to determine and validate the RNA quantity. The TaKaRa PrimeScript® 1st Strand cDNA Synthesis Kit (Dalian, China) was employed to synthesize cDNA according to the manufacturer's procedure. The cDNAs were stored at −20 °C for subsequent experiments.

Based on the whole-genome sequence of the sister species *Tetranychus urticae* (bioinformatics.psb.ugent.be/orcae/overview/Tetur), we designed and synthesized gene-specific primers to gain the full-length cDNA sequence of the Vg gene, as shown in Table S3. PCR was performed in a final reaction volume of 25 μL including 2.5 μL of 10× TaKaRa PCR buffer, 2.0 μL of TaKaRa dNTPs, 2.5 μL of $MgCl_2$, 1.0 μL of cDNA template, 1 μL of F/R primers, 14.8 μL of ddH_2O, and 0.2 μL of TaKaRa rTaq™ polymerase. The PCR programme was as follows: 95 °C for 3 min, followed by thirty-five cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s, with a final extension for 10 min at 72 °C. Next, we used the Tiangen Gel Extraction Mini Kit (Beijing, China) to separate the PCR products to obtain the fragment of the target gene, which was ligated into the TaKaRa pMD™ 19-T vector (Dalian, China), and finally transfected into Tiangen *Escherichia coli* DH5α-competent cells (Beijing, China) for sequencing (Beijing Genomics Institute, Beijing, China).

2.5. TcVg gene characteristic and phylogenetic analyses

We used Lynnon Biosoft DNAMAN software (version 5.2.2, USA) to edit the gene sequences, followed by the ClustalW program to align amino acid sequences of Vg genes (Urbino and Dalmon, 2007). We computed the isoelectric points and molecular weights (kDa) of the Vg proteins using ExPASy (Expert Protein Analysis System) software (http://www.expasy.org) (Elisabeth et al., 2003). We used the neighbour-joining (NJ) method to construct a phylogenetic tree of Vg genes using MEGA (Molecular Evolutionary Genetics Analysis) software (version 7.0.26) *via* 1000 bootstrap replicates (Alan et al., 2014).

2.6. RNAi assay

We designed and synthesized special T7 promoter primers of the Vg gene to amplify target sequence, as shown in Table S3. A GFP (green fluorescent protein, accession number: *ACY56286*) construct was used for the negative RNAi group. The amplified PCR products were gel-purified, and dsRNA was prepared using a Thermo Scientific T7 High Yield Transcription Kit (Lithuania, EU). We performed 1% agarose gel electrophoresis and spectrophotometry analysis to measure the lengths and concentrations of the dsRNA products, respectively. The purified dsRNA was stored at −80 °C for subsequent experiments.

The *TcVg* gene expression was knocked down using a leaf-disc-mediated RNAi method following our previous study (Zhou et al., 2021). Briefly, the cowpea leaves were cut to the feeding ground with a 2.0-cm diameter, followed by placement in an oven at 60 °C for 5 min. Each dried leaf was exposed to 20 μL of DEPC-treated water, dsRNA-*GFP* (1000 ng/μL), or dsRNA-*TcVg* (1000 ng/μL) for 4 h at 25 °C. After the

solution was completely absorbed, the leaves were placed on water-soaked filter paper to make leaf discs, followed by placement on a wet sponge to prevent mites from escaping. Each dsRNA-permeated leaf disc contained 30 female adult mites after 24 h of starvation. Subsequently, *T. cinnabarinus* ingested dsRNA in the leaves through its piercing and sucking mouth parts. Finally, after 48 h of dsRNA feeding, the exposed *T. cinnabarinus* were gathered for subsequent experiments.

2.7. qPCR assay

Gene-specific primers for qPCR (Table S3) were designed using Primer 5.0 and synthesized by the Beijing Genomics Institute (Beijing, China), and a ribosomal protein S18 (*RPS18*) gene was employed as the reference gene (Sun et al., 2010). qPCR was performed in a final reaction volume of 20 μL containing 10 μL of Hercules BIO-RAD iQ™ SYBR® Green Supermix (CA, USA), 1 μL of cDNA template, 1 μL of F/R primer, and 7 μL of ddH_2O. The qPCR programme was as follows: 95 °C for 120 s, followed by forty cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, and the relative expression level of the Vg gene was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The melting curve of qPCR was analysed at 60 °C ~ 95 °C. Each separate test contained 3 technical and biological replicates.

