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Transcription profiling of two cytochrome P450 genes potentially involved in acaricide metabolism in citrus red mite *Panonychus citri*

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ABSTRACT

Acaricide resistance in citrus red mite, *Panonychus citri* (McGregor), has been a growing problem in the management of this pest globally. As a member of main detoxification enzymes, cytochrome P450 monooxygenases (P450s) play important roles in the development of acaricide resistance in arthropods. In this study, two novel P450 genes (named CYP4CF1 and CYP4CL2) were cloned from *P. citri*. CYP4CF1 and CYP4CL2 had open reading frames of 1527 and 1758 nucleotides encoding 508 and 585 amino acids, respectively. The putative proteins shared 34% identity with each other. Phylogenetic analysis showed that CYP4CF1 and CYP4CL2 were most closely related to CYP4CF2 and CYP4CL1 from *Tetranychus urticae*, respectively. The transcriptional activities of CYP4CF1 and CYP4CL2 were also investigated. Quantitative RT-PCR revealed that the expression level of CYP4CF1 was increased after induction by pyridaben, while that of CYP4CL2 increased after induction by abamectin, azocyclotin, pyridaben, and spirodiclofen. The mRNA levels of both CYP4s were significantly higher in larval stages, as well as in a field resistant population (BB) than in laboratory susceptible strain (LS). The identification of this detoxification enzyme activity showed that the activity of P450s was higher than the control after exposure to azocyclotin, pyridaben and spirodiclofen. The results suggested that two CYP4 genes might play distinct but significant roles in larvae of *P. citri*. The results further indicate that CYP4CF1 is associated with pyridaben metabolism, while CYP4CL2 may be involved in detoxification of abamectin, azocyclotin, pyridaben, and spirodiclofen.

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1. Introduction

The citrus red mite, *Panonychus citri* (McGregor) (Acari: Tetranychidae), has a worldwide distribution and is regarded as one of the most important citrus pests in many countries [1]. It is a serious mite pest on citrus and can feed on 112 different plant species including peach, pear, and Chinese prickly ash [2]. Injury to the plants caused by mite feeding results in extensive defoliation leading to a pale appearance of green immature fruits, which affects citrus harvest quantity and quality (weight, sugar content, and appearance) [3]. Spraying of synthetic acaricides is regarded as the most common control practice of this pest. However, because of several factors associated with biology of this mite and high possibility of acaricide exposure, *P. citri* rapidly develops resistance to acaricides [4,5]. To date, the citrus red mite is on the third place of species that evolved severe resistance from the family Tetranychidae [6]. It has shown resistance to organophosphates, pyrethroids, organotin miticide, organochlorine, carbamates, ketoenols, mitochondrial electron transport inhibitor (METI) acaricides, organic nitrogen acaricides, biological acaricides, and bifenazate in the fields [5–9]. Acquired acaricide resistance presents the greatest challenge in the control of *P. citri*, which is responsible for economic losses in citrus agriculture.

Similar to insects, enhanced enzymatic detoxification and target site insensitivity are major causes of development of resistance in Acari [4]. The majority of cases involved sequestration/metabolism of the pesticides before it reaches the target site due to quantitative or qualitative changes in major detoxification enzymes such as cytochrome P450 monooxygenases (P450s), carboxylesterase (CarE) and glutathione *S*-transferases (GSTs) [10–12]. Arthropod P450s have been subdivided into four major clades (clans or subclasses) [13]. Each clade has been further subdivided into various CYP families, based on the degree of amino acid sequence similarity [14]. P450s are important metabolic enzymes catalyzing a number of lipophilic compounds, and are involved in metabolism of a wide range of endogenous and exogenous chemical substances such as hormones, pheromones, pesticides, and plant secondary compounds [14,16]. Overexpression of P450s leading to increased metabolism of pesticides has been demonstrated in many insects such as in *Musca domestica* (CYP6A1 [17], CYP6A12 [18], and CYP6D1 [19]), *Drosophila melanogaster* (CYP6G1) [20], *Helicoverpa armigera* (CYP9A12 and CYP9A14) [21], and *Culex quinquefasciatus* (CYP9M10, CYP4H34 and CYP6Z10) [15]. Recently, transgenic

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expression of CYP6P9a and CYP6P9b in *D. melanogaster* demonstrated that elevated expression of either of these genes confers resistance to both permethrin and deltamethrin [22].

According to earlier studies, CYP4 is an important P450 family in insects, for the genes can be induced by xenobiotics, overexpressed in resistant strains and participate in the detoxification of pesticides [23,24]. Additionally, an increased metabolism mediated by CYP4s is associated in acaricide resistance of Acari. For example, CYP4-d of *Amblyseius womersleyi* is overexpressed in methidathion-resistant strain and is identified as a metabolizing methidathion [25]. With the availability of genome sequence of *Tetranychus urticae*, it was also shown that the host plant affected expression of the gene CYP4CF2 [26]. In this study, our objectives are to: (1) identify differential transcription of two putative P450 genes CYP4CF1 and CYP4CL2 at different life stages as well as male and female samples, (2) investigate potential differences in transcript expression levels that might be associated with acaricide metabolism and induction, and thus (3) predict function of P450 genes in *P. citri* by comparing with P450s known in other arthropods.

2. Materials and methods

2.1. Mite preparation, bioassay and inductions

The laboratory colony of *P. citri* used in this study was originally collected from a field population from the citrus nursery in the Citrus Research Institute, Chinese Academy of Agricultural Sciences, Chongqing, China. The colony was maintained on seedlings of *Citrus reticulate* in incubators at 25 ± 1 °C, 75–80% RH with 14-h L/10-h D light cycle. The mites were maintained under acaricide-free conditions and regarded as relative susceptible strain (LS). A field population (Beibei, BB) was collected from the citrus orchards in Beibei, Chongqing, China, in 2012. As a result of bioassay (ascertained by leaf-dip bioassay method as described by Hu *et al.* [8]), the LC_{50} of Beibei population to pyridaben was 22-fold higher than that of LS.

