CYP4CL2 confers metabolic resistance to pyridaben in the citrus pest mite *Panonychus citri*

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Abstract

The citrus red mite *Panonychus citri* has developed strong resistance to acaricides. Cytochrome P450 monooxygenases (P450s) can detoxify pesticides, and are involved in pesticide resistance in many insects. Here, a pyridaben-resistant *P. citri* strain showed cross-resistance to cyenopyrafen, bifenazate, fenpyroximate, and tolfenpyrad. Piperonyl butoxide, a P450 inhibitor, significantly increased the toxicity of pyridaben to resistant (Pyr_Rs) and susceptible (Pyr_Control) *P. citri* strains. P450 activity was significantly higher in Pyr_Rs than in Pyr_Control. Analyses of RNA-Seq data identified a P450 gene (*CYP4CL2*) potentially involved in pyridaben resistance. Consistently, it was up-regulated in two field-derived resistant populations (CQ_WZ and CQ_TN). RNA interference-mediated knockdown of *CYP4CL2* significantly decreased pyridaben resistance in *P. citri*. Transgenic *Drosophila melanogaster* expressing *CYP4CL2* showed increased pyridaben resistance. Molecular docking analysis showed that pyridaben could bind to several amino acids at substrate recognition sites in CYP4CL2. These findings shed light on P450-mediated pyridaben resistance in pest mites.

Key words: Pyridaben cross-resistance, enzymatic characteristics, RNAi, transgenic flies, molecular model

1 Introduction

Insecticide resistance is an ongoing challenge in sustainable pest management. One of the molecular mechanisms implicated in insecticide resistance is increased activity of metabolic enzymes that detoxify or sequester insecticides before they reach their target sites. The key metabolic enzyme cytochrome P450 (P450) plays a crucial role in the detoxification of various xenobiotics, including insecticides.[1] Numerous studies have shown that the induced or constitutive expression of P450 genes in insecticide-resistant insect strains and/or in response to insecticide exposure confers insecticide resistance.[1-2] Investigations of the molecular basis of neonicotinoid resistance in whitefly, *Bemisia tabaci,* revealed that several P450 genes (including *CYP6DW3*, *CYP6CM1* and *CYP4C64*) are expressed at high levels in resistant strains and their encoded products play pivotal roles in resistance.[3-5] Another study identified a cluster of nine CYP6AE subfamily genes encoding products that are capable of detoxifying several xenobiotics (xanthotoxin, 2-tridecanone, and indoxacarb) *in vivo*.[6] Overexpression of *CYP6P9a* and *CYP6P9b* in *Anopheles funestus* conferred resistance to carbamates and their encoded products were demonstrated to be able to metabolize carbamates.[7]

Pyridaben inhibits complex I (NADH-coenzyme Q oxidoreductase) of the oxidative phosphorylation pathway, thereby disrupting mitochondrial electron transport.[8] It targets the ubiquinone binding site in the nuclear-encoded PSST, the 49 kDa subunit of mitochondrial complex I.[9-10] PSST is the carrier of iron-

sulfur cluster N2, a proposed direct electron donor for ubiquinone reduction.[11] Inhibition of electron transport by pyridaben eventually causes death.

Because of its effectiveness against various mite species, pyridaben has been used globally as an acaricide.[12] However, its extensive use has resulted in strong pyridaben resistance in some mite species, and this threatens crop production.[13-15] Target-site mutations in various proteins are associated with high or very high acaricide resistance levels in mites.[16] For example, the H92R and A94V point mutations located within the PSST subunit are associated with strong pyridaben resistance.[17-18] Most studies exploring the molecular basis of pyridaben resistance have focused on target site mutations, while fewer have focused on the detoxifying enzymes that contribute to pyridaben resistance.