2.8. Prokaryotic expression of the TcVg gene

Escherichia coli expression systems were employed to express the *TcVg* gene following our previously described procedure (Zhou et al., 2021). Briefly, we used PCR to amplify the cDNA sequences of the *Vg* gene with a pair of specific primers and the BamH I and *Sma* I restriction enzyme sites, as shown in Table S3. Next, we used the TaKaRa restriction enzymes BamH I and Sma I to digest the PCR product, which was further gel purified, ligated into the pGEX-6P-1 vector, and transfected into BL21 cells (*DE3*) for Vg gene expression. The cells were shaken at 180 rpm for 12 h at 37 °C, diluted with LB (Luria Bertani) medium containing 0.1 mg/mL of Amp (ampicillin) at a ratio of 1:100, and finally incubated at 37 °C with an OD_{600} value of 0.6–0.8. IPTG (isopropyl β-D-1-thio-galactopyranoside, 0.024 mg/mL) was used to induce the suspended cells, which were incubated at 180 rpm for 12 h at 37 °C. The Beyotime Biotechnology BeyoGold™ GST-tag Purification Resin (Beijing, China) was employed to purify the fusion protein, which was then analysed by SDS-PAGE. The Bradford method (Bradford, 1976) was used to determine the Vg protein concentration.

2.9. Isothermal titration calorimetry assay

The purified Vg protein was dialysed in ITC buffer (pH 7.5, 25 mM HEPES, 100 mM NaCl) at 4 °C. We first dissolved scoparone in DMSO to 10 mM and then diluted it in the same buffer. The titrations included 20 injections of 400 μL of 1 mM scoparone into the cells, which contained 10 μM ~ 10 mM of Vg protein or control buffer at 25 °C. Water containing 7% DMSO was used to fill the reference cell to ensure the same heat capacities of the two cells. The experiments were performed using a Malvern PEAQITC instrument with a stirring speed of 750 rpm.

2.10. Statistical analysis

All the experiments were performed in three replicates, and the data were expressed as mean ± SD (standard deviation). SPSS 17.0 software (United States) was employed to perform statistical analysis. For all the experimental data, statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Significant differences were considered at $P < 0.05$.

3. Results

3.1. Egg-laying capacity difference of T. cinnabarinus

To explore the effect of scoparone on the egg-laying capacity of *T. cinnabarinus*, the egg-laying of female mites in four consecutive generations (G0, G1, G2 and G3) was determined by G0 individuals exposed to the LC_{40}, LC_{50} and LC_{60} doses of scoparone. Compared with the control groups, as shown in Fig. 2 and Fig. S3, the egg-laying capacity of the G0, G1, and G2 generations indicated that the eggs laid per mite decreased significantly as follows: 38.35%, 42.52%, and 36.86%, respectively, at the LC_{40} doses of scoparone; 47.43%, 50.56% and 44.59%, respectively, at the LC_{50} doses of scoparone; and 57.97%, 63.92% and 51.78%, respectively, at the LC_{60} doses of scoparone. Thus, during the whole life span, they produced (respectively) 35, 60, and 48 fewer eggs at the LC_{40} dose; 43, 71, and 58 fewer eggs at the LC_{50} dose; and 52, 90, and 68 fewer eggs at the LC_{60} dose. However, no significant differences were observed for the egg-laying capacity of the G3 generation at the LC_{40}, LC_{50}, and LC_{60} doses between the acaricide and control groups (Fig. S4).

3.2. Vg content after scoparone exposure

To further investigate whether scoparone-mediated inhibition of mite egg-laying was mediated by Vg protein, the total Vg protein content at the 3-day-old adult stage (3A) of female adult *T. cinnabarinus* in the G0, G1 and G2 generations was detected in G0 individuals exposed to the LC_{40}, LC_{50} and LC_{60} doses of scoparone. Compared with the control groups, at LC_{40}, LC_{50}, and LC_{60} doses of scoparone, the Vg protein content was significantly lower (2.22-, 3.43-, and 7.06-fold in the G0 generation; 2.88-, 4.17-, and 15.00-fold in the G1 generation; and 2.20-, 3.01-, and 4.81-fold in the G2 generation, respectively; Fig. 3).

3.3. Sequence analysis of TcVg

To further explore the molecular functions of *TcVg*, we cloned and characterized its cDNA. Table S4 presents the lengths of the deduced amino acid sequence, accession number, theoretical isoelectric point, and predicted protein molecular weight. *TcVg* was predicted to comprise Vitellogenin_N, DUF1943 (unknown function), and VWD (von Willebrand factor type D) superfamily domains (Fig. 4A).

We performed phylogenetic analysis according to the amino acid sequence of the Vg protein from arachnids and insects. As shown in Table S5, we obtained the complete ORF sequences of Vg from the *T. urticae* genome (bioinformatics.psb.ugent.be/orcae/overview/Tetur) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). As shown in Fig. 4B, the results demonstrated that the *TcVg* groups with other amino acid sequences from Arachnida possess the closest genetic distance with the Vg of *T. urticae* (*TuVg*), implying possibly similar biological functions between *TcVg* and *TuVg*.