In order to collect mites of different stages and with different induction regimes, more than 700 leaf discs were prepared. Fully expanded leaves were collected randomly from *Camellia reticulate* Blanco trees grown in orchards. The leaves were confirmed to have no prior pesticide exposure. Leaves were washed thoroughly, and water was removed from the surface. Leaf discs with a diameter of 4 cm each were placed on a 5 mm layer of water-saturated cotton in Petri dishes (9 cm diameter). Total 2700 adult females were transferred to 90 leaf discs and allowed to lay eggs for 12 h before being removed. Thirty Petri dishes with eggs were brushed into three centrifugal tubes and stored at −80 °C for RNA extraction. There were 60 discs left for collecting the larvae, nymphs (mixture of the protonymph and deutonymph), female adults, and male adults after hatching of the eggs on the surface.

Abamectin (purity, 97%), azocyclotin (purity, 95%), pyridaben (purity, 95%), and spirodiclofen (purity, 97%) (analytical standard, purchased from Sigma–Aldrich, St. Louis, MO, USA) were used to treat the adult female mites. The sub-lethal concentrations (LC_{30}) of these four acaricides against *P. citri* were defined as treatment concentration. First, 20.6 mg abamectin, 21.1 mg azocyclotin, 21.1 mg pyridaben, and 20.6 mg spirodiclofen were dissolved in 2 mL acetone. The resulting solutions were used as stock solutions with a concentration of 10,000 mg/L. Then, the four stock solutions were diluted to 0.070, 97.421, 2.997, and 0.055 mg/L with distilled water as working solutions for abamectin, azocyclotin, pyridaben, and spirodiclofen, respectively. Each leaf disc with 30 mites on the surface was dipped for 5 s in the solution. In addition, there were 120 leaf discs contained in each acaricide induction and leaf discs treated with distilled water (containing corresponding concentration of acetone alone) were used as a control. After 24 h, the surviving mites were collected and frozen at −80 °C for RNA and enzyme extraction.

2.2. Total RNA extraction and reverse transcription

Total RNA used for cloning the full length of P450 cDNAs and analyzing the profiles of two P450 genes was extracted using RNeasy® plus Micro Kit (Qiagen GmbH, Hilden, Germany) and treated with a gDNA elimination column supplied by the kit to remove genomic DNA. The total RNA was dissolved in 20 μL DEPC treated H_2O and stored at −80 °C for future use. To check the RNA quantity, the absorbance at 260 nm and the absorbance ratio of $OD_{260/280}$ were measured with a Nanovue UV–Vis spectrophotometer (GE Healthcare, Fairfield, CT). The RNA integrity was further confirmed by 1% agarose gel electrophoresis. The reverse transcription was carried out using PrimeScript® 1st Strand cDNA Synthesis Kit (Takara Biotechnology Dalian Co., Ltd., Dalian, China) and the synthesized cDNA was stored at −20 °C. The 5′ and 3′ regions of the cDNAs were obtained using the SMARTer™ RACE cDNA Amplification kit (Clontech, CA, USA) following the manufacturer's instructions.

2.3. Cloning of the full-length P450 cDNAs

2.3.1. 3′ RACE and 5′ RACE

Two CYP4 fragments CYP4N1 and CYP4N2 were obtained using degenerate PCR from our previous work [27]. Gene-specific primers (Table 1) were designed to amplify the 3′- and 5′-ends. Nested PCR was then conducted to amplify the 3′-ends. The PCR was conducted in a C1000™ Thermal Cycler (Bio-Rad Laboratories, Inc., USA). The total volume of PCR was 25 μL that contained 15.25 μL double distilled water (ddH_2O), 2.5 μL 10× PCR buffer (Mg^{2+} free), 2.0 μL Mg^{2+}, 2.0 μL dNTP (2.5 mM), 1 μL 3′ RACE templates or first round PCR product, 1 μL each primer (10 μM), and 0.25 μL rTaq™ polymerase (Takara). The PCR program was 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 62–65 °C for 30 s, and 72 °C for 30–60 s and final extension at 72 °C for 10 min. The amplification of 5′ ends was similar to 3′ RACE.

The RACE product was analyzed on a 1% agarose gel and the band of interest was purified by Gel Extraction Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). Finally, the purified DNA was cloned into a pGEM-Teasy vector (Promega (Beijing) Biotech Co., Ltd., Beijing, China). Recombinant plasmids were isolated from eight positive clones and their inserts were sequenced (Invitrogen Life Technologies, Shanghai, China).

2.3.2. Confirmation of full-length cDNA sequences

The 5′-, 3′-ends and the two P450 cDNA fragments (CYP4N1 and CYP4N2) were assembled to produce putative full-length P450 sequences by using the DNAMAN software (DNAMAN 5.2.2, Lynnon BioSoft). Based on the 5′ untranslated regions (UTR) and 3′ UTR of the cloned cDNA fragments obtained from the 5′ and 3′ RACE, a pair of primers (Table 1) were designed to amplify the open reading frames (ORF) of two P450 genes. The PCR program was as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 30 s, 54–56 °C (according to the anneal temperature of primer) for 30 s, and 72 °C for 2 min 10 s, and final extension at 72 °C for 10 min. The PCR products were recycled with the Gel Extraction Mini Kit. The recovery products were cloned into a pGEM-Teasy vector (Promega) and sequenced by the same methods as described above.

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