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Over-expression of *CYP6A2* is associated with spirotetramat resistance and cross-resistance in the resistant strain of *Aphis gossypii* Glover

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ABSTRACT

A laboratory-selected spirotetramat-resistant strain (SR) of cotton aphid developed 579-fold and 15-fold resistance to spirotetramat in adult aphids and 3rd instar nymphs, respectively, compared with a susceptible strain (SS) [26]. The SR strain developed high-level cross-resistance to alpha-cypermethrin and bifenthrin and very low or no cross-resistance to the other tested insecticides. Synergist piperonyl butoxide (PBO) dramatically increased the toxicity of spirotetramat and alpha-cypermethrin in the resistant strain. RT-qPCR results demonstrated that the transcriptional levels of *CYP6A2* increased significantly in the SR strain compared with the SS strain, which was consistent with the transcriptome results [30]. The depletion of *CYP6A2* transcripts by RNAi also significantly increased the sensitivity of the resistant aphid to spirotetramat and alpha-cypermethrin. These results indicate the possible involvement of *CYP6A2* in spirotetramat resistance and alpha-cypermethrin cross-resistance in the cotton aphid. These together with other cross-resistance results have implications for the successful implementation of resistance management strategies for *Aphis gossypii*.

1. Introduction

The cotton or melon aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is one of the most economically important insect pests in agriculture and has developed different levels of resistance to broad-spectrum insecticides, such as organophosphates, pyrethroids and carbamates, due to the extensive use of insecticides in China [1–5]. To control aphid resistance, it is important to implement resistance management strategies based on the sequential application of insecticides with different modes of action [6].

Spirotetramat, a spirocyclic tetronic/tetramic acid derivative (spirodiclofen, spiromesifen and spirotetramat), is a systemic insecticide developed for the control of sucking pests [7,8]. Spirotetramat is an inhibitor of acetyl-CoA carboxylase (ACC) and causes a significant reduction in total lipid biosynthesis in a manner similar to the tetronic acid derivatives spirodiclofen and spiromesifen [9–11]. Spirotetramat has been registered in China since 2011 under the trade name Movento and is applied as a foliar spray to control a range of sucking insects including aphids. Spirotetramat is instantly transformed to spirotetramat-enol in the plant after leaf uptake, which is distributed throughout the plant due to the insecticide's favorable physicochemical properties. Spirotetramat-enol inhibits *Myzus persicae*, *Spodoptera frugiperda*, as well as *Tetranychus urticae* ACC by interfering with the carboxyl transferase partial reaction [12]. Research indicated that one amino acid substitution (E645K) in the biotin carboxylase domain of the ACC gene is associated with spiromesifen resistance in *Trialeurodes vaporariorum* [13]. In addition to target insensitivity, cytochrome P450 monooxygenase (P450)-mediated detoxification may contribute to high level insecticide resistance. Resistance mediated by P450 monooxygenases could be the most frequent type of metabolism-based insecticide resistance [14–16]. The up-regulated transcription of P450 genes appears to be the general mechanism for increasing enzyme levels in resistant individuals [17–19]. As reported in *T. urticae*, the overexpressed cytochrome P450 (*CYP392E10*) metabolizing spirodiclofen accounts for this resistance [20]. Until now, the mechanisms of spirotetramat resistance employed by the resistant *A. gossypii* have been unclear. Recent reports showed that resistance to the tetronic acid derivative spirodiclofen was found in populations of mite *T. urticae* [21–23], *Panonychus citri* [24] and *Panonychus ulmi* [25]. The resistance ratio of UK and European populations of *T. vaporariorum* to spiromesifen was up to 26-fold [13]. These reports imply the potential resistance risk of spirotetramat in cotton aphids. The mode of action of spirotetramat is novel and lacks cross-resistance to existing chemical classes of insecticides. However, the cross-resistance situation in the SR strain warrants investigation.

In the present study, a spirotetramat-resistant strain of *A. gossypii* established in the laboratory via continuous selection with spirotetramat was used to evaluate the resistance risk and elucidate a potential

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resistance mechanism. The synergistic effects of PBO against this strain, the associated cross-resistance, and the effect of *CYP6A2* suppression on spirotetramat toxicity in this resistant strain were investigated.

2. Materials and methods

2.1. Insect

Two cotton aphid (*A. gossypii*) strains were used for this study: one spirotetramat-resistant (SR) strain and one susceptible (SS) strain [26]. The cotton aphid SS strain was collected in the field in Changchun city of Jilin province in July 2008 where limited insecticides have been applied. The aphid species was maintained without any insecticide treatment since its collection. The SR strain was established from the SS population via consecutive selection for 58 generations with increased concentrations of spirotetramat (LC_{30}) via the leaf-dipping method [27]. Because the spirotetramat has long-lasting effect against aphids, and the aphid population needs more time to recover its population density, thus a low dose (LC_{30}) was used for resistance selection. Both resistant and susceptible strains were reared on cotton plants (*Gossypium hirsutum* (L.)) in the laboratory at 20–23 °C with a photoperiod of 16:8 (L:D) h.

2.2. Chemicals and reagents

The spirotetramat formulation (Movento®, 22.4% SC) was obtained from Bayer Crop Science (Germany). Omethoate (98%), chlorpyrifos (97%), malathion (98%), acephate (90%), methidathion (40% EC), carbofuran (93%), methomyl (92%), alpha-cypermethrin (98%), cyfluthrin (97%), esfenvalerate (97%), bifenthrin (96.8%), chlorfenapyr (97%), imidacloprid (96%), and acetamiprid (97.8%) were supplied by Qingdao Hansen Biologic Science Co., Ltd. (China). Sulfoxaflor (50% WDG) was obtained from Dow AgroSciences (USA), and thiamethoxam (25% WDG) was purchased from Syngenta (Switzerland). Piperonyl butoxide (PBO, 90% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA). *Ex-Taq* DNA polymerase, RNase-free DNase I and DNA Marker DL2000 were purchased from Sangon Company (Shanghai, China). The PrimeScript™ First-Strand cDNA Synthesis kit, SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus), oligo $(dT)_{18}$ and agarose were purchased from Takara (Dalian, China). pGEM-T vector and the T7 RiboMAX™ Express RNAi System were purchased from Promega (USA).

