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VGSC Mutations and P450 Overexpression are Associated with Beta-Cypermethrin Resistance in *Aphis gossypii* Glover from a Chinese Wolfberry (*Lycium barbarum* L.) Field

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Abstract

Chinese wolfberry (*Lycium barbarum* L.), a specialty crop of ecological, medical and economic values in Ningxia province of China, is subjected to severe *Aphis gossypii* Glover damage. Currently, *A. gossypii* populations showing extremely high-level resistance to beta-cypermethrinin in the major wolfberry planting areas in Ningxia. The specific resistance mechanisms, however, are still not known. In this work, we collected a field *A. gossypii* strain (HSP) from a wolfberry orchard of Ningxia in 2021 using a single-time sampling method and its resistance to beta-cypermethrin was determined to be extremely high (994.74–fold) as compared with a susceptible strain (SS). Then we explored the potential resistance mechanisms from two aspects of metabolic detoxification and target-site alterations. Bioassay of beta-cypermethrin with or without the synergist showed that piperonyl butoxide (PBO) significantly increased the toxicity of beta-cypermethrin (4.72–fold) to the HSP strain while triphenyl phosphate (TPP) and diethyl maleate (DEM) exhibited no significant synergistic effects. Correspondingly, the *O*-demethylase activity of the cytochrome P450s in the HSP strain was 1.68–fold higher than that in the susceptive strain (SS), whereas changes of carboxylesterases and glutathione *S*-transferases in their activities were unremarkable. Also, fifteen upregulated P450 genes were identified by both RNA-Seq and qRT-PCR technologies, containing eleven CYP6 genes, three CYP4 genes and one CYP380 gene. Especially, five CYP6 genes of high relative expression levels (> 3.00–fold) were intensively expressed by the beta-cypermethrin induction in the HSP aphids. These metabolism-related results indicate the key role of the P450-mediated metabolic detoxification in the HSP resistance to beta-cypermethrin. Sequencing of voltage-gated sodium channel (*VGSC*) genes identified a prevalent M918L mutation and a new G1012D mutation in the HSP *A. gossypii*. Moreover, heterozygous 918M/L and 918M/L+G1012D mutations were the dominant genotypes with frequencies of 60.00% and 36.67% in the HSP population, respectively. Overall, *VGSC* mutations along with P450-mediated metabolic resistance were contributed to the extremely high resistance of the HSP wolfberry aphids to beta-cypermethrin, providing support for *A. gossypii* control and resistance management in the wolfberry planting areas of Ningxia using insecticides with different modes of action.

Keywords: Chinese wolfberry; *Aphis gossypii*; beta-cypermethrin resistance; cytochrome P450s; *VGSC* mutations

1. Introduction

Chinese wolfberry, a genus of deciduous perennial shrub (*Lycium barbarum* L.) of the Solanceae family, distributes in the northwest, arid regions of China, which is a crop of high ecological values

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with capacities of ameliorating wind erosion and improving soil structure [1]. Moreover, the wolfberry fruits have also been widely used as a foodstuff, as well as an herbal medicine [2], which makes it a crop of economic importance. Currently, Ningxia wolfberry represents the largest cultivated area in China, due to the suitable natural conditions producing wolfberries of the best nutraceutical quality with the highest market value [3,4]. However, in recent years, Chinese wolfberry has been suffering from severe *Aphis gossypii* Glover damage in the Ningxia province of China [5,6]. Chemical insecticides have been intensively employed to control *A. gossypii* in many agricultural areas of China [7]. Beta-cypermethrin is a representative member which was also commonly used to control *A. gossypii* on the Chinese wolfberry plants in Ningxia. Due to the long-term over-application, *A. gossypii* has developed different levels of resistance to beta-cypermethrin on multiple crops in China [7–9]. Our previous study revealed that the *A. gossypii* populations in 8 major wolfberry planting areas of Ningxia regions had all developed extremely high-level resistance to beta-cypermethrin, with resistance ratios ranging from 2539.70 to 31916.00[10]. However, few studies have focused on the resistance mechanisms of *A. gossypii* populations to beta-cypermethrin in the Chinese wolfberry fields of Ningxia. Thus, in this study, we expand our previous study to explore the deeper molecular characterization underling the beta-cypermethrin resistance of a field highly resistant population, in terms of metabolic detoxication and discovery of new target-site mutations.

A. gossypii can be resistant to insecticides by four major ways [7,11,12], including target-site insensitivity, enhanced detoxification, reduced penetration, and behavioral avoidance or apastia. Notably, metabolic mechanism governed by the increased activity or overexpression of detoxifying enzymes, as well as target-site mechanism in terms of insect sodium channel mutations, has been frequently reported to account for pyrethroid resistance in multiple aphid species [7,13–15]. Three groups of detoxifying enzymes, cytochrome P450 monooxygenases (P450s) [16–19], carboxylesterases (CarEs) [20–22] and glutathione *S*-transferases (GSTs) [23–25], were demonstrated to play key roles in beta-cypermethrin resistances to diverse pest insects, including aphid species [7,18,26]. Besides, P450s were verified to potentially contribute to the pyrethroid resistance by the increased activity in *A. gossypii*, including three cypermethrin pesticides of beta-cypermethrin [7], cypermethrin [27] and alpha-cypermethrin [8]. Of note, the major mechanism responsible for the pyrethroid resistance in insects is attributed to point mutations in the target protein of the voltage-gated sodium channel (VGSC), reducing the sensitivity to pyrethroids [14,15,28]. There are up to now more than 50 *VGSC* mutations identified to be associated with pyrethroid resistance in various invertebrates, and the majority mutation sites locate in the domain II, a potential pyrethroid-binding site within the VGSC [14,15]. Among those, the super knockdown resistance (*kdr*) mutation, M918L in the linker connecting DIIS4 and DIIS5, has been widely detected in pyrethroid-resistant *A. gossypii* populations [7,29–31], producing extremely high resistance levels to beta-cypermethrin (> 2000.00) [7]. Apart from existing independently, the M918L mutation also occurs in combination with other mutation sites to enhance the pyrethroid-resistant levels in some insect species [14,32]. It is noteworthy that the co-occurrence of M918L with other point mutations has been scarcely observed in the field populations of *A. gossypii*. Therefore, one of the objectives of this study is to explore whether the co-occurring VGSC mutations are involved in the beta-cypermethrin resistance in wolfberry *A. gossypii*. Yet, to our knowledge, the roles of the three detoxification enzymes and *VGSC* mutations remain unclear in the beta-cypermethrin resistance of the Chinese wolfberry aphids in Ningxia regions.

In the present study, we collected a beta-cypermethrin resistant *A. gossypii* strain (HSP) from a Chinese wolfberry orchard in a major growing area of Ningxia wolfberry (Wuzhong city). Subsequently, to elucidate the potential roles of P450s, CarEs and GSTs in beta-cypermethrin resistance in the *A. gossypii* strain, we performed synergistic bioassays, as well as enzyme activity assays, to confirm their effects. Further, we carried out a comparative transcriptome analysis to identified the overexpression of detoxification enzyme genes associated with the beta-cypermethrin resistance. According to the transcriptome variations, we also measured the expression levels of the upregulated P450s genes involved in beta-cypermethrin resistance in the *A. gossypii* resistant strain,

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using a quantitative real-time PCR assay. Moreover, the potential mutations in *VGSC* genes and their frequencies were detected to reveal the *VGSC* genotype of the resistant strain. Our findings provide valuable insights into the potential resistance mechanisms of Ningxia wolfberry *A. gossypii* to pyrethroids and will also contribute to the design of the improved strategies for the resistance management.