Citrus red mite, *Panonychus citri*, is one of the major cosmopolitan citrus pest mites.[18] The mites feed on the leaves, branches, and even the fruits of citrus species.[19] The extensive use of insecticides together with the short life cycle and high reproduction of this mite has promoted the development of insecticide resistance in *P. citri*.[20-21] Monitoring revealed a high level of pyridaben resistance among this mite in several citrus orchards.[14] Here, we focused on a pyridaben-resistant near-isogenic line of *P. citri* that was constructed in a previous study.[14] We analyzed the role of detoxification as a resistance mechanism by detecting the activities of detoxification enzymes and conducting synergism experiments. In addition, the cross-resistance to several acaricides with different modes of action was determined using bioassays. Transcriptome

profiling was used to identify the differentially expressed genes (DEGs) between pyridaben-resistant and susceptible strains. The role of *CYP4CL2,* a candidate gene in the pyridaben resistance of *P. citri*, was assessed by RNA interference and Gal4/UAS overexpression system in *Drosophila*. A molecular model of CYP4CL2 was obtained using AlphaFold2 and molecular docking analyses were performed to predict the binding mode and key amino acids. The results of these analyses reveal the mechanism by which *CYP4CL2* contributes to the pyridaben resistance of *P. citri*.

2 Methods and materials

2.1 Mites and chemicals

A relatively susceptible strain (Pyr_Control, pyridaben LC_{50} value of 1.649 mg/L) and a resistant stain (Pyr_Rs, the LC_{50} value of 1016.406 mg/L) were used in this study. These strains were obtained by crossing and generational screening between a laboratory susceptible strain (Lab_S) and a pyridaben-resistant strain (Pyr_R).[14] Both strains were maintained in an incubator at 27 ± 1°C, with 60 ± 5% relative humidity (RH) under a 16:8 h light/dark photoperiod. Two field populations (CQ_WZ and CQ_TN, with LC_{50} values of 186.845 and 160.006 mg/L, respectively, Table S1) were collected from Wanzhou and Tongnan, Chongqing, China in 2023 (E107°55′37″, N30°46′39″; E105°45′13″, N30°04′52″). The collected mites were fed on sweet orange leaves until they produced offspring. Adult mites of the first generation were used for functional analyses. All of the technical-grade acaricides used in functional validation

111 analyses are listed in Table S2.

112 **2.2 Bioassays**

113 Acaricide bioassays were performed as described previously.[14] Briefly, 3- to 5-
114 day-old females were transferred to leaf discs cut from fresh sweet orange
115 leaves. After 2–3 h, leaf discs with mites were dipped into acaricide solutions
116 (at least five concentrations) for 5 s and then placed on wet sponges. The
117 superfluous solution on the surface of leaf discs and around the mites was
118 removed with absorbent paper. Leaf discs were dipped in blank solution (ddH_2O,
119 containing 1% acetone and 0.1% Triton X-100) as the control. After dipping, the
120 leaf discs were incubated at 27 ± 1°C, with 60 ± 5% RH under a 16:8 h light/dark
121 photoperiod for 24 h. The mites that did not move when touched with a soft
122 brush were considered as dead. PoloPlus v.2.0 (LeOra Software 2008) was
123 used to calculate the median lethal concentration (LC_{50}) and 95% confidence
124 limits (95% CL) in the bioassays of *P. citri*.

125 **2.3 Enzyme assays**

126 *Enzyme extract preparation and protein assays*

127 Two hundred 3- to 5-day-old female adults of the Pyr_Control or Pyr_Rs strains
128 were collected for enzymatic assays. All samples were homogenized with
129 phosphate-buffered saline (PBS) (0.04 M, pH=7.4) on ice. The homogenate
130 was centrifuged at 12,000 g for 10 min at 4°C and the supernatant was collected
131 as the enzyme extract. For each strain, four biological replicates and two
132 technical replicates were analyzed.

Glutathione S-transferase activity

The activity of glutathione-S-transferase (GST) was determined in a reaction with the substrate 1-chloro-2,4-dinitrochloroben-zene (CDNB) (Sigma-Aldrich, St Louis, MO, USA). The 0.3-mL reaction mixture consisted of 100 µL 0.6 mM CDNB, 100 µL enzyme solution, and 100 µL 6 mM glutathione (Sigma-Aldrich). For the control, PBS (0.04 M, pH=7.4) was added instead of the enzyme extract. The absorbance value at 340 nm was determined every 30 s for 5 min using an xMark^{TM} microplate spectrophotometer reader (Bio-Rad, Hercules, CA, USA). Each group was analyzed with four biological replicates and two technical replicates.