3.4. Expression patterns of TcVg

To further investigate the molecular function of *TcVg*, we detected the expression modes of *TcVg* during different developmental stages and scoparone exposure groups. The expression levels of *TcVg* genes during different developmental stages [egg, larvae, nymph, 0A (preoviposition stage), 3A (3-day adults), 10A (10-day adults), and female adult] and in the G0, G1, and G2 generations at the 3A stage of female *T. cinnabarinus* by the G0 individuals exposed to different scoparone treatment groups were evaluated by qPCR and RT-PCR, respectively. The relative *TcVg* expression was barely measurable from the egg stage until 0A, reaching a peak at 3A, and then being reduced at the 10A stage (Fig. 5).

As shown in Fig. 6, the results indicated that the Vg gene expression in the G0, G1, and G2 generations at the 3A stage of female

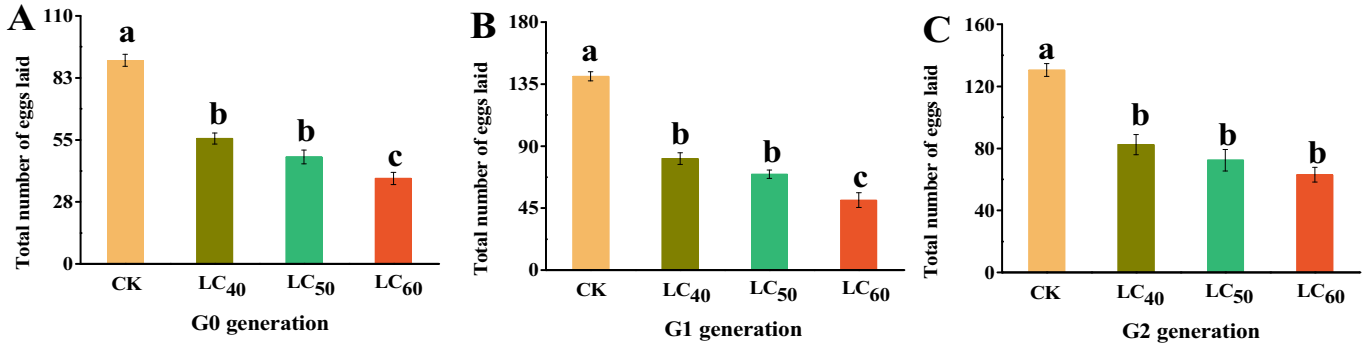

Fig. 2. Total egg-laying of *T. cinnabarinus* in the G0 (A), G1 (B) and G2 (C) generations by the G0 individuals exposed to the $LC_{40,}$ LC_{50} and LC_{60} of scoparone. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$).

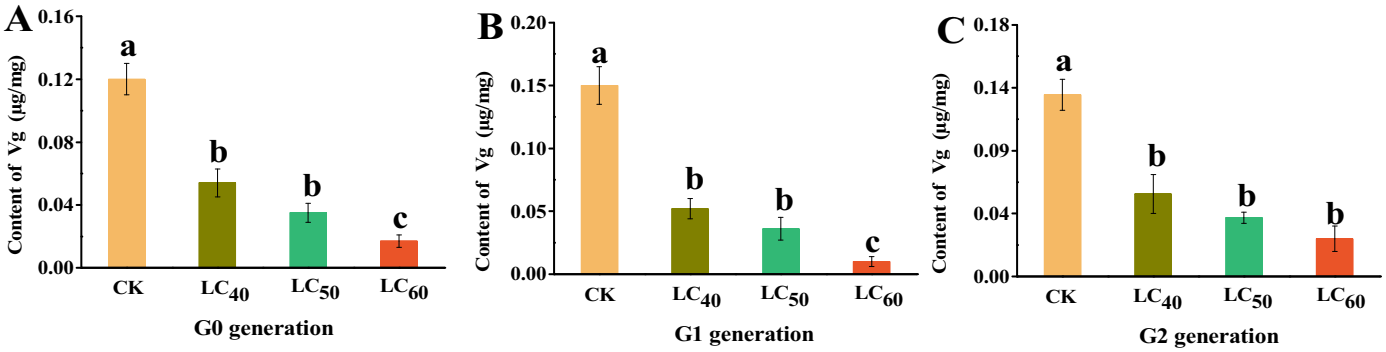

Fig. 3. Total vitellogenin (Vg) content at three-day adult stage (3A) of female *T. cinnabarinus* in the G0 (A), G1 (B) and G2 (C) generations by the G0 individuals exposed to the $LC_{40,}$ LC_{50} and LC_{60} of scoparone. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$).