2. Materials and Methods

2.1. Chemicals

Beta-cypermethrin (96.6% purity) was obtained from Shaanxi Meibang Pesticide Co., Ltd. (Xi'an, China). Piperonyl butoxide (PBO, 95.0% purity), triphenyl phosphate (TPP, 99.0% purity) and diethyl maleate (DEM, 99.0% purity) were purchased from Sun Chemical Technology Co., Ltd. (Shanghai, China). Tween 80 (99.0% purity), ethylenediamine tetraacetic acid (EDTA, 99.5% purity), dithiothreitol (DTT, ≥ 97.0% purity), phenylmethylsulfonyl fluoride (PMSF, ≥ 98.0% purity), glycerol (99.0% purity), α-naphthyl acetate (α-NA, 98.0% purity), 1-chloro-2,4-dinitrobenzene (CDNB, ≥ 99.0% purity), and p-nitroanisole (PNOD, 98.0% purity) were purchased from Shanghai Aladdin Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Insects

The relatively susceptible colony of *A. gossypii* (SS) was originally collected in 2021 from the wild Chinese wolfberry plants in Yinchuan area of Ningxia (latitude 38.47N, and longitude 106.21E), China. This population was ascertained to be relatively susceptible to beta-cypermethrin compared with the field population by laboratory bioassays. The resistant *A. gossypii* field population was collected in 2021 from a Chinese wolfberry plantation in the Hongsipu district of Wuzhong City in the Ningxia province of China (termed HSP strain, latitude 37.37N, and longitude 106.07E). These two *A. gossypii* populations were reared separately on Wolfberry plants in mesh cages (60 × 60 × 150 cm) without exposure to any insecticide under the controlled conditions with 25±1ºC, and 16:8-h light/dark photoperiod. After collection, each aphid strain was reared indoors for three generations (about 40 days) under standardized conditions without any selection pressure before being subjected to experiments.

2.3. Bioassays

The toxicity of beta-cypermethrin to *A. gossypii* was determined using a previously described leaf-dipping method [7]. Prior to the bioassays, beta-cypermethrin was dissolved in acetone and diluted with distilled water containing 0.01% (v/v) tween 80 to prepare the stock solutions. Preliminary range-finding assays were conducted to define the concentration range of beta-cypermethrin. Five beta-cypermethrin concentrations (625, 1250, 2500, 5000 and 10000 μg·mL^{-1}) were used to evaluate its toxicity to the HSP *A. gossypii*. To determine the toxicity of beta-cypermethrin to the SS *A. gossypii*, the final beta-cypermethrin concentrations were set as 0.5, 1.0, 2.5, 5.0 and 7.5 μg·mL^{-1}, respectively. Wolfberry leaves of relatively identical morphology were dipped for 15 s in an insecticide solution of a desired concentration or in distilled water containing 0.01% (v/v) tween 80 as a control. Treated leaves were air dried naturally, then placed upside down in 90-mm Petri dishes (five leaves per Petri dish), with each bottom lined with a sheet of 9-cm moist filter paper. Uniform apterous *A. gossypii* adults were selected and transferred onto the treated leaves (five individuals per leaf). Each Petri dish was then covered with a layer of transparent plastic wrap with pinholes, and incubated in a growth cabinet at 25±1ºC with a 16:8-h light/dark photoperiod. Each treatment was carried out in triplicate, and at least 30 aphids were monitored for each repetition. Aphid mortality was checked after 24-h incubation. The median lethal concentration (LC_{50} value) was calculated using the probit package of SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). The confidence intervals of

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LC_{50} values were compared statistically to assess significance between the two strains at $P < 0.05$. Resistance ratio (RR) was estimated using the LC_{50} value of the SS strain as the factor divisor [16,17].

2.4. Synergistic Bioassays

To reveal the enzyme inhibitor synergism effects, stocks of PBO, TPP, and DEM were prepared in acetone and diluted with distilled water containing 0.01% (v/v) tween 80. The maximum dose that generated no more than 10% mortality in both HSP and SS strains was utilized as the testing concentration in this study [7]. The maximum sublethal doses of PBO, TPP and DEM for the SS strain were determined to be 50 mg·L^{-1}, 20 mg·L^{-1}, and 30 mg·L^{-1}, respectively, according to the bioassay method depicted in Section 2.3. Synergistic bioassays were performed by a previously described method [12]. Briefly, apterous *A. gossypii* adults were exposed to wolfberry leaves that were pretreated with desired insecticide solutions containing an enzyme inhibitor at its maximum sublethal concentration. The subsequent operations were performed as described in Section 2.3. The synergistic ratio (SR) was defined as the ratio of the LC_{50} value without synergist to the LC_{50} value with synergist [7,12].

2.5. Enzyme Activity Analysis

Aliquots of *A. gossypii* samples (50 mg) from different strains were separately homogenized on ice in the corresponding buffer solutions of 4 mL of 40 mM phosphate buffer (pH 7.0) for the CarEs and GSTs assay [33,34], 1 mL of 100 mM phosphate buffer (pH 7.6, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% glycerol) for the P450s assay [33]. The obtained homogenates were centrifuged at 12,000 rpm for 20 min at 4°C, and then the supernatants were collected as the enzyme sources. The protein content of each enzyme source was determined according to the Bradford method [35], using a PC0010 Bradford Protein Assay Kit (Solarbio, Beijing, China).

CarEs activity was determined using α-NA as a substrate according to the procedures described by Shi et al. [33]. GSTs activity was measured using CDNB as a substrate following the method described by Shi et al [33]. Following the procedures described by Shi et al. [33], the activity of P450s was assayed by detecting the *O*-demethylation of PNOD. Each detoxification enzyme activity assay was performed with three biological replicates and three technical replicates.

2.6. Transcriptome Sequencing and Analysis

100 mg of apterous adults of SS and HSP strains with the identical conditions were used for total RNA extraction. Total RNA samples were prepared using a RNAiso Plus kit (Takara, Dalian, China) following the manufacturer's instructions. The total RNA quality was monitored by 1% agarose gel electrophoresis, as well as a Thermo Nanodrop 2000 spectrophotometer (Wilmington, USA) *via* the values of $OD_{260/280}$ and $OD_{260/230}$. Three replicates were carried out for each strain.

Library construction and sequencing were completed at Biomarker Technologies Company Ltd. (Qingdao, China) with three replicates for each strain. The cDNA library sequencing was performed by an Illumina HiSeq 2500 sequencing platform generating paired-end reads. High-quality clean reads were obtained by removing sequencing adapters, poly-N regions, and low-quality reads from the raw sequencing data. The obtained high-quality sequencing data was mapped to the *A. gossypii* genome ASM401081v1 [36] as a reference using HISAT2 [37]. StringTie [38] was then applied to the assembly of the mapped reads to reconstruct a unigene library. To eliminate effects of gene lengths and sequencing levels, read counts were standardized with fragments per kilobase of transcript per million mapped fragments (RPKM). The DEseq2 package was used to remove the possible batch effects prior to DEG analysis, and then to identify the differentially expressed genes (DEGs) between the SS and HSP strains, with false discovery rate (FDR) values ≤ 0.01 and absolute values of $|\log_2\text{Fold Change}| \geq 1.0$ as the threshold for significantly differential expression [39]. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to predict the functions and molecular pathways of DEGs [39]. DEGs encoding cytochrome P450

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enzymes were screened according to the DEGs function annotations. Then the obtained P450 DEGs sequences were realigned by blastx in NCBI [40]. Finally, the amino acid sequences of the P450 DEGs and their respective highly homologous protein sequences of identified families were used to construct a phylogenetic tree to determine the preliminary classification of the P450 DEGs [41], using the MEGA 12.0 program.