2.3. Bioassays

A stock solution of insecticides (except the formulations) was prepared in acetone and diluted to a series of concentrations (seven concentrations) with distilled water containing 0.05% (v/v) Triton X-100 and 1% acetone. Leaves were dipped for 15 s in the required concentration of insecticide or into 0.05% (v/v) Triton X-100 water for controls and placed in the shade to air dry. Bioassays were carried out by transferring at least 35 aphids (adult or third instar nymph) onto the treated cotton leaves on each whole seeding. Bioassays were maintained in the laboratory at 20–23 °C with a photoperiod of 16:8 (L:D) h. Each concentration had three replications, and mortality was assessed (7 days for spirotetramat; 2 days for organophosphates, pyrethroids and carbamates; 3 days for neonicotinoid) and used to estimate the toxicity of the insecticides to both strains. LC_{50} values were calculated via probit analysis using POLO-PC software (LeOra Software Inc., Berkeley, CA). The resistance ratios (RRs) were estimated at the LC_{50} level as RF = LC_{50} of the SR strain/LC_{50} of the SS strain, and the 95% CLs for the RFs were calculated according to Robertson and Preisler [28].

2.4. Synergism bioassays

Insecticide toxicity in the presence or absence of synergist PBO was evaluated using the bioassay method described above. The maximum sublethal doses of PBO for the susceptible strain were determined using the bioassay method described in Section 2.3. At least five concentrations of PBO and a control were used. The maximum dose that led to zero mortality in the susceptible strain was adopted as the maximum sublethal concentration in our study. Cotton aphids (adult or third instar nymph) were exposed to cotton leaves that were treated with the PBO (final concentration of 80 mg/L) and insecticide mixtures. Mortality was recorded after 2 days for alpha-cypermethrin and 7 days for spirotetramat. The synergistic ratio was calculated using the conventional approach of dividing the LC_{50} without the synergist by the LC_{50} with PBO. The probit analysis was conducted using POLO software (LeOra Software Inc., Berkeley, CA).

2.5. Total RNA isolation, cDNA synthesis, qPCR and data analysis

Total RNA was extracted from the SR and SS aphids with TRIzol (Invitrogen, USA) according to the manufacturer's instructions and was treated with RNase-free DNase I (Takara, Japan). The RNA samples were quantified by measuring the absorbance at 260 nm, and the quality was checked via agarose gel electrophoresis. First-strand cDNA was synthesized from the total RNA (1.0 μg) using the PrimeScript™ First-Strand cDNA Synthesis Kit (Takara, Japan) with oligo $(dT)_{18}$ as a primer.

Quantitative real time PCR was performed on ABI 7500 (Applied Biosystems) using the SYBR® Premix *Ex Taq*™ II (Tli RNaseH Plus) kit (Takara, Japan). Gene primers (Table 1) were designed using Primer Premier 5.0 and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The thermal cycling protocol included an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The fluorescence signal was measured at the end of each extension step at 60 °C. After the amplification, a dissociation step cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s was performed to confirm that only the specific products were amplified. The experiment was conducted three times independently for each strain. The transcript levels of the target genes were expressed as normalized transcript abundance using GAPDH and EF1α as internal reference genes. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [29]. Significant differences between the SS and SR strains were analyzed using Student's t-test via the GraphPad InStat 3 statistical software (GraphPad Software, 2000).

2.6. Rearing on artificial diet and dsRNA feeding

Our former research indicated that *CYP6A2* was the only up-regulated P450 gene among the differentially expressed genes (DEGs) in the spirotetramat-resistant strain according to a transcriptomic survey [30]. We designed the specific primers with DNAMAN 6.0 software based on the *CYP6A2* sequences and the predicted interference site

Table 1
Primers used in real-time RT-PCR and dsRNA synthesis.

Primer name	Sequence (5′–3′)	Application
GAPDH-F	AACAGTTTTTTGAGTGGCGGT	Real-Time PCR
GAPDH-F	TGGTGTCAACTTGGATGCGTA	Real-Time PCR
EF1a-F	GAGATGCACCACGAAGCTTTAGTAGA	Real-Time PCR
EF1a-R	GAAACCACGTCTCAATTCTTTGACTG	Real-Time PCR
6A2-F	AAACACAGAATACCAACGAG	Real-Time PCR
6A2-R	ACCTAAATAAGTCCACAAGC	Real-Time PCR
Ds6A2-F1	GGTG taatacgactcactataggTACTACTGTCAGTGCCGACG	dsRNA synthesis
Ds6A2-R1	TAAGAACCGCTTGCCCA	dsRNA synthesis
Ds6A2-F2	TACTACTGTCAGTGCCGACG	dsRNA synthesis
Ds6A2-R2	GGTG taatacgactcactataggTAAGAACCGCTTGCCCA	dsRNA synthesis
DsECFP-F1	GGTG taatacgactcactataggTTACGCCAAGCTTGCATGCCT	dsRNA synthesis
DsECFP-R1	ACTCCAGCAGGACCATGTGATC	dsRNA synthesis
DsECFP-F2	TTACGCCAAG CTTGCATGCCT	dsRNA synthesis
DsECFP-R2	GGTG taatacgactcactataggACTCCAGCAGGACCATGTGATC	dsRNA synthesis