T. cinnabarinus was downregulated by scoparone treatment in the G0 individuals compared with that in the control groups. Based on data analysis, at the LC_{40}, LC_{50}, and LC_{60} doses of scoparone, the relative transcript level of *TcVg* was remarkably lower (1.78-, 2.35-, and 3.23-fold in the G0 generation; 2.04-, 2.55-, and 4.02-fold in the G1 generation; and 1.76-, 2.12-, and 2.52-fold in the G2 generation, respectively) at the 3A stage than that in the control groups.

3.5. RNAi via dsRNA knockdown

To explore the molecular mechanism by which scoparone inhibits the egg-laying of mites, we employed RNAi technology to knock down the transcription levels of *TcVg* in *T. cinnabarinus* (Figs. S5–S6). The transcript level of *TcVg* was remarkably reduced by 57% after feeding dsRNA-*TcVg* compared with the control groups (Fig. 7A). Additionally, compared with the control groups, at the 3A stage of female *T. cinnabarinus* after 48 h of pretreatment with dsRNA-*TcVg*, the Vg content was significantly lower (2.43-fold) (Fig. S7). No remarkable difference was found between the DEPC-water and dsRNA-*GFP* control groups (Figs. 7A and S7). Thus, leaf disc-mediated RNAi successfully knocked down the transcription of *TcVg* in *T. cinnabarinus*.

3.6. Effect on egg-laying by RNAi

The eggs laid per mite were remarkably reduced by 32.21% after dsRNA-*TcVg* feeding for 48 h compared with those of the control (Figs. 7B and S8). No remarkable difference was found in egg-laying between the DEPC-water and dsRNA-*GFP* control groups (Fig. S8). These results indicated that *TcVg* RNAi decreased the egg-laying of female mites.

3.7. Egg-laying assay of T. cinnabarinus to scoparone after RNAi of TcVg

Compared with the DEPC-water control groups, at the 3A stage of female *T. cinnabarinus* on the G0 individuals exposed to the LC_{50} of scoparone, the egg-laying inhibition of female mites by scoparone was significantly increased by 47.43% when *TcVg* was knocked down by RNAi in *T. cinnabarinus* (Fig. 8A and B). No remarkable difference was observed in egg-laying between the DEPC-water and dsRNA-*GFP* control groups (Fig. 8A and B). These results demonstrated that the egg-laying inhibition of female *T. cinnabarinus* by scoparone was mediated by the *TcVg* gene.

3.8. Scoparone binding to recombinant Vg protein

To test the interaction between scoparone and the Vg protein, we employed an *E. coli* heterologous expression system to obtain the Vg protein. As shown in Fig. 9A, SDS-PAGE analysis indicated that a new specific band (approximately 200 kDa) was found in the whole-cell lysate and purified protein. We then performed an isothermal titration calorimeter (ITC) assay to determine the ability of scoparone to bind purified Vg protein. Scoparone displayed effective binding to the Vg protein, and its binding constant (K_d) value was 218.9 μM (Fig. 9B and C; Table S6). The negative value of ΔG (−5777.03 cal/mol) indicated an automatic interaction between Vg and scoparone.

4. Discussion

Here, we analysed the Vg gene from *T. cinnabarinus*, referred to as *TcVg*. The sequence of the Vg protein contains Vitellogenin_N, DUF1943 (unknown function), and VWD superfamily domains. Vitellogenin_N is