2.7. Reverse Transcriptase Quantitative PCR (qRT-PCR) Analysis

RNA extraction was carried out according to the protocols described above (Section 2.6). First-strand cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). qRT-PCR was performed in an Applied Biosystems StepOnePlus Real-Time System (Waltham, MA, USA), using the SYBR Select Master Mix (Applied Biosystems). Ten DEGs were selected randomly for qRT-PCR to validate the transcriptome results. The relative expression of 17 up-regulated P450s genes (termed as *AgoCYPup1* to *AgoCYPup17*) was also determined by this method. Beta actin (*β-ACT*), a house keeping gene, was used as the internal reference gene for *A. gossypii* [42]. Gene primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, USA) and the optimal primers are listed in Table S1 *via* primer optimization. The amplification program is present in Table S2. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Three independent biological replicates were carried out for each experiment.

2.8. VGSC Mutations Analysis in A. gossypii

Total RNA was extracted from apterous adults of *A. gossypii* as described in Section 2.6. The full-length of *VGSC* genes (*VGSC1* and *VGSC2*) were amplified with primer pairs listed in Table S3 using a Takara PrimeSTAR® GXL DNA Polymerase (Dalian, China). The major mutant regions of *VGSC1* (DIIS1–DIIS6) and *VGSC2* (DIIIS6–DIVS4) fragments were also amplified with specific primer pairs (Table S3). The PCR amplification program for *VGSC* mutation analysis is listed in Table S4. PCR products were directly subjected to paired-end sequencing [15] by TSINGKE Biological Technology (Beijing, China).

2.9. Determination of VGSC Mutation Frequencies in A. gossypii

Genomic DNA was extracted from 30 individual adults of the HSP population with an Omega Bio-tek EZNA® Insect DNA Kit (Norcross, GA, USA). A specific primer (Table S3) was used to amplified the genomic DNA fragment of *VGSC1* gene covering the detected mutation sites (DIIS4–DIIS6 containing intron sequences) using the Takara PrimeSTAR® GXL DNA Polymerase (Dalian, China). PCR products were obtained according to a recommended procedure in the instruction manual (Table S4), and directly subjected to sequencing in both directions by TSINGKE Biological Technology (Beijing, China).

2.10. Statistical Analysis

Significant differences were analyzed in SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) using the Student's t-test, with a significance level set at $P \leq 0.05$. The classification of resistance levels referred to the standards of the World Health Organization (WHO): extremely high resistance ≥160-fold ≥high resistance ≥40-fold ≥moderate resistance ≥10-fold ≥low resistance ≥5-fold ≥decreased susceptibility ≥3-fold ≥susceptibility [15,43]. Nucleotide sequences were analyzed using Chromas 2.6.6 and GeneDoc 2.7.

3. Results

3.1. Resistance of the HSP A. gossypii To Beta-Cypermethrin

The susceptibilities of the field-collected *A. gossypii* population HSP and SS strain to beta-cypermethrin were evaluated with results listed in Table 1. The SS strain showed a very low LC_{50}

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value of 2.33 μg·mL^{-1} to beta-cypermethrin. The LC_{50} value of the HSP strain (2317.74 μg·mL^{-1}), however, was determined to be significantly higher than that of the SS, indicating the extremely high-level resistance of HSP strain to beta-cypermethrin with a RR (resistance ratio) value of 994.74.

Table 1. Resistance of HSP and SS *A. gossypii* populations against beta-cypermethrin and the synergistic effects of PBO, TPP and DEM on the beta-cypermethrin toxicity in resistant HSP *A. gossypii.*

Strain	Insecticide/synergist[1]	Slope ± SE	LC_{50} (μg·mL^{-1}) (95% CL)	Chi-square	RR[2]/SR[3]
SS	beta-cypermethrin	1.11 ± 0.12	2.33 (1.54 – 3.18)*4	0.42	—
HSP	beta-cypermethrin	2.02 ± 0.16	2317.74 (2025.90 – 2644.88)	5.30	994.74
	beta-cypermethrin + PBO	1.74 ± 0.18	491.23 (363.19 – 611.38)*	2.26	4.72
	beta-cypermethrin + TPP	2.04 ± 0.19	2169.48 (1864.02 – 2533.14)	4.85	1.07
	beta-cypermethrin + DEM	1.63 ± 0.16	2441.71 (2044.55 – 2919.19)	3.00	0.95

[1] The final concentrations of PBO, TPP and DEM were 50 mg·L^{-1}, 20 mg·L^{-1} and 30 mg·L^{-1}, respectively. [2] RR refers to resistance ratio relative to the SS strain. [3] SR refers to synergistic ratio within the HSP strain. Asterisk (*) represents significant difference in the LC_{50} value of a treatment as compared with that of beta-cypermethrin alone against the HSP strain at $P < 0.05$.

3.2. Effect of Synergists on A. gossypii Resistance to Beta-Cypermethrin

The toxicity of beta-cypermethrin with a synergist (PBO, TPP or DEM) to the HSP population was monitored with results shown in Table 1. Only PBO significantly increased the beta-cypermethrin toxicity, displaying a much lower LC_{50} value of 491.23 μg·mL^{-1}. The SR (synergistic ratio) value was determined to be 4.72 with PBO treatment. Nevertheless, both TPP and DEM exerted no distinct effects on the toxicity of beta-cypermethrin against the HSP *A. gossypii*, exhibiting equivalent LC_{50} values to the SS strain with 2169.48 μg·mL^{-1} for TPP and 2441.71 μg·mL^{-1} for DEM, respectively. These results indicate that the metabolic resistance oriented by cytochrome P450 enzymes was involved in the beta-cypermethrin resistance of the HSP *A. gossypii*.

3.3. Detoxification Enzyme Activities

The differences in three metabolic enzyme activities were compared between the SS and HSP strains. As illustrated in Figure 1, there were no significant differences observed in both the carboxylesterases (CarEs) and glutathione *S*-transferases (GSTs) activities between the susceptible and resistant strains, whereas the cytochrome P450 *O*-demethylase activity of the HSP strain was 1.68–fold higher than that of the SS strain. These findings provide evidence for the key role of cytochrome P450 metabolism in the HSP *A. gossypii* resistance to beta-cypermethrin, consistent with the PBO synergistic effect on the toxicity of beta-cypermethrin.

