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Cross-resistance pattern and basis of resistance in a thiamethoxam-resistant strain of *Aphis gossypii* Glover

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ABSTRACT

A thiamethoxam-resistant strain of cotton aphid (ThR) displayed a 13.79-fold greater resistance to thiamethoxam than a susceptible cotton aphid (SS) strain. Piperonyl butoxide (PBO) and triphenyl phosphate (TPP) synergistically increased the toxicity of thiamethoxam in the resistant strain, whereas diethyl maleate (DEM) did not exhibit significant synergistic effects. Bioassay results indicated that the ThR strain developed increased levels of cross-resistance to bifenthrin (11.71 fold), cyfluthrin (17.90 fold), esfenvalerate (6.85 fold), clothianidin (6.56 fold), methidathion (5.34 fold) and alpha-cypermethrin (4.53 fold) but did not show cross-resistance to malathion, omethoate, acephate, chlorpyrifos, methomyl, sulfoxaflor or imidacloprid. PBO and TPP increased bifenthrin toxicity in the resistant strain by 2.38 and 4.55 fold, respectively. Quantitative real-time PCR results indicated that the mRNA expression levels of the α1, α4-1, α4-2, α5 and α7 subunits decreased significantly by 3.32, 1.60, 2.05, 5.41 and 1.48 fold, respectively, in the resistant strain compared with those in the susceptible strain. However, significant differences were not observed in the expression of the α2, α3 and β1 subunits. No target-site mutations within the α1, α2 and β1 subunits of nicotinic acetylcholine receptors (nAChRs) were detectable in the ThR strain. In conclusion, the levels of thiamethoxam resistance and cross-resistance to other insecticides observed in the ThR strain are likely regulated by two mechanisms, which include the overexpression of detoxification-related P450s and esterase. These results should be useful for the understanding thiamethoxam resistance mechanism and the management of insecticide-resistant cotton aphids in China.

1. Introduction

The cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) is one of the most economically important insect pests in agriculture. These insects cause damage via direct feeding, plant virus transmission and photosynthesis inhibition via the facilitation of black sooty mold that grows on the honeydew secreted by aphids [1]. Additionally, *A. gossypii* Glover has developed different levels of resistance to broad-spectrum insecticides, including organophosphates, pyrethroids, carbamates and neonicotinoids [2–4].

Neonicotinoid insecticides are highly effective at controlling strains of *A. gossypii* resisting to insecticides, such as organophosphate and carbamate. Thiamethoxam and imidacloprid belong to the family of systemic pesticides classified as neonicotinoid insecticides, which are the most widely applied. These insecticides irreversibly bind to the nicotinic acetylcholine receptors (nAChR) of cells in the nervous system and interfere with the transmission of nerve impulses in insects [5,6]. The extensive and repetitive use of neonicotinoids has led to the development of resistance in the planthopper *Nilaparvata lugens* [7], *Bemisia tabaci* [8–10], *Myzus persicae* [11,12] and *A. gossypii* [13]. Chen et al. [14] reported that the field populations of *A. gossypii* have developed high levels of resistance to imidacloprid, acetamiprid and sulfoxaflor in China (the LC_{50} values were >5000 mg L^{-1}, >5000 mg L^{-1} and 868.19-mg L^{-1} for imidacloprid, acetamiprid and sulfoxaflor, respectively) [14]. Since the 1990s, neonicotinoid insecticides have been used to control a range of sucking insects, including aphids. These reports demonstrate that cotton aphids may also be at risk of developing resistance to thiamethoxam.

Target-site resistance is an important mode of adaptation in which the activities of altered target proteins are blocked by various toxins, and it is involved in synthetic toxin tolerance in insect pests [15]. Target-site insensitivity of nAChRs is conferred by a mutation (Y151S) in the α1 and α3 subunits of imidacloprid-resistant *N. lugens* [7] or a mutation (R81T) in the β1 subunit of imidacloprid-resistant *Myzus persicae* and *A. gossypii* [14,16]. Two novel mutations in the β1 subunit, V62I and K264E, were first detected in imidacloprid-resistant *A. gossypii* [14].

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Beyond noting the qualitative changes in insecticide target sites, few reports have described the quantitative changes in target proteins associated with resistance. Studies have also indicated that the reduced expression of the α2 and α8 nAChR subunits was correlated with imidacloprid resistance in *Musca domestica* and *N. lugens*, respectively [17,18]. The expression levels of the α10 and β1 subunits increased and the β2 subunit decreased in imidacloprid-, thiamethoxam-, or clothianidin-treated *Acyrthosiphon pisum* [19]. In addition to target-site insensitivity, metabolic mechanisms related to resistance include cytochrome P450 monooxygenases (P450s), esterases (especially carboxylesterases, CarEs), and glutathione S-transferases (GSTs) [20]. The enhanced oxidative detoxification related to P450 overexpression is an important biochemical mechanism for neonicotinoid resistance. P450s belong to a diverse class of enzymes with many functions that range from biosynthesis to xenobiotic metabolism [21,22]. The significant overexpression of *CYP6ER1*, *CYP6CM1*, and *CYP6CY3* accounts for high levels of resistance to neonicotinoids in *N. lugens* [23], *B. tabaci* [10,24,25] and *M. persicae* [11]. Whether *A. gossypii* employs similar strategies to increase insecticide tolerance is unknown.

