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The fenpropathrin resistant *Tetranychus cinnabarinus* showed increased fecundity with high content of vitellogenin and vitellogenin receptor

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ABSTRACT

Carmine spider mite, *Tetranychus cinnabarinus* (Boisduval), an agricultural pest of economically important crops, has developed resistance to a group of pesticides. We have selected a fenpropathrin-resistant strain (FeR) of *T. cinnabarinus* from the isogenous and susceptible strain (SS), and found that the FeR not only showed resistance but its fecundity also increased. According to the numbers of eggs laid per day of both strains, the FeR was more fertile than SS throughout the life span. To investigate the underlying reason, the protein contents of vitellogenin (vg) and vitellogenin receptor (vgr) were detected, and the results showed both of them were significantly higher in FeR than in SS. Then, the mRNA-expressions of vg and vgr genes were compared between FeR and SS. From the transcriptome data of *T. cinnabarinus*, we classified two vg genes (designated as *Tcvg1* and *Tcvg2*, respectively) and a vgr gene (designated as *Tcvgr*). The expressions of *Tcvg1*, *Tcvg2* and *Tcvgr* were highly associated with the fecundity of the mites that their mRNAs were extremely abundant at the adult stage, but hardly detectable during the developmental period (from egg to deutonymph). In accordance with the protein content, the expression levels of the three genes were all significantly higher in FeR than they were in SS. These results suggested that after resistance selection with fenpropathrin in *T. cinnabarinus*, the fecundity and the expression of reproduction-related genes (vg and vgr) were significantly higher in fenpropathrin resistant strain than that in susceptible strain.

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1. Introduction

Resurgence of arthropod pests induced by chemical pesticides is the result of a combination of various factors, from the homeostatic modulation in the pests itself to the elimination or reduction of natural enemies and/or competitors [1]. Among these factors, resistance and enhanced reproduction are thought to contribute to the resurgence phenomenon [2]. In *Nilaparvata lugens*, stimulation of insecticides on the reproduction appeared to be of more significance than destruction of natural enemies [3]. Evidences suggested that low doses of deltamethrin-induced hormesis in the southern red mite *Oligonychus ilicis* led to significant increase of its population [4]. In the study of *Podisus distinctus* females, pyrethroid-application with sublethal dose was found to have positive effects on the oviposition ability [5].

Carmine spider mite, *Tetranychus cinnabarinus* (Boisduval), which is also considered as the red form of *Tetranychus urticae* sometimes [6], is known as a widely distributed agricultural pest of significantly economic importance in China [7]. It feeds on a broad spectrum of vegetables, fruits and other crops like cotton, eggplant, cowpea, resulting in declined yield and quality of agricultural products [8]. Its resistance has developed quickly owing to the typical arrhenotoky genetics, highly effective reproduction, extremely short life cycle as well as the frequent application of chemical acaricides over the years [7,9,10]. Resistance monitoring showed that field strains of *T. cinnabarinus* have developed resistance against fenpropathrin [11]. In the lab, under continuous stress of fenpropathrin, we also selected a fenpropathrin-resistant strain of *T. cinnabarinus* (FeR) from the homologous and susceptible strain (SS) with 100-fold resistance, and we observed that the FeR obtained a higher fecundity than the SS, which might contribute to the resurgence of *T. cinnabarinus* in the field after chemical control. Thus, we intend to identify the important reason that resulted in this phenomenon.

Vitellogenin (vg) and vitellogenins receptor (vgr) both play indispensable roles in the reproductive process of oviparous species. Vg is the precursor of the major yolk protein vitellin (vn) in insects and many other oviparous species [12]. In insects, vgs are synthesized in the fat body and secreted into the hemolymph. Then they are taken up by developing oocytes and become vitellin, which is the major provider of nutrient for the embryo [12–14]. Vgs are transported into oocytes by vgr through endocytosis [15–18]. Vgr is of vital importance for egg-laying species since it mediates a key step of oocyte maturation, which is a prerequisite of reproduction [16]. Hence, we intended to investigate whether there was an underlying connection between the expression of reproduction-related genes (vg and vgr) and the

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enhancement of the reproductive capacity of fenpropathrin-resistant *T. cinnabarinus*.

In this study, we confirmed that the daily and total fecundity of *T. cinnabarinus* was significantly higher in FeR than SS. The protein contents of vg and vgr were detected, and the results showed either of them was significantly higher in FeR than that in SS. To investigate the molecular mechanism, we identified and characterized two *vg* genes and one *vgr* gene in *T. cinnabarinus*, and examined their expression profiles at different developmental stages, especially the expression differences between FeR and SS. The expression levels of the *Tcvgs* and *Tcvgr* were associated with the fecundity of *T. cinnabarinus*, and the mRNA levels were all significantly higher in FeR than in SS.

2. Materials and methods

2.1. Strains of mites

2.1.1. Susceptible strain

T. cinnabarinus were originally collected around 15 years ago in the field in Beibei District, Chongqing, China, and have been reared on fresh potted cowpea leaves in artificial climate chamber (pesticide free) under conditions as follows: a photoperiod of 14:10 h (L:D), temperature at 26 ± 1 °C, 35–55% relative humidity(RH). These mites are considered as the susceptible strain (SS).

2.1.2. Fenpropathrin -resistant strain (FeR)

The FeR was selected by continuous pressure of fenpropathrin from a sub-strain of susceptible *T. cinnabarinus* (SS), and these two strains were kept under the same environment condition. After >70 generations' selection, the resistance ratio of FeR was over 100-fold [19]. Further details on the FeR strain selection refers to He et al. [20].