Carboxylesterase activity

The activity of carboxylesterase (CarE) was determined as described by Vanasperen,[22] using α-naphthyl acetate (α-NA) (Sigma-Aldrich) as the substrate. The substrate solution consisted of 1 mM α-NA and 0.1 mM physostigmine (Dr. Ehrenstorfer GmbH, Ausburg, Germany). For the assay, 75 µL enzyme solution and 100 µL substrate solution were added to the well of a 96-well plate and then incubated for 10 min at 30°C. The reaction was terminated by adding 25 µL of a mixture of 5% (w/v) sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and 1% (w/v) fast blue B salt (Sigma-Aldrich). The absorbance value of the reaction mixture was determined at 600 nm using a microplate spectrophotometer reader. For each group, four biological replicates and two technical replicates were analyzed.

Cytochrome P450 activity

Based on the method described in previous studies, an insect CYP450 ELISA kit (Hengyuan Biotechnology Co., Ltd., China) was used to determine P450 activity.[23] According to the manufacturer's protocols, the absorbance value of each sample at 450 nm was determined using a microplate spectrophotometer reader and then activity was calculated from a standard curve (y=0.0292x+0.1689). For each group, four biological replicates and two technical replicates were analyzed.

Synergism assays

Next, we investigated the roles of detoxification enzymes in the resistance of *P. citri* to pyridaben using detoxification enzyme inhibitors. Three inhibitors were used in these analyses: the esterase-inhibitor S, S, S tributyl-phosphorotrithioate (TPP, TRC, Toronto, Canada); the cytochrome P450-dependent monooxygenase-inhibitor piperonylbutoxide (PBO) (Bioway Bioruler, Connecticut, USA); and the glutathione-based detoxification inhibitor diethylmaleate (DEM) (AbMole, Houston, USA). The final concentration of each inhibitor (TPP, PBO and DEM) resulted in a maximum rate of 5% mortality of *P. citri*. Each detoxification enzyme inhibitor was dissolved in acetone and diluted to the final concentration with ddH_2O. For the assays, 30–40 female adult mites were transferred onto citrus discs with a diameter of 1.5 centimeter. Then, the discs with mites were dipped into the detoxification enzyme inhibitor solutions and the superfluous liquid was absorbed using filter paper. All treated mites

were transferred to new fresh citrus leaf discs and maintained in artificial incubator for 4 h. After treatments with enzyme inhibitors, the leaf discs with mites were subjected to an LC_{30} pyridaben treatment. Each analysis was conducted four times. The mortality rates of mites (%) were assessed after 24 h. Mites exposed to ddH_2O containing the same concentration of acetone as that in the treatments served as the control.

Enzyme assay data analyses

Significant differences in detoxification enzyme activity between Pyr_Control and Pyr_Rs strains were determined by Student's *t*-test using GraphPad 8.0 statistical software. Student's *t*-test was also used to detect significant differences in mortality between the control group and groups treated with detoxification enzyme inhibitors.

2.4 RNA extraction, cDNA synthesis, and quantitative real-time PCR

For each group, about 100 female adult mites were collected and total RNA was isolated using the MicroElute®Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's protocols. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA). The absorbance ratio of optical density (OD260/280 and OD260/230) was used to assess the purity of the RNA, and 1% w/v agarose gel electrophoresis confirmed its integrity. All RNA samples were stored at

199 −80°C until further use. The PrimeScript RT reagent Kit (TaKaRa Biotechnology,
200 Dalian, China) was used to synthesize cDNA.
201 All primers for quantitative real-time PCR (qPCR) were designed using
202 tools at the National Center for Biotechnology Information (NCBI) PrimerBlast
203 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table S3. The
204 10 μL qPCR reaction mixture consisted of 5 μL 2×NovoStar® SYBR qPCR
205 SuperMix Plus (Novoprotein, Shanghai, China), 3 μL cDNA, 0.5 μL each primer
206 (forward and reverse), and 1 μL RNase-free water. The PCR analyses were
207 performed on a CFX384TM Real-Time System (Bio-Rad). The thermocycling
208 conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s
209 and 60°C for 30 s. Gene transcript levels were standardized based on the
210 transcript levels of the reference genes *PcGAPDH* and *PcELF1A* using qBase[24].
211 qPCR analyses were conducted to determine the transcript levels of *CYP4CL2*
212 in different field and laboratory strains and in the *CYP4CL2*-silenced strains.
213 The experiment consisted of three treatments with three biological replicates in
214 a completely randomized design. Each replicate was analyzed with two
215 technical replicates.
216 **2.5 Transcriptome sequencing and data analysis**
217 Total RNA was extracted from 200 female adults of the Pyr_Control or Pyr_Rs
218 strains using the MicroElute®Total RNA Kit (Omega Bio-Tek), according to the
219 manufacturer's instructions. After the quality and quantity of RNA were
220 determined using a 2100 Bioanalyzer (Agilent) and a NanoDrop 2000

instrument (ThermoFisher Scientific), 1 μg total RNA per sample was used to construct a cDNA library using the VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme, Nanjing, China). The cDNA libraries were sequenced on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) following the protocols of the NovaSeq 6000 S4 Reagent kit (Illumina) and 150-bp paired-end reads were generated. Four biological replicates were sequenced for each strain.