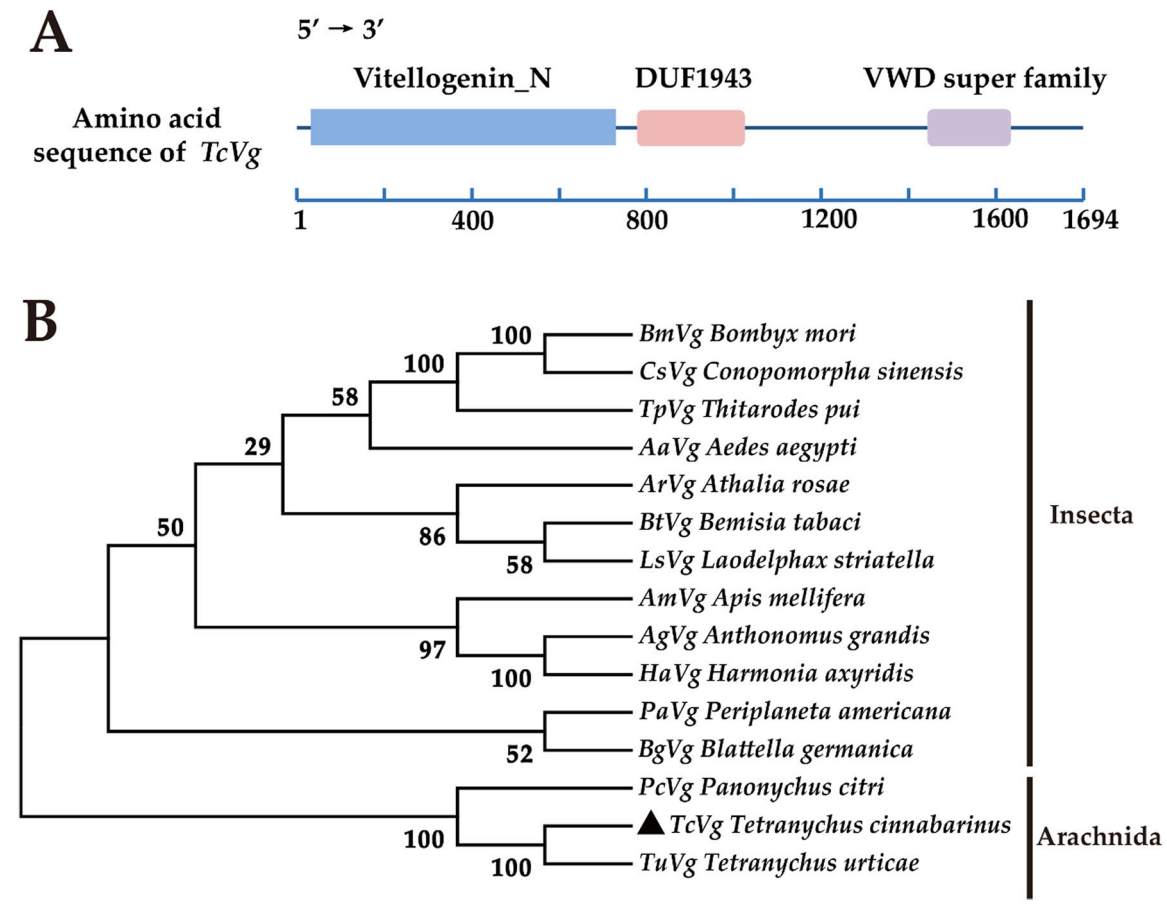

Fig. 4. Conserved domains of deduced amino acid sequences of Vg gene (*TcVg*) from the *T. cinnabarinus* (A); and phylogenic analysis of *TcVg* (B). (A) The putative protein sequence of *TcVg* contains domains of Vitellogenin_N, DUF1943 (domain of unknown function), and VWD (von Willebrand factor (vWF) type D domain) super family. (B) Maximum likelihood tree constructed by MEGA 7.0.26. Phylogeny testing was conducted *via* the bootstrap method with 1000 replications. The sequences used for constructing the tree are listed in Supplementary Table S5.

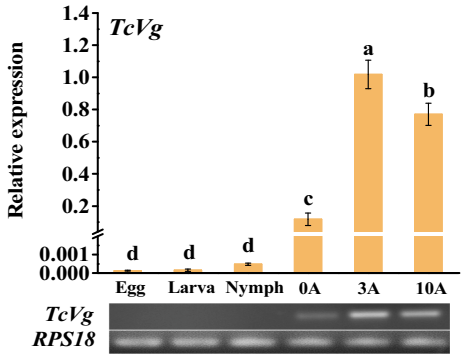

Fig. 5. Expression profiles of *TcVg* transcript in different developmental stages of *T. cinnabarinus*. The following life stages were analysed: egg, larvae, nymph, 0A (preovipositional stage), 3A (3-day adults), 10A (10-day adults), and female adult. Error bars represent the standard error of the calculated mean based on three biological replicates. The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$). *RPS18* was used as the reference gene.

the amino terminal region of lipoproteins, and VWD, an abbreviation for von Willebrand factor, contains several D-type domains: D1 and D2 exist in the *N*-terminal propeptide, while the remaining D domains are used for polymerization (Liu et al., 2016). Moreover, the expression levels of the Vg gene in *T. cinnabarinus* resemble those in *T. urticae* (Ning et al., 2017), being lowly expressed in eggs until the 0A stage but reaching a peak at 3A, followed by a reduction at the 10A stage, a finding that is consistent with the trend of daily egg-laying curves of mites. Thus, the egg-laying capacity of *T. cinnabarinus* exhibits a positive link with the transcript levels of the *TcVg* gene.