In the present study, a thiamethoxam-resistant strain of *A. gossypii* established in the laboratory via continuous selection with thiamethoxam was used to evaluate the risk of resistance and elucidate potential resistance mechanisms. The synergistic effects of PBO against this strain, the associated cross-resistance, and the transcriptional levels of nAChRs and potential resistance-associated mutations were investigated. These results should be useful for the understanding thiamethoxam resistance mechanism and will help promote the efficient control of this pest and provide important insights for the successful implementation of resistance management strategies for *A. gossypii*.

2. Materials and methods

2.1. Insects

Two cotton aphid (*A. gossypii*) strains were used for this study. One strain was resistant to thiamethoxam (ThR), and the other was susceptible (SS) [26]. The SS strain was collected in 2008 from Jilin Province, 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 with increased concentrations of spirotetramat (LC_{30}) via the leaf-dipping method. Both the resistant and susceptible strains were reared on cotton plants [*Gossypium hirsutum* (L.)] in the laboratory at 20 °C–23 °C with a photoperiod of 16 h:8 h (light:dark).

2.2. Chemicals

Piperonyl butoxide (PBO), triphenyl phosphate (TPP), and diethyl maleate (DEM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Omethoate (98%), chlorpyrifos (97%), malathion (98%), acephate (90%), methidathion (40% EC), methomyl (92%), alpha-cypermethrin (98%), cyfluthrin (97%), esfenvalerate (97%), bifenthrin (96.8%), imidacloprid (96%), and clothianidin (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). All other chemicals and solvents were reagent grade. 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).

2.3. Bioassays

A stock solution of insecticides (except the formulations) was prepared in acetone and diluted to a series of concentrations (six or 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 the control treatment and then placed in the shade and allowed to air dry. Bioassays were conducted by transferring at least 25 apterous adult aphids onto the treated cotton leaves on each whole seeding. Bioassays were maintained in the laboratory at 20 °C–23 °C with a photoperiod of 16 h:8 h (light:dark). Three replicates were performed for each concentration, and mortality was assessed (after 1 day for pyrethroids; after 2 days for organophosphates and carbamates; and after 3 days for neonicotinoid) and used to estimate the toxicity of the insecticides to both strains. The LC_{50} values were calculated via the probit analysis using POLO-PC software (LeOra Software Inc., Berkeley, CA, USA). Resistance factors (RFs) were estimated at the LC_{50} level. RF = LC_{50} of the ThR strain/LC_{50} of the SS strain. The 95% CIs for the RFs were calculated according to Robertson and Preisler [27].

2.4. Synergism bioassays

The maximum dose that led to zero mortality in the SS strain was considered the maximum sublethal concentration in our study. The maximum sublethal doses of PBO, DEM and TPP for the SS strain were determined using the bioassay method described in Section 2.3. A minimum of six concentrations, which included a control (acetone alone), were used for each synergist. The aphids were exposed to cotton leaf discs (15-mm diameter) treated with PBO, DEM or TPP (at the maximum sublethal dose). The final concentrations of PBO, DEM and TPP were 80 mg L^{-1}, 40 mg L^{-1}, and 40 mg L^{-1}, respectively. Mortality was recorded after different times for the insecticides as described in Section 2.3. The synergistic ratio was calculated using the conventional approach that divides the LC_{50} without the synergist by the LC_{50} with the synergist. The probit analysis was conducted using POLO software (LeOra Software Inc., Berkeley, CA).

2.5. Total RNA isolation, cDNA synthesis, nAChRs cloning and sequencing

Total RNA was extracted from the ThR and SS aphids using TRIzol (Invitrogen, USA) according to the manufacturer's instructions and then 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 using the PrimeScript™ First-Strand cDNA Synthesis kit (Takara, Japan) with oligo$(dT)_{18}$ as a primer.

To identify the potential nAChR mutations, three pairs of primers (Table 1) were designed based on the α1, α2 and β1 subunit sequences of the cotton aphid (Supplementary data 1). The PCR products of nAChRs cDNA from both strains were eluted from the gel using the TIANgel Midi Purification Kit (Tiangen) and then directly cloned into the pGEM-T easy vector (Promega). The positive clones were selected for sequencing (10 clones from the ThR strain and 10 clones from the SS strain derived from each primer pair).

2.6. Quantitative RT-PCR and data analysis

Quantitative real-time PCR was performed on the ABI 7500 platform (Applied Biosystems) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan). Gene-specific primers for real-time PCR (Table 1) were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The thermal cycling protocol included initial denaturation at 95 °C for 30 s and then 40 cycles at 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 amplification, one dissociation step cycle of 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 housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1-alpha (EF1a) were used as internal reference genes for *A. gossypii*. Relative gene expression