To obtain clean reads, Trimmomatic (version 0.36) was used to remove the low-quality reads and adaptor sequences. The remaining high-quality reads were mapped to the complete *P. citri* genome (GeneBank: JAAABK000000000) using HISAT2. All uniquely mapped reads were used to calculate the gene RPKM (reads per kilobase of exon per million mapped sequenced reads) values, which indicated the normalized gene transcript levels. The DEGs between Pyr_Control and Pyr_Rs strains were selected using the bioconductor package DESeq2, and met the following criteria: *P*-value < 0.05 and fold change >2. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs was conducted using KOBAS software (version 2.1.1) with a *P*-value < 0.05.

2.6 RNA interference

To explore the role of the P450 gene in the pyridaben resistance of *P. citri*, RNAi was used to suppress its expression. The mites were soaked in dsRNA solution as described elsewhere.[25] Briefly, dsRNA primers containing a T7 promoter at

the 5′ end for the *P450* gene and GFP were designed using PrimerBlast (Table S3). The dsRNA was synthesized *in vitro* using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific). Then, the quality of the dsRNA was determined by 1% agarose gel electrophoresis and the concentration was measured using a NanoVue UV-Vis spectrophotometer (Thermo Fisher Scientific).

Three-day-old female mites were soaked in the dsRNA solution (1000 ng/μL) at 25°C for 24 h according to a recent study.[32] The mites were transferred to fresh citrus leaves and left for 4–6 h before RNA isolation and pyridaben bioassays. The bioassays were conducted as described above and differences between the dsRNA-treated and control groups were determined by probit analysis (PoloPlus v.2.0, LeOra Sofware, 2008). The hypotheses of equality and parallelism, and the methods used to calculate the lethal dose ratio at LC_{50} (LCR_{50}) and the 95% confidence limits (CL) were as described elsewhere.[26]

2.7 Mortality bioassay of transgenic *Drosophila melanogaster*

The Gal4/UAS system[27] was used to overexpress P450 in *D. melanogaster*. The open reading frames of the CYP4CL2 genes were each subcloned into pJFRC28-10XUAS-IVS-GFP-p10 (Addgene Plasmid #36431). Each plasmid was then microinjected into *D. melanogaster* embryos. Using the PhiC31 system, the plasmid DNA was integrated at the attP40 docking site on chromosome 2 to generate the transgenic line, UAS-CYP4CL2. Virgin females

from the Act5C-GAL4 strain were crossed with males carrying UAS-CYP4CL2 to obtain a heritable transgenic line, Gal4/UAS-CYP4CL2. Each line was reared under artificial conditions at 25°C with 60% RH under 12:12 h light/dark photoperiod. The transcript level of CYP4CL2 in transgenic flies was determined by qPCR, relative to that of the housekeeping gene *RPL11*[28] (Table S3). Flies with the same background as the UAS-CYP4CL2 transgenic lines were used as controls.

Bioassays to assess the toxicity of pyridaben to transgenic lines were carried out as described previously.[28] Filter papers impregnated with pyridaben (1000 mg/L, a lethal concentration of pyridaben) were rolled up and placed in 30-cc glass vials, covering the entire wall. All vials were plugged with cotton soaked in 10% (w/v) sucrose. Then 25–30 flies were transferred into each vial, and knockdown rate was scored after 24 h of exposure to pyridaben. For all assays, five replicates were analyzed. Student's *t*-test was used to compare knockdown rate of each experimental group with that of the control group.