Low expression of the Vg gene underlines its significance in the action mechanism of scoparone in inhibiting the egg-laying ability of mites (Zhou et al., 2019). However, it remains unclear how low-expression Vg genes mediate the acaricide phenotype. To explore the molecular mechanism of the compound, we further employed RNAi technology to validate the connection between target gene function and low expression. RNAi has been widely employed to verify or identify target genes of pesticides in mites and insects (Xu et al., 2016). Oral and microinjection of dsRNA are effective means to perform RNAi in insects (Kwon et al., 2013). However, the size of *T. cinnabarinus* is relatively small, only approximately 0.5 mm, and it is difficult to achieve microinjection without influencing its survival rate. Thus, in the present study, we used the leaf disc-mediated feeding dsRNA-*TcVg* method to reduce the expression levels of the Vg gene such that the variations in egg-laying capacity after gene knockdown could be determined. Clear gene silencing was observed through a significant reduction in mRNA expression and Vg content. Silencing of the Vg gene by RNAi may impair the function and development of the ovary, leading mites towards female infertility. Similar results have also been reported in *Romalea microptera* (Tetlak et al., 2015; Tokar et al., 2014). A recent study reported that Vg plays a key role in egg development of the ovaries (Salmela et al., 2015). Another study showed that Vg participates in transgenerational immune priming and exerts dual functions-immunity and fecundity (Havukainen et al., 2011). In honey bees, Vg maintains

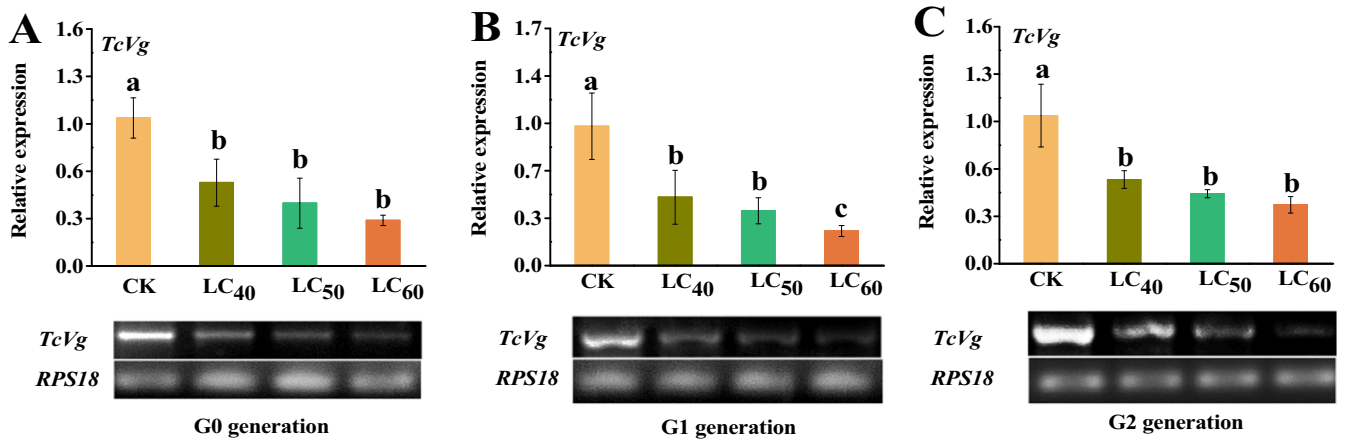

Fig. 6. Expression profiles of *TcVg* transcript in the G0, G1 and G2 generations at 3A stage of female *T. cinnabarinus* by the G0 individuals exposed to LC_{40}, LC_{50} and LC_{60} of scoparone, respectively. (A, B, C) Relative expression of *TcVg* of *T. cinnabarinus* in the G0, G1, and G2 generation exposed to 0.137, 0.279, and 0.569 mg/mL scoparone (LC_{40}, LC_{50} and LC_{60}) in 0.1% (v/v) Tween-80 and 3% (v/v) acetone at the adult stage for 48 h using a slip-dip bioassay were analysed using qRT-PCR, respectively. Error bars represent the standard error of the calculated mean based on three biological replicates. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$). *RPS18* was used as the reference gene.