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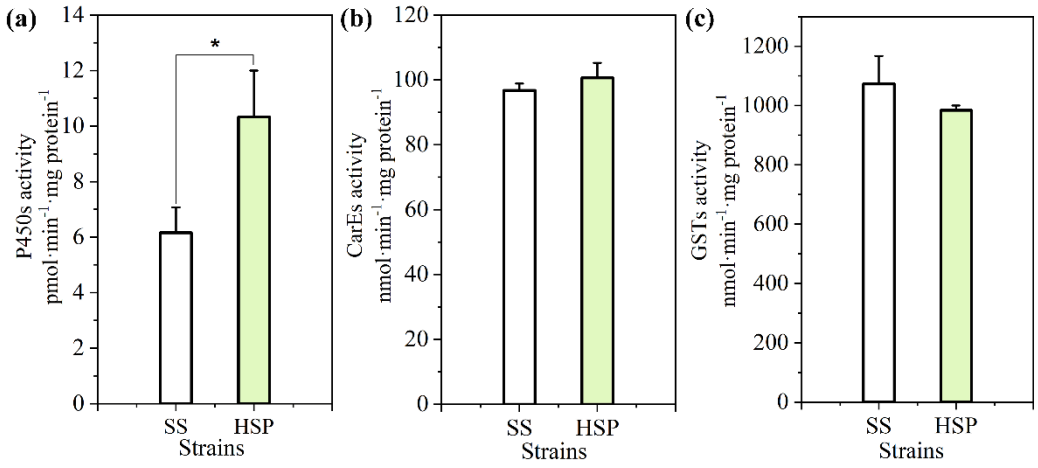

Figure 1. Detoxification enzyme activities of cytochrome P450 monooxygenases (P450s) (a), carboxylesterases (CarEs) (b) and glutathione *S*-transferases (GSTs) (c) in the SS and HSP strains of *A. gossypii*. The enzyme activities were expressed as nanomoles or picomoles of substrate converted per minute per milligram of extracted protein, and 50 mg of each *A. gossypii* sample was used for detection. The error bars represent means ± standard deviations. The asterisk (*) in plot represents significant difference between the SS and HSP strains at $P < 0.05$.

3.4. Comparative Transcriptomics of the SS and HSP Strains

To gain insights into the molecular variations underlying the beta-cypermethrin resistance of the HSP *A. gossypii*, transcriptome profiling assays were performed to identify the relative differentially expressed genes (DEGs) as compared with the SS strain. Each cDNA library produced a data pool containing clean reads ranging from 39722998 to 63552688, and mapped reads ranging from 37260918 to 56654337 with mapped ratios above 88.74% (Table S5). The Q30 percentages were over 94.72% for all the sample reads, indicating that the Illumina RNA-Seq data obtained are reliable. A total of 913 DEGs were identified in the HSP strain, with 766 up-regulated genes and 147 down-regulated genes (Table S5). The expression level difference and statistically significant degree of all DEGs were illustrated by a volcano plot (Figure 2a). To verify the accuracy of the transcriptome sequencing results for DEGs, five up-regulated and five down-regulated genes were randomly selected and their expression levels were measured by the qRT-PCR technology. As depicted in Figure 2b, the expression patterns of these ten DEGs were highly consistent by these two methods with a high correlation (r = 0.94), indicating that the RNA-Seq data are credible. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was further performed to determine the specific biochemical pathways of the identified DEGs with results shown in Figure 2c. Among the top 20 enriched KEGG pathways, those related to substance metabolism account for 65%. Moreover, two P450- related KEGG pathways, metabolism of xenobiotics by cytochrome P450 and drug mentalism-cytochrome P450, were significantly enriched in the top 10 KEGG pathways. These results imply that the detoxification effect of cytochrome P450 metabolism may participate in the development of the beta-cypermethrin resistance in the HSP wolfberry aphids.

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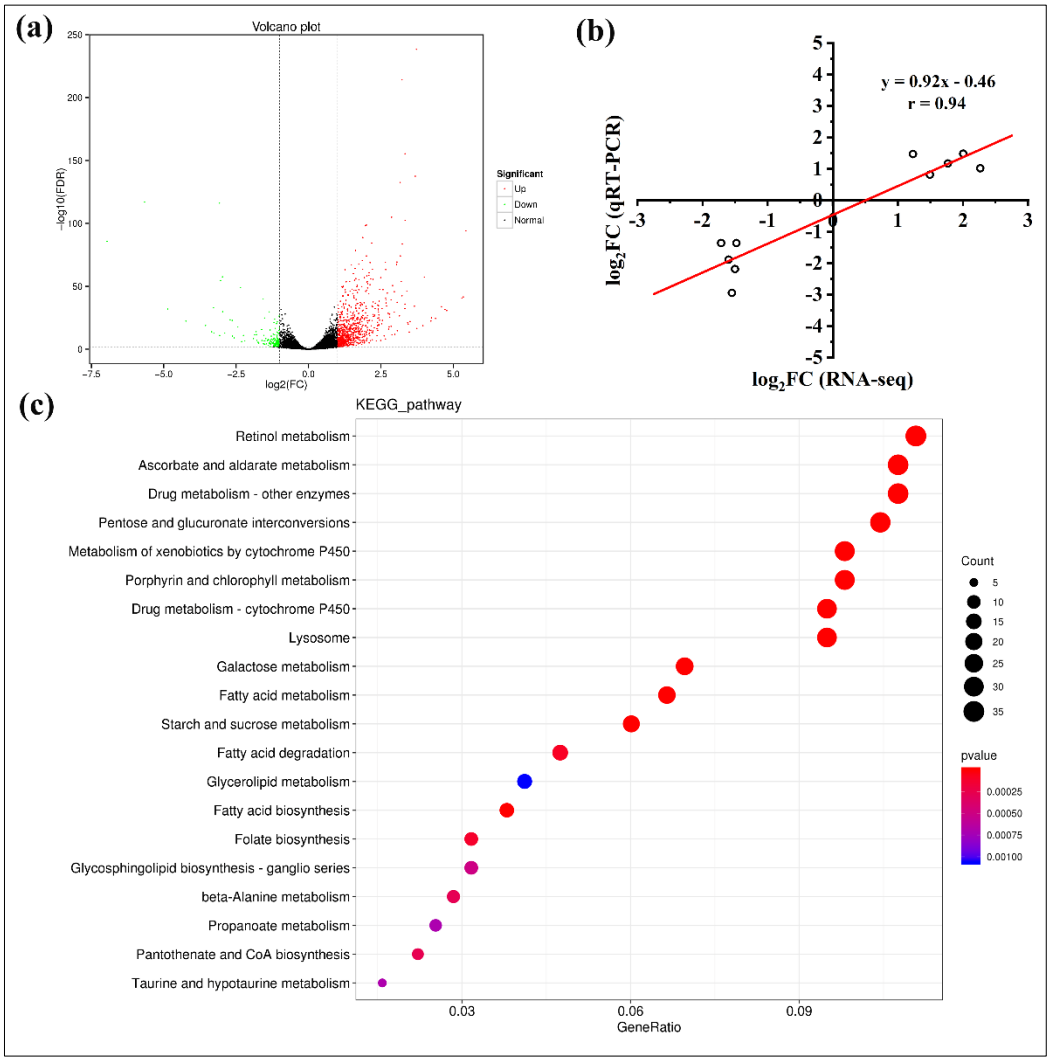

Figure 2. (a) Volcano plot of all differentially expressed genes (DEGs) between the SS and HSP strains with green spots showing down-regulated DEGs, and red spots showing up-regulated DEGs. (b) Correlation between the logarithmic fold changes of qRT-PCR (y-axis) and RNA-Seq (x-axis) in the gene expression levels. (c) Top 20 of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. DEGs were identified using false discovery rate (FDR) values ≤ 0.01 and absolute values of |log2Fold Change| ≥ 1.0 as the cutoff thresholds. Ten DEGs were selected randomly for the correlation analysis.