2.8 CYP4CL2 modelling and pyridaben docking

To identify the amino acids within each substrate recognition site (SRS) of CYP4CL2, the primary sequence was aligned with that of HaCYP6AE19[29] using Clustal Omega.[30] A model of the CYP4CL2 enzyme was generated using AlphaFold2[31] by submitting the primary sequence to the ColabFold website[32] and running AlphaFold2 with default options. The generated model lacked the heme group; therefore, this co-factor was introduced into the model by copying

the atom coordinates of heme from a high-resolution human P450 structure as described elsewhere,[33] followed by 100 steps of conjugate-gradient energy minimization in SWISS-PdbViewer.[34]

A 3D structure file for pyridaben (ZINC1543237) was retrieved from the ZINC database (ZINC20, A Free Ultralarge-Scale Chemical Database for Ligand Discovery).[35] Automated docking of pyridaben with the CYP4CL2 model was performed by first using AutoDockTools (v1.5.7) (Molecular Graphics Laboratory, Scripps Research Institute, La Jolla, CA, USA) to define rotatable bonds and merge non-polar hydrogens. Then, AutoDock Vina (v1.2.3)[36] was used to dock pyridaben within a grid of 20 × 22 × 24 points (1 Å spacing) that covered the CYP4CL2 active site. The figure was produced using UCSF ChimeraX version 1.5.

3 Results

3.1 Cross-resistance to other pesticides in the pyridaben-resistant strain

The cross-resistance of the pyridaben-resistant strain (Pyr_Rs), which shows 616.377-fold resistance[14] compared with that of the relatively susceptible strain (Pyr_Control), to ten different acaricides was determined. As shown in Table 1, the 95% confidence limits of the LC_{50} values of cyenopyrafen, bifenazate, fenpyroximate, and tolfenpyrad in Pyr_Rs and Pyr_Control did not overlap, indicating that there were significant differences in resistance to these acaricides between Pyr_Rs and Pyr_Control.

The Pyr_Rs strain showed cross-resistance to fenpyroximate (63.369-fold

that of Pyr_Control), and low cross-resistance to cyenopyrafen, bifenazate, and tolfenpyrad (3.282-fold, 4.787-fold, and 7.278-fold that of Pyr_Control, respectively). There was no obvious cross-resistance to cyflumetofen, abamectin, fenpropathrin, propargite, tebufenpyrad, and fenazaquin, given the overlap of the 95% confidence limits of the LC_{50} values.

3.2 Detoxification enzyme activity and synergism bioassays

The specific enzyme activities of CarEs, GST, and P450 are shown in Table 2. The P450 activity was significantly higher in Pyr_Rs than in Pyr_Control (1.550-fold higher, P=0.006), at 0.262 and 0.169 nmol/mg pro/min, respectively. There were no significant differences in CarEs, and GST activity between Pyr_Rs and Pyr_Control.

The results of synergism studies using the enzyme inhibitors PBO, TPP, and DEM on Pyr_Rs and Pyr_Control strains are shown in Fig. 1. Pretreatment with PBO significantly synergized pyridaben toxicity, with 2.613- and 1.807-fold synergism in the Pyr_Rs and Pyr_Control strains, respectively. Pretreatment with DEM and TPP did not significantly affect pyridaben toxicity in either Pyr_Rs or Pyr_Control.

3.3 Differentially expressed genes (DEGs) identification and functional annotation

In total, eight independent cDNA libraries constructed from Pyr_Rs and Pyr_Control were sequenced on the Illumina sequencing platform. These analyses generated a mean of 7.67 Gb clean data for each library (Table S4).

The Q30 value was 94.5% and the GC content ranged from 38% to 41%, indicating that the quality of the data was sufficient for transcriptome analysis. The raw data was uploaded to NCBI database with the biosample accessions SAMN37473146-SAMN37473153.

To identify the potential detoxification genes involved in *P. citri* pyridaben resistance, we detected DEGs between Pyr_Rs and Pyr_Control strains (Fig. 2). In total, there were 204 DEGs with an absolute fold-change of ≥ 2 and *P*-value (FDR) < 0.05. Of these, 91 genes were significantly up-regulated and 113 were down-regulated in Pyr_Rs *versus* Pyr_Control (Fig. 2A). To identify the function of the up-regulated genes, they were were annotated at the KEGG database. Most of them were attributed to metabolic pathways, especially the drug metabolism pathway (Fig. 2B). This result suggested that genes in the drug metabolism pathway may be involved in pyridaben metabolism in the pyridaben-resistant *P. citri* strain.