2.2. Measurement of fecundity using life-table

Thirty petri dishes were prepared for each strain, and two freshly-cut cowpea leaves (diameter: 2 cm) were put on each petri dish. Then, females were transferred to a leaf-disc to lay eggs, respectively. Six hours later, the mites and redundant eggs were removed, and only one egg was kept on the leaf disc for further observation. Observation of the development of the eggs was conducted with dissecting microscope SZ660 (Chongqing Optec Instrument, Chongqing, China) at an interval of 12 h. When the egg developed to deutonymph stage and could be distinguished as female, one male mite from the same strain was added to each disc for mating. When the male is found dead, we replaced it with another one. Those female mites that entered adult stage at the same time were screened out and each day, we recorded the number of eggs they laid. The eggs were gently removed with a soft tiny brush after counting without disturbing the mites. All the leaf discs were kept under the same rearing conditions as previous described. The leaves were replaced every week, and the mites were transferred to the fresh leaf gently with a soft tiny brush. The total number of eggs was calculated when a female mite died naturally, and this was recorded as valid data (the data of unnatural death was not include), finally, the data of 24 females from FeR and 25 females from SS were collected for analysis.

The difference of total fecundity between SS and FeR strain was determined by independent-sample *t*-test with a *p*-value < 0.05 in SPSS 16.0 (SPSS Inc., Chicago, USA).

2.3. Vitellogenin and vitellogenin receptor content detection in SS and FeR

Two hundred female mites (at three-day adult stage) were collected as one sample, then, homogenized with 1 mL 0.5 mol/L pH 7.5 Tris-HCl buffer on ice. The fluid was transferred into a 2.5 mL centrifuge tube and centrifuge the samples at 10,000 *g*, 4 °C for 10 min. The supernatant was collected for protein detection. The content of total protein was detected using Bradford's method [21], and the content of vg and vgr was detected according to the instructions of ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd. Product number: ml104703 for vitellogenin; ml104704 for vitellogenin receptor). Briefly, standard curves of vg and vgr were constructed with the standards provided by the kit to calculate the content in the sample, then, the 50 μL enzyme extracts were added into the wells and incubated at 37 °C for 30 min, after that, the wells were washed with wash buffer for five times. 50 μL of the enzyme conjugate solution was added, and the wells were incubated, and washed in the same condition. After coloration at 37 °C, in the dark, for 10 min, the absorbance was detected at 450 nm, and the contents of vg and vgr were calculated according to the standard curves. Data of the proportions of vg and vgr in the total protein was analyzed using SPSS 16.0 applying Student's *t*-test for significance ($p < 0.05$). Three replicates were set for analysis.

2.4. Sequence analysis of Tcvg1, Tcvg2 and Tcvgr

Full sequences of *Tcvg1*, *Tcvg2* and *Tcvgr* have been acquired in the transcriptome data of *T. cinnabarinus* [22]. Deduced sequences of amino acids of the three genes were obtained through Primer Premier 5 (Version 5.00) (PREMIER Biosoft International, Palo Alto, CA, USA).

The structural features of the putative protein sequences of *Tcvgs* and *Tcvgr* were compared to the vitellogenins and vitellogenin receptors in other representative organisms in the conserved domain database on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Deduced amino acids of vitellogenin and vitellogenin receptor from *T. urticae*, *Panonychus citri*, and some other model insects were downloaded from the genome database of *T. urticae* and the NCBI database (http://www.ncbi.nlm.nih.gov/). The similarity of amino acids for the two *Tcvgs* was determined using the DNAMAN version 8 (Lynnon Corporation). The corresponding phylogenetic trees were constructed with MEGA 5.0, applying the maximum likelihood method, bootstrapping with 1000 pseudoreplicates [23].

2.5. RNA extraction and cDNA construction

When collecting mite samples of different developmental stages, a dozen of leaf discs were prepared with 3–4 leaves on it. Put 30–50 female mites to each leaf and remove them 12 h later. When the majority of the eggs had developed to the stage demanded, collect them gently with a soft brush into a 1.5 mL centrifuge tube. We managed to collect seven different developmental stages of mites of the two strains, i.e. eggs (E), larva (L), protonymphs (1N), deutonymphs (2N), preoviposition stages (0A), 3-day adult (3A) and 10-day adult (10A).

Total RNA was extracted from whole body mites (100–200 mg) which had been smashed thoroughly in a mortar in liquid nitrogen, using TRIzol® Reagent (Life Technologies, Frederick, MD, USA) following the manufacturer's instructions. The extracted RNAs were dissolved in RNase-free water and stored at −80 °C before use. 1% agarose gel electrophoresis of the total RNAs was conducted instantly once the RNA extraction and dissolution is finished to ensure the RNAs' integrity. The concentration and purity of the extracted RNAs were examined using a NanoVue UV–Vis spectrophotometer (GE Healthcare Bio-Science, Uppsala, Sweden) by measuring the absorbance at 260 nm and the absorbance ratio at OD260/280 and at OD260/230 respectively [19].

Next, cDNAs were constructed using PrimeScript® RT reagent kit (Takara, Shiga, Japan) after the RNAs had been treated with DNase I (Promega, Madison, WI, USA) to eliminate any potential genomic DNA that could interfere with RT-qPCR. The transcription of RNA was operated with the C1000TM Thermal Cycler (Bio-Rad, Foster City, CA, USA)

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