3.5. Cytochrome P450 DEGs and phylogenetic Analysis

Given the facts of the significant synergistic effect of PBO on the beta-cypermethrin toxicity to the HSP strain, the enhancement of P450 *O*-demethylase activity in the HSP strain, and two P450-related metabolism pathways enriched in the top 10 KEGG pathways, we analyzed the *A. gossypii* transcriptome data of the SS and HSP strains, and obtained 17 up-regulated cytochrome P450 genes (*AgoCYPup1–AgoCYPup17*) (Table S6). No down-regulated cytochrome P450 DEGs were screened by the gene function annotations of Nr, Pfam and Swiss-Prot databases. Phylogenetic analysis was further carried out using the putative amino acid sequences of these P450 DEGs and other P450 amino acid sequences from multiple insects (*Myzus persicae* Sulzer, *Rhopalosiphum padi* L., *Aphis craccivora* Koch, *Acyrthosiphon pisum* Harris and *Aphis glycines* Matsumura), to determine their specific family classification. Figure 3 shows that these P450 DEGs were classified into three clades and four families of P450 genes [41]. Eleven of P450 DEGs, *AgoCYPup1* to *AgoCYPup6*, *AgoCYPup11*, and *AgoCYPup14* to *AgoCYPup17*, belong to clade CYP3 family CYP6. *AgoCYPup7* belongs to clade CYP2 family CYP305. Four of P450 DEGs, *AgoCYPup8* to *AgoCYPup10*, and *AgoCYPup13*, belong to clade CYP4 family CYP4. *AgoCYPup12* belongs to clade CYP4 family CYP380.

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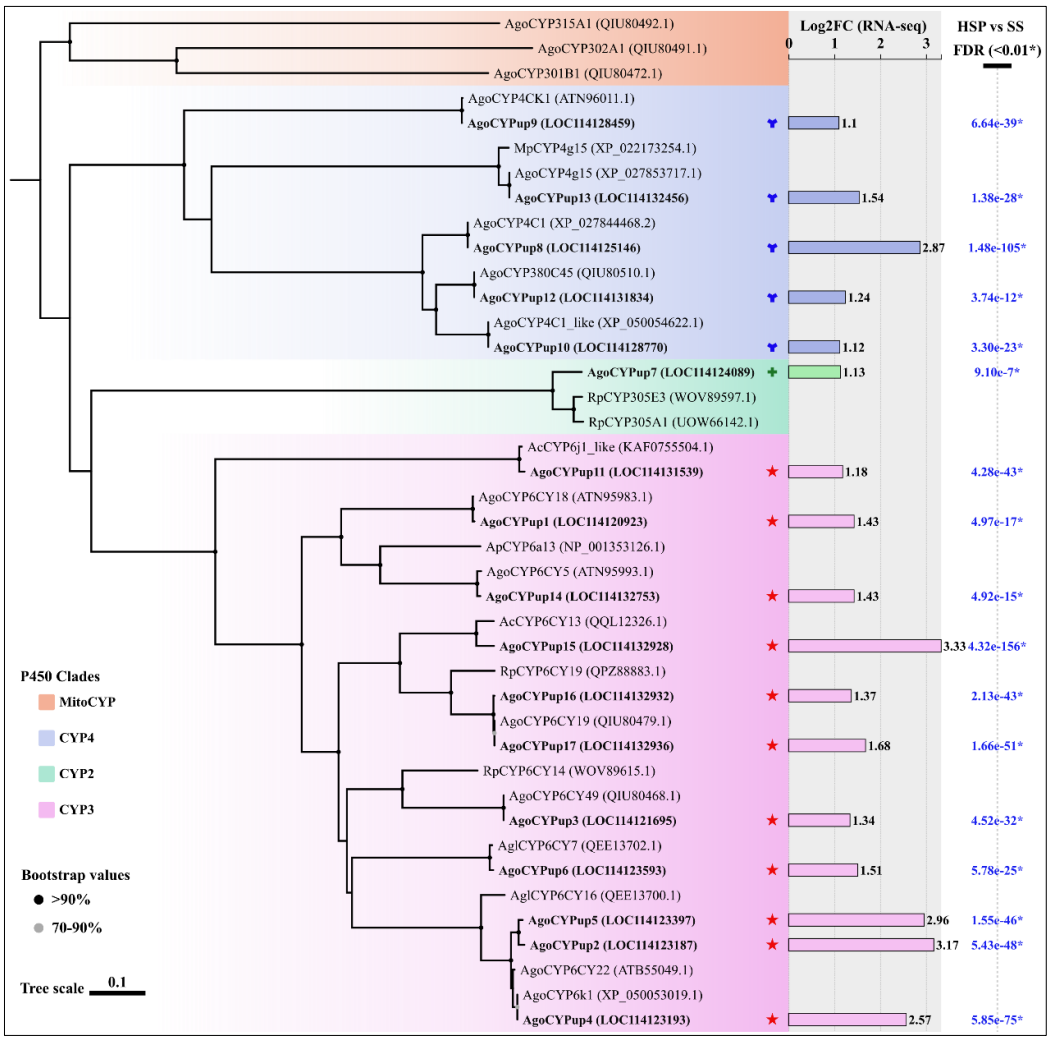

Figure 3. Phylogenetic analysis of 17 up-regulated cytochrome P450 DEGs (*AgoCYPup1–AgoCYPup17*) and their differential expression folds with FDR value (<0.01) from the transcriptome data. The phylogenetic tree was constructed by the neighbor-joining (NJ) method using amino acid sequences of *Aphis gossypii* Glover (Ago), *Myzus persicae* Sulzer (Mp), *Rhopalosiphum padi* L. (Rp), *Aphis craccivora* Koch (Ac), *Acyrthosiphon pisum* Harris (Ap) and *Aphis glycines* Matsumura (Agl). Bootstrap analysis was performed for the assignment of phylogeny with 1000 replications and the values > 70% are marked on each node of the tree. Bootstrap values below 70% are simply not shown. Green, pink, blue, and orange backgrounds represent the CYP2, CYP3, CYP4, and mitochondrial clades of cytochrome P450s, respectively, according to the similarity of their amino acid sequences.

3.6. Relative Expression Levels of the Cytochrome P450 DEGs

The qRT-PCR method was employed to confirm the relative expression levels of the seventeen cytochrome P450 DEGs. As shown in Figure 4a, the expression patterns of these genes measured by qRT-PCR were equivalent to those obtained by the transcriptome data (Figure 3), in spite of the differences in the fold changes of each gene. Except for *AgoCYPup7* and *AgoCYPup9*, other fifteen genes were significantly up-regulated in the HSP strain. Of note, the expression levels of five P450 genes (*AgoCYPup3* to *AgoCYPup5*, *AgoCYPup15*, and *AgoCYPup17*) in the HSP strain were over 3–fold higher than those in the SS strain. Thus, to further explore the relationship between the P450 genes and beta-cypermethrin resistance in the HSP *A. gossypii*, the expression levels of these five genes were observed under the beta-cypermethrin treatment at LC_{50} for 12 h in the surviving HSP individuals. The mRNA in dead individuals generally degrades rapidly so that only the surviving HSP individuals were used to measure the induction of beta-cypermethrin in P450 gene expression regardless of the possible slight enhancement effect caused by the partial sampling method. As

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illustrated in Figure 4b, the expression levels of all the five P450 genes increased significantly in the HSP strain after beta-cypermethrin treatment. *AgoCYPup4* exhibited a highest expression level (6.04–folds) in the HSP strain after the induction of beta-cypermethrin. These results show that overexpression of the 15 P450 genes is associated with the beta-cypermethrin resistance in the HSP *A. gossypii*, and that the five P450 genes of *AgoCYPup3*, *AgoCYPup4*, *AgoCYPup5*, *AgoCYPup15* and *AgoCYPup17* may play key roles in formation of the HSP *A. gossypii* resistance to beta-cypermethrin due to their high-level expressions with and without beta-cypermethrin treatment.