Based on the up-regulated genes from comparative transcriptomics between Pyr_Rs and Pyr_Control strains, one p450 gene and three ABC transporter genes were identified (Table S5). The previous synergism analyses indicated that P450s contributed to pyridaben resistance in *P. citri*. Therefore, the P450 gene, EVM0004731(*CYP4CL2*), was considered as a candidate gene involved in resistance to pyridaben. Subsequently, gene function analysis were performed *in vivo* and *in vitro*.

3.4 Functional analysis of *CYP4CL2*

353 The qPCR results showed that *CYP4CL2* was significantly up-regulated in the
354 pyridaben-resistant *P. citri* strains (Pyr_R and Pyr_Rs) and two resistant field
355 populations (CQ_WZ and CQ_TN) (Fig. 3), as compared with the susceptible
356 strains Lab_S and Pyr_Control. We generated RNAi lines by feeding mites with
357 a dsRNA solution to suppress the expression of *CYP4CL2.* The transcript levels
358 of *CYP4CL2* and the silencing efficiency in different *P. citri* strain/populations
359 were confirmed by qPCR (dsCYP4CL2 significantly reduced *CYP4CL2*
360 expression as compared to dsGFP.) (Fig. S1). As the 95% CL of LCR_{50} >1, the
361 tests for the hypotheses of equality revealed significant differences in the LC_{50}
362 values to pyridaben among test groups (Table 3).[33] Consequently, the LC_{50}
363 values were significantly reduced in all test strain/populations with silenced or
364 low *CYP4CL2* expression (the LC_{50} values were reduced from 2.535 to 1.185
365 mg/L, 1276.617 to 637.595 mg/L, 174.709 to 91.028 mg/L, and 215.237 to
366 93.766 mg/L in the RNAi-treated Pyr_Control, Pyr_Rs, CQ_TN, and CQ_WZ,
367 respectively) (Table 3). These results suggest that *CYP4CL2* has played an
368 important role in the development of pyridaben resistance in *P. citri*.

369 To determine whether *CYP4CL2* confers cross-resistance, we evaluated
370 the susceptibility of *CYP4CL2*-silenced lines to bifenazate, fenpyroximate, and
371 tolfenpyrad (Fig. S2). No obvious differences in susceptibility were observed
372 between CYP4CL2-silenced lines and the controls, indicating that *CYP4CL2*
373 did not participate in cross-resistance to bifenazate, fenpyroximate, and
374 tolfenpyrad (Fig. S2).

3.5 Pyridaben resistance of *Drosophila melanogaster* overexpressing CYP4CL2

Next, we determined whether expression of CYP4CL2 alone was sufficient to confer pyridaben resistance *in vivo*. For these analyses, *CYP4CL2* was overexpressed in transgenic *D. melanogaster* using the Gal4/UAS system. We constructed UAS-CYP4CL2 and Gal4/UAS-CYP4CL2 transgenic flies. The results of qPCR analyses confirmed that *CYP4CL2* was overexpressed in the Gal4/UAS-CYP4CL2 line, but not in the control lines (UAS-CYP4CL2) (Fig. 4A). The knockdown rate of flies treated with a lethal concentration (1000mg/L) of pyridaben were recorded to determine their resistance to pyridaben. Bioassays carried out with pyridaben on the transgenic Gal4/UAS-CYP4CL2 line indicated significantly increased resistance in this line (Fig. 4B), with a significantly lower knockdown rate (68.2% after 24 h; $P < 0.001$) than that of the control line (94.4% for UAS-CYP4CL2).

3.6 Molecular modeling and pyridaben docking analysis

The SRSs of CYP4CL2 were identified using the sequence of another P450 (HaCYP6AE19)[29] as reference (Fig. S3). A model of CYP4CL2 was then generated to determine if pyridaben could be accommodated within its active site. Fig. 5 shows the top-scoring docking prediction (estimated free energy of binding (ΔG_b) of −4.115 kcal/mol), which placed pyridaben in proximity to the heme group. This docking position allowed pyridaben to have possible binding interactions with amino acid side chains from four of the six SRSs (SRS1, 4, 5,

and 6). Because none of these amino acids were predicted to form a hydrogen bond with the ligand, van der Waals interactions were predicted to be the main type of ligand-binding interaction.