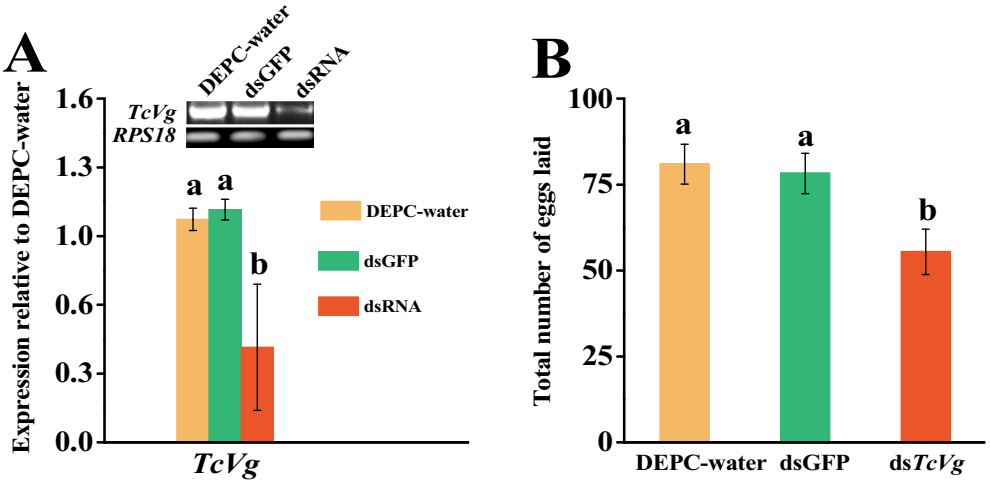

Fig. 7. qRT-PCR and RT-PCR analysis of *TcVg* expression at 3A stage of female *T. cinnabarinus* after RNAi treatment at 48 h post dsRNA feeding relative to the expression levels after DEPC treated-water and dsGFP treatment (A), and the total egg-laying of *T. cinnabarinus* after 48 h of pre-treatment with dsRNA (B). The water- and double-stranded green fluorescent protein (dsGFP)-treated group was regarded as the control. The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$). *RPS18* was used as the reference gene.

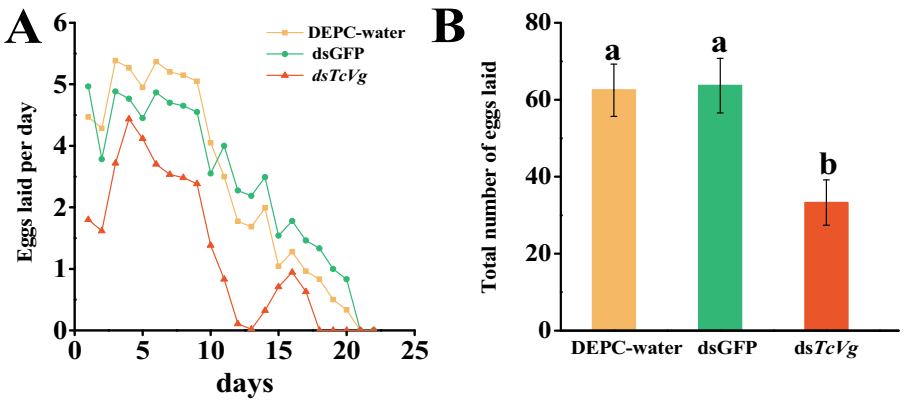

Fig. 8. Knockdown of *TcVg* expression increased the egg-laying inhibition of mites by scoparone at 3A stage of female *T. cinnabarinus* by the G0 individuals exposed to LC_{50} of scoparone. Daily egg-laying curves (A) and total egg-laying (B) of *T. cinnabarinus* after 48 h of pretreatment with dsRNA-*TcVg* by the G0 individuals exposed to LC_{50} of scoparone. The water- and double-stranded green fluorescent protein (dsGFP)-treated group was regarded as the control. The bars represent the average (± SD). Different lowercase letters above the columns indicate significant differences (Tukey's test, $P < 0.05$).

the longevity of workers and queens and manages behavioural activity by regulating juvenile hormones (Corona et al., 2007). These findings indicate that Vg functions beyond serving as an energy reserve for developing embryo nourishment. However, our results from RNAi and egg-laying assays showed the involvement of the Vg gene of *T. cinnabarinus* in the egg-laying capacity of scoparone towards mites. In our study, the egg-laying inhibition of female mites by scoparone was significantly increased when *TcVg* was downregulated *via* RNAi by G0

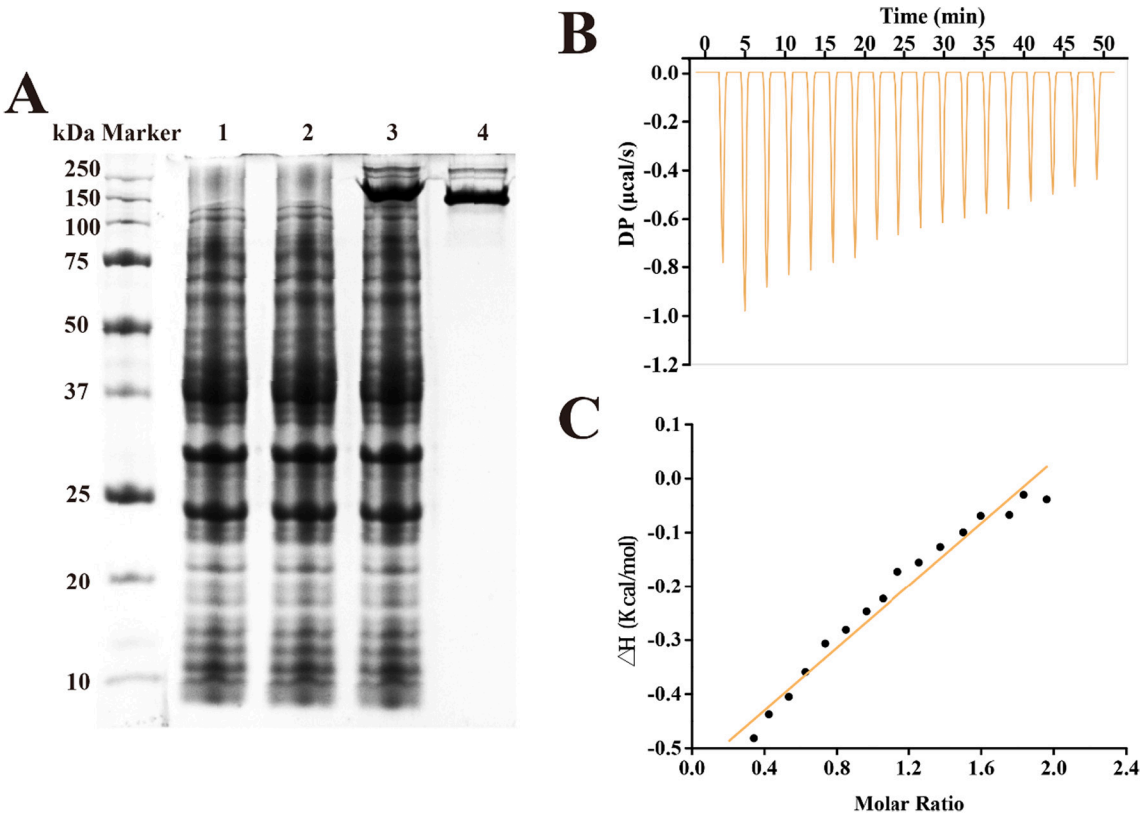

Fig. 9. Effect of scoparone on recombinant Vg protein. (A) Expression of recombinant Vg protein by *E. coli*. Lane 1: supernatant of pGEX-6p-1 (no insert). Lane 2: recombinant Vg protein without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Lane 3: recombinant Vg protein with IPTG induction. Lane 4: purification of recombinant Vg protein. (B, C) ITC binding isotherms show the interaction between scoparone (1 mM as titrant) and Vg protein (1 mM) and the measured K_d is 218.9 μM.

individuals exposed to the LC_{50} of scoparone, indicating that RNAi-mediated low expression of the Vg gene increased the egg-laying inhibition of *T. cinnabarinus* by scoparone. In summary, these results suggested that inhibition of the egg-laying of female mites by scoparone is mediated by the low expression of the Vg gene of *T. cinnabarinus*.

We further employed a heterologous expression system to explore the function of the mite Vg gene. Heterologous expression is not only a universal method to obtain specific gene products but also offers an opportunity to explore the functions of specific genes *in vitro* (Zhou et al., 2021). For the first time, we successfully overexpressed the Vg gene in an *E. coli* expression system and obtained purified Vg protein (approximately 200 kDa). However, direct proof of the interaction between the Vg protein and scoparone is limited. ITC (isothermal titration calorimeter) technology can detect the direct interaction between proteins and small molecules by real-time monitoring of thermal changes. Thus, in the present study, an ITC assay was used to determine the ability of scoparone to bind purified Vg protein. Scoparone bound to the Vg protein, and its K_d value was 218.9 μM, implying its potential function in inhibiting the egg-laying of mites by directly targeting the Vg protein.

5. Conclusion

Scoparone showed excellent acaricidal activity with a significant effect on inhibiting egg-laying and can be employed as a sustainable green acaricide candidate for controlling *T. cinnabarinus* with strong fecundity. Additionally, the inhibition of the egg-laying capacity of female *T. cinnabarinus* by scoparone is mediated by the low expression of the Vg gene. More importantly, scoparone may function in inhibiting the egg-laying of mites by directly targeting the Vg protein. The current study advances our understanding of the mechanism of scoparone inhibiting the egg-laying of *T. cinnabarinus* and clarifies novel strategies that control pest mites with strong fecundity by inhibiting egg-laying, facilitating the use of scoparone in crop protection.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2021.109055.

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