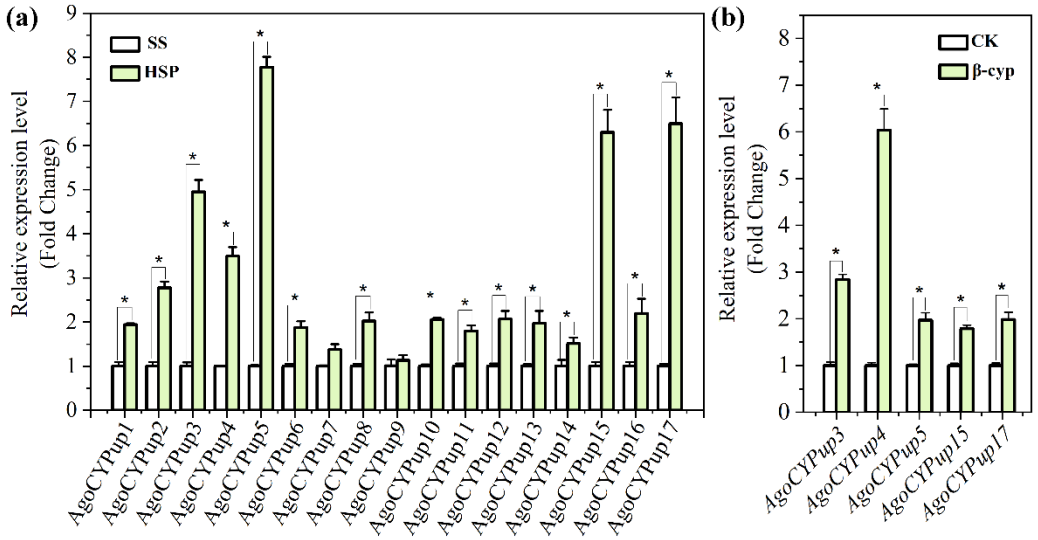

Figure 4. (a) Relative expression of 17 up-regulated cytochrome P450 DEGs in the HSP strain as compared with those in the SS strain. (b) Relative expression of 5 highly up-regulated cytochrome P450 DEGs (over 3–fold) with (β-cyp) and without (CK) beta-cypermethrin treatment in the HSP strain. The HSP aphids were treated with beta-cypermethrin at 2317.74 μg·mL^{-1} (LC_{50} value) for 12 h and then only the survival individuals were used for the qRT-PCR analysis. The error bars represent means ± standard deviations. The asterisks (*) in plots represent significant difference at $P < 0.05$.

3.7. Target-Site Mutations and Frequencies

To identify non-synonymous mutations in the target *VGSC* gene, the full length of *VGSC* genes (*VGSC1* and *VGSC2*), as well as their major mutant fragments (DIIS1–DIIS6 and DIIIS6–DIVS4), were amplified and sequenced. Compared with the susceptible strain (SS), the cDNA sequences of *VGSC* in the HSP *A. gossypii* population varied at two positions, resulting in amino acid substitutions in the putative polypeptide (Figure 5). The first nucleotide point mutation was determined as M918L, a change from ATG to TTG corresponded to the non-synonymous substitution of methionine with leucine at the amino acid residue 918 (Figures 5 and 6). The second nucleotide mutation was a change from GGT to GAT in terms of the replacement of 1012 glycine with aspartic acid (G1012D) (Figures 5 and 6), which, to our knowledge, has not been found yet in any insect species. To further explore the genotypes and their mutation frequencies in the HSP field population, a genomic DNA fragment of *VGSC* covering the two mutation sites (M918L and G1012D) was amplified from individual adults. The representative nucleotide sequence chromatograms of the *VGSC* gene fragment covering the 918 and 1012 sites were shown in Figures S1, S2 and S3. At codon 918, only the heterozygous allele 918L mutation was observed in the HSP strain with a mutation frequency of 96.67% (Table 2). At codon 1012, a total of 36.67% of the HSP individuals were heterozygous allele 1012D mutation, whereas no homozygous 1012D mutation was detected (Table 2). Therefore, two mutation genotypes including two heterozygous mutations of 918M/L and 918M/L+1012G/D were detected in the HSP *A. gossypii* strain, with frequencies of 60.00% and 36.67%, respectively, suggesting its key role in the development of the resistance of the HSP wolfberry aphids to beta-cypermethrin.

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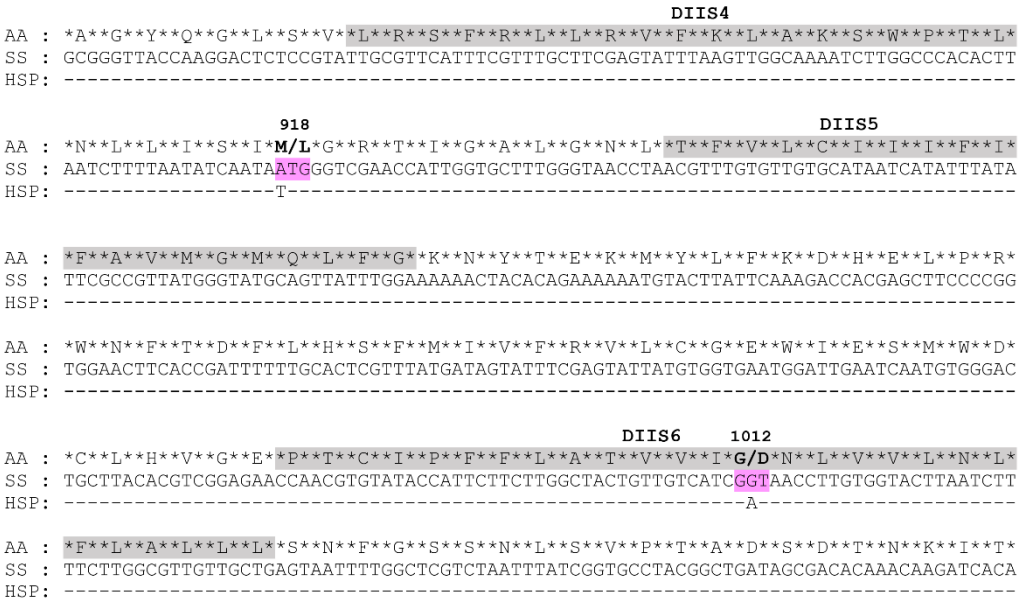

Figure 5. Partial cDNA sequences and their deduced amino acid sequences (AA) of VGSC in the SS and HSP strains. Two mutated codons are highlighted.

Table 2. The potential genotypes of *VGSC* and their frequencies in the HSP strain.

Genotype of *VGSC*		Genotype frequency (%)[5]
918	1012	
M/M[1]	G/G[3]	3.33
	G/D[4]	0.00
	D/D	0.00
M/L[2]	G/G	60.00
	G/D	36.67
	D/D	0.00
L/L	G/G	0.00
	G/D	0.00
	D/D	0.00

[1] M represents methionine. [2] L represents leucine. [3] G represents glycine. [4] D represents aspartic acid. [5] Thirty adult individuals were used to detect the genotype frequency of the HSP strain.

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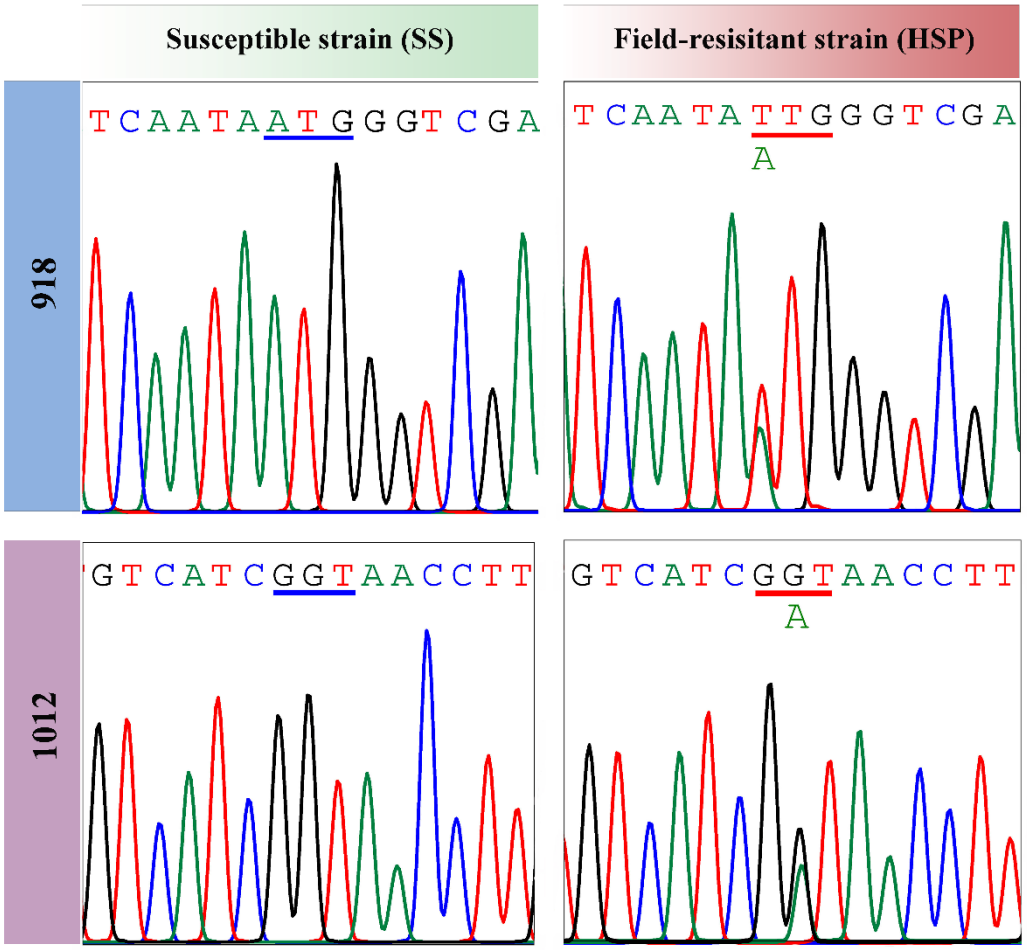

Figure 6. Partial nucleotide sequence chromatograms of the 918 and 1012 loci of VGSC in the SS and HSP strains.

4. Discussion

A. gossypii is one of the most destructive pest insects in Chinese wolfberry fields, facing the challenges of insecticide resistance and population management [44]. Long-term frequent insecticide applications have caused pyrethroid resistance in many field populations of *A. gossypii* [7,10,29,30] and many other aphid species [15,30]. Beta-cypermethrin is a major pyrethroid that has been widely used to control aphids in multiple crop fields of China in the last several decades [7,10,26,45,46], due to its low price and high efficacy. Chen et al. (2017) [7] found that *A. gossypii* field populations had evolved extremely high levels of resistance to beta-cypermethrin in the major cotton growing areas of China, with resistance ratios (RRs) ranging from 2159 to over 40 000. Our previous resistance monitoring data indicated that eight field populations of wolfberry aphids had all developed very high resistance to beta-cypermethrin in the major producing areas of Ningxia in China, with RRs ranging from 2539.70 to 31916.00 [10]. Nowadays, elucidating the resistance mechanisms to pyrethroids of pest insects is particularly important for the design of appropriate management strategies. However, few studies have focused on the resistance mechanism exploration of wolfberry aphids to beta-cypermethrin. Thus, in current work, we expand our previous study to investigate the beta-cypermethrin resistance mechanism of a HSP field-resistant population with a 994.74–fold resistance level as compared with the susceptible strain.

Enhanced metabolic detoxification has been reported as a direct resistance mechanism in many pyrethroid resistant insects. Currently, two methods, the synergistic effect determination of detoxifying enzyme inhibitors and detoxifying enzyme activity assay, are commonly used to determine the metabolic capacity of detoxifying enzymes [7,40]. Cytochrome P450 enzymes are important enzymes for detoxifying exogenous toxic chemicals including insecticides, contributing to

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the resistance of aphids to pyrethroids [7,30,47,48]. A previous study revealed that the P450 inhibitor piperonyl butoxide (PBO) significantly increased the toxicities of both α-cypermethrin (24.47-fold) and cyfluthrin (11.06-fold) against an *A. gossypii* resistant strain [48]. Chen et al. (2017) found that the synergistic effect of PBO on the toxicity of beta-cypermethrin (3.50-fold) was significant to a pooled *A. gossypii* population comprised of various field populations [7]. The activity of P450 enzymes in multiple insecticide-resistant populations has been also reported to be significantly increased compared with that in their respective susceptible strains [19,40,49]. Our results of this study showed that the synergistic ratio (SR) of PBO to the HSP *A. gossypii* was 4.72-fold, while other two detoxifying enzyme inhibitors of TPP and DEM showed no synergistic effects on the HSP strain with SR values of 1.07 and 0.95, respectively. Consistently, the *O*-demethylase activity of the cytochrome P450s in the HSP strain was 1.68–fold higher than that in the SS strain, whereas changes of the other two detoxifying enzymes of CarEs and GSTs in their activities were not remarkable. These results reveal that the activity increasing of cytochrome P450s is a very important factor in the HSP *A. gossypii*.

Besides activity increases of specific P450s, it has also been widely demonstrated that overexpression of P450 genes is also an important molecular mechanism for metabolic resistance [16,19,30,40,47], because it can up-regulate the amount of P450 enzymes. For instance, seven of P450 genes of CYP6CY19, CYP6CZ1, CYP6CY51, CYP6DA1, CYP6DC1, CYP4CH1 and CYP4CJ5, were significantly upregulated in a *R. padi* resistant strain to lambda-cyhalothrin [47]. In our present study, 17 of up-regulated cytochrome P450 genes were identified by the comparative transcriptomics analysis between the SS and HSP strains. The qRT-PCR method was also used to confirm the results of the transcriptome data. Although the expression trends of these 17 up-regulated genes were identical between these two methods, the expression levels of two P450 DEGs identified by transcriptome were not statistically significant by the qRT-PCR method. These divergences in the gene expression levels may attribute to the differences between the RPKM and $2^{-\Delta\Delta Ct}$ methods [50,51]. Besides the constitutive overexpression, inducible expression by insecticides can also contribute to the increased expression of P450 genes [16]. Therefore, we further detected the inducible expression of five CYP6 family genes of high relative expression levels (> 3-fold), and the results showed that these five P450 genes were all significantly expressed under the induction of beta-cypermethrin in the HSP wolfberry aphids. Overall, 15 of up-regulated P450 genes identified by both RNA-Seq and qRT-PCR, especially five CYP6 family genes of high relative expression levels, may play a key role in beta-cypermethrin resistance in wolfberry aphids. The individual contributions of these genes to the metabolic resistance, however, still need to be further explored by RNA interference (RNAi) or heterologous expression studies.

Target-site alteration is another major resistance mechanism to pyrethroid insecticides. Pyrethroids including beta-cypermethrin target VGSCs, and then modify the gating transitions by altering the activation/inactivation kinetics of the channel, finally disrupting the normal nerve function and leading to paralysis and death [15,32]. The major mechanism responsible for pyrethroid resistance in pest insects is caused by VGSCs mutations that reduce the sensitivity to pyrethroids [15,32]. Currently, over 50 pyrethroids-resistance mutations have been identified in various insect species, with various unique and multiple substitutions [14,15,29]. In *A. gossypii*, however, the *kdr* (L1014F) and *s-kdr* (M918L) mutations in the target *VGSC* are the most studied and widely regarded as the primary mechanism of resistance to pyrethroids. The mutation M918L in the linker connecting DIIS4 and DIIS5 was first identified in a fenvalerate resistant strain of *A. gossypii* with an extremely high resistance level of 199.54-fold. Subsequently, this mutation was described in multiple pyrethroid-resistance field strains of *A. gossypii* [7,27,29,30]. For example, a field *A. gossypii* strain from Cameroon was reported to carry the heterozygous M918L mutation with extremely high resistance to cypermethrin (473.00-fold). Chen et al. (2017) found that 96.8–100% of individuals with the M918L mutation were observed in multiple *A. gossypii* populations that evolved extremely high resistance levels to beta-cypermethrin ranging from 2159-fold to over 40000-fold [7]. In addition to the commonly reported M918L mutation, another substitution of M918V was also identified in *A. gossypii* populations collected from Xinjiang province of China [52]. However, other substitutions at

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M918 (T/I) have been only described in resistant populations of other aphid species [15,29,30]. In this study, only heterozygous substitution of M918L was identified in the HSP wolfberry aphids and no L1014F was observed. These results are in line with the previous research findings that M918L and L1014F are yet to be found together in field *A. gossypii* populations, and that the heterozygous M918L mutation is prevalent in field populations in China and Cameroon in terms of a potential fitness cost associated with this mutation in the homozygous form [29,30]. Interestingly, another new heterozygous mutation of G1012D was also found in the HSP strain which, to our best, has never screened against existing *VGSC* mutation databases or aligned with *VGSC* sequences from related aphid species. So far, most of mutation sites occur in the domain II that potentially constitutes the pyrethroid-binding sites with the domain I and domain III, respectively [14]. The L1014F is the first reported *kdr* mutation in pyrethroid-resistant populations across evolutionarily divergent insect species [14,15]. In DIIS6, adjacent to the 1014 locus, mutations, I1011M and V1016G, were reported to reduce the sodium channel sensitivity to two pyrethroids permethrin and deltamethrin [14,52]. Since G1012 is also located in the pyrethroid receptor sites, we speculate that G1012D mutation might affect the action of pyrethroids like beta-cypermethrin. Furthermore, co-occurrence of more than one mutation often leads to a higher resistance level to pyrethroids than the individual ones [14]. For instance, the double mutations L1014F+M918T almost abolished the VGSC sensitivity to deltamethrin, whereas the L1014F or M918T mutation alone only caused about a 5–10 –fold reduction in the sensitivity [14,54,55]. Therefore, the double mutations M918L+G1012D may also act synergistically to increase the beta-cypermethrin resistance in the HSP wolfberry aphids with a high mutation frequency of 36.67%. Yet, the relationship between the G1012D mutation and resistance, as well as the synergistic effect of M918L and G1012D mutations on resistance, still need to be further investigated.

The development of extremely high insecticide resistance is commonly associated with multiple resistance mechanisms. Generally, resistance mechanisms to insecticides are complicate in terms of target-site variations, enhancement of detoxification, reduced transportation and penetration, and behavioral avoidance or apastia [26,30]. Among those, metabolic and target-site mechanisms have been frequently reported to account for pyrethroid resistance [26]. The combined effect of these two mechanisms can usually lead to extremely high insecticide resistance levels in insects including aphids [7,26,27,45,47]. For example, only several P450 genes of overexpression were identified in the *A. gossypii* strains with medium to high level of resistance to dinotefuran (74.7-fold), whereas both the target alteration and P450 genes overexpression were found in an *A. gossypii* strain with extremely high resistance (> 2300-fold) to a same type of insecticide thiamethoxam [47]. The target-resistance associated *VGSC* mutation along with enhanced detoxification, was also demonstrated to contribute to the extremely high resistances to beta-cypermethrin in *A. gossypii* populations from China (> 2000-fold) [7], and to cypermethrin in a Burk *A. gossypii* population from Cameroon (473.00-fold) [27]. In our current work, the *O*-demethylase activity of the HSP strain was only 1.68-fold higher than that of the SS strain, and the resistance of the HSP strain to beta-cypermethrin still reached to an extremely high level (210.83–fold) with the PBO inhibition. These results couldn't explain the extremely high resistance to beta-cypermethrin of the HSP wolfberry aphids (994.74–fold) thoroughly. Therefore, based on the discussion above, we can conclude that the *VGSC* mutations in combination with metabolic detoxification confer the extremely high resistance on the HSP wolfberry aphids to beta-cypermethrin. Generally, metabolic detoxification can confer pesticide resistance ranging from low to high levels while target-site alteration can cause extremely high-level resistance. Thus, we speculate that the *VGSC* mutations might be the main-acting metabolism. The specific contribution of P450-mediated metabolism and target alterations to resistance, however, still needs to be further studied, to fully understand the relationship between different resistance mechanisms.

5. Conclusions

In conclusion, a wolfberry aphid population HSP of extremely high-level resistance to beta-cypermethrin was collected and used as a model to uncover the potential resistance mechanisms.

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Cytochrome P450-mediated enhancement of metabolic detoxification follows a resistance-associated pattern. Heterozygous 918M/L and 918M/L+ G1012D mutations contribute to the beta-cypermethrin resistance of the HSP wolfberry aphids. Both the *VGSC* mutations and the enhanced P450 metabolism resistance were found as the resistance mechanisms for the extremely high beta-cypermethrin resistance of the HSP aphids. These results indicate that pyrethroid insecticides should not be employed for control of *A. gossypii* in the wolfberry planting areas of Ningxia, and rotation application of other insecticides with different action targets will beneficial to the resistance management.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Figure S1: Representative nucleotide sequence chromatogram of the VGSC gene fragment covering the 918 and 1012 sites without any mutations; Figure S2: Representative nucleotide sequence chromatogram of the VGSC gene fragment covering the 918 and 1012 sites with a 918M/L heterozygous mutation; Figure S3: Representative nucleotide sequence chromatogram of the VGSC gene fragment covering the 918 and 1012 sites with both 918M/L and 1012G/D heterozygous mutations. Table S1: Primers for qRT-PCR assay; Table S2: Amplification program for qRT-PCR assay; Table S3: Primers for amplifying VGSC gene of *Aphis gossypii*; Table S4: Amplification program for *VGSC* mutation analysis; Table S5: Summary RNA-Seq data of HSP and SS *A. gossypii* populations; Table S6: List of up-regulated cytochrome P450 enzyme DEGs in the *A. gossypii* resistant population of HSP.

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