Discussion

Insecticides are powerful weapons in the control of insect pests that threaten crop production. Whilst maintaining the efficacy of these insecticides and the integrity of their targets is vital, the evolution of insecticide resistance in target pests due to strong selection pressure imposed by widespread insecticide use is a serious problem.[37] In addition, cross-resistance to compounds in the same insecticide group is a frequently observed phenomenon. Cross-resistance among groups of insecticides that differ in their structural and functional characteristics can be extremely unpredictable in the field. Many studies have focused on cross-resistance between pyridaben and other acaricides in different spider mites. For example, fenpyroximate-resistant strains selected in the laboratory showed moderate level of cross-resistance to pyridaben.[38] In *Tetranychus cinnabarinus* (Boisduval), *CYP389C16* directly contributes to the cross-resistance between cyflumetofen and pyridaben by hydroxylation.[39] Another study selected for cyenopyrafen resistance in *Tetranychus urticae*, and bioassay results confirmed the development of cross-resistance to pyridaben.[40] Similarly, the development of resistance to bifenazate in *T. urticae* was found to be accompanied by cross-resistance to pyridaben and other acaricides.[41] In the current study, the *P. citri* pyridaben-resistant strain exhibited cross-resistance

to several tested acaricides (cyenopyrafen, bifenazate, fenpyroximate, and tolfenpyrad), consistent with the results of the studies mentioned above.

The *P. citri* pyridaben-resistant strain showed higher P450 activity compared with that of the susceptible strain. Moreover, the toxicity of pyridaben to the resistant and susceptible strains of *P. citri* was significantly increased by PBO (a known inhibitor of P450), suggesting that P450 may be involved in the detoxification of pyridaben. This result was in accordance with the results of a previous study on pyridaben-selected *T. urticae* strains.[15] Overexpression of their respective genes was found to be the cause of increased enzyme activities in resistant strains. In general, genetic phenomena are responsible for resistance to metabolic-based insecticides.[1] Genomic changes lead to the amplification, overexpression, and variations in the coding sequences of major groups of genes encoding metabolic enzymes. Of these, P450s play critical roles in the development of resistance to synthetic insecticides.[42] In insects, the induction and constitutive overexpression of genes encoding P450s are responsible for the detoxification of insecticides.[43] A large number of insect P450s involved in insecticide resistance have been identified and functionally analyzed. Based on the DEGs detected from the RNA-seq data, *CYP4CL2* was identified as a potentially important gene in detoxification in our study. Consistent with this, a previous study reported that this P450 gene was significantly up-regulated in *P. citri* exposed to sublethal concentration of pyridaben.[44] We also detected up-regulation of *CYP4CL2* in different resistant

populations, providing further evidence for its important role in the development of pyridaben resistance in *P. citri*.

To confirm the function of P450 in pyridaben resistance, we performed gene-silencing using the RNAi method. When the expression of *CYP4CL2* was suppressed by dsRNA delivery, the resistance of *P. citri* significantly decreased both in laboratory strains and field populations. These RNAi results demonstrate the importance of *CYP4CL2* in pyridaben resistance. The knockdown of *CYP4CL2* did not affect the responses of *P. citri* to LC_{30} treatments with bifenazate, fenpyroximate, and tolfenpyrad, indicating that this P450 gene was not responsible for the cross-resistance against these acaricides, even though it confers resistance to pyridaben.

The transgenic *Drosophila* system for *in vivo* gene expression has proved to be powerful tool for exploring the genetic basis of insecticide resistance over the last 60 years.[45] Transgenic overexpression of P450s in *D. melanogaster* using the Gal4/UAS system has been used successfully to evaluate the function of many P450 genes from different insects. For example, transgenic *D. melanogaster* overexpressing the *Plutella xylostella* (L.) *CYP6BG1* gene showed significantly increased tolerance to chlorantraniliprole.[46] Similarly, *NlCYP6CS1* was introduced into *D. melanogaster* to clarify its contribution to pymetrozine resistance in *Nilaparvata lugens*.[47] Transgenic *D. melanogaster* overexpressing two *N. lugens* P450 genes (*CYP6ER1vA* and *CYP439A1*) exhibited significant resistance to buprofezin.[48] Another study reported that the

duplication and divergence of *CYP6ER1* in *N. lugens* has led to resistance to several neonicotinoids.[33] The transgenic flies have been frequently applied in P450 function analysis when the active recombinant protein can't be achieved,[46-48] which could provide evidence in P450 mediated resistance development in combination with the RNAi assay. Therefore, we introduced *CYP4CL2* into *Drosophila* to verify the function *in vitro*. Here, overexpression of the *P. citri* P450 gene *CYP4CL2* in *D. melanogaster* significantly increased its pyridaben resistance. These results provide further evidence that the up-regulation of *CYP4CL2* plays an important role in pyridaben resistance in *P. citri*.

P450 proteins share a conserved protein fold with six highly variable SRSs that frame the catalytic pocket and substrate access channels, and this variability contributes to the catalytic specificity of the enzyme.[49] Site-directed mutagenesis studies have shown that SRSs constitute several important domains of the tertiary structure of P450 proteins and contribute to their function.[50] The P450 catalytic site is mainly formed by SRS1, SRS4, SRS5, and SRS6 and the substrate access channel is shaped mainly by SRS2 and SRS3.[51] Although mutations both within and outside[36] SRS regions can confer insecticide resistance by increasing ligand affinity or active-site accessibility for a substrate. In addition, our transgenic *Drosophila* study shows that *P. citri* CYP4CL2 significantly promotes the tolerance of fly to pyridaben. An increase in CYP4CL2 expression therefore appears to be the prevalent mechanism in current populations of resistant *P. citri*. We predict that there may be selective

pressure for the *CYP4CL2* gene to duplicate further boosts its activity towards pyridaben to increase resistance.

In summary, our bioassay results provide evidence of cross-resistance between pyridaben and other acaricides in a pyridaben-resistant strain of *P. citri*. Enzyme activity and synergism analyses suggested that P450s contribute to pyridaben resistance. Analyses of RNA-Seq data revealed an up-regulated P450 gene, *CYP4CL2,* in a pyridaben-resistant strain of *P. citri*. Expression analyses revealed the up-regulation of *CYP4CL2* in several different resistant strains/populations. RNAi-mediated knockdown of this P450 gene increased the susceptibility of *P. citri* to pyridaben, whereas transgenic *D. melanogaster* expressing *CYP4CL2* showed significantly increased pyridaben resistance. Molecular modelling indicated the pyridaben could be accommodated within the CYP4CL2 active site. These results provide useful information about the mechanism of P450-mediated resistance to pyridaben in *P. citri* and identify the CYP4CL2 gene as an important target for future genetic screening of resistant *P. citri* populations.

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Notes

The authors declare no competing financial interest.

References

1. Li, X.; Schuler, M. A.; Berenbaum, M. R., Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol*. **2007,** *52* (1), 231-253.

2. Daborn, P. J.; Yen, J. L.; Bogwitz, M. R.; Le Goff, G.; Feil, E.; Jeffers, S.; Tijet, N.; Perry, T.; Heckel, D.; Batterham, P.; Feyereisen, R.; Wilson, T. G.; ffrench-Constant, R. H., A single P450 allele associated with insecticide resistance in Drosophila. *Science* **2002,** *297* (5590), 2253-2256.

3. Yang, X.; Wei, X. G.; Yang, J.; Du, T. H; Yin, C.; Fu, B.; Huang, M. J; Liang, J. J.; Gong, P. P.; Liu, S. N.; Xie, W.; Guo, Z. J.; Wang, S. L.; Wu, Q. J.; Nauen, R.; Zhou, X. G; Bass, C.; Zhang, Y. J., Epitranscriptomic regulation of insecticide resistance. *Sci. Adv.* **2021,** *7* (19), eabe5903.

4. Yang, X.; Deng, S.; Wei, X. G.; Yang, J.; Zhao, Q. N.; Yin, C.; Du, T. H.; Guo, Z. J.; Xia, J. X.; Yang, Z. Z.; Xie, W.; Wang, S. L.; Wu, Q. J.; Yang, F. S.; Zhou, X. G.; Nauen, R.; Bass, C.; Zhang, Y. J., MAPK-directed activation of the whitefly transcription factor *CREB* leads to P450-mediated imidacloprid resistance. *Proc. Natl. Acad. Sci. U. S. A.* **2020,** *117* (19), 10246-10253.

5. Xue, H.; Fu, B.; Huang, M. J.; He, C.; Liang, J. J.; Yang, J.; Wei, X. G.; Liu, S. N.; Du, T. H.; Ji, Y.; Yin, C.; Gong, P. P.; Hu, J. Y.; Du, H.; Zhang, R.; Wang, C.; Khajehali, J.; Su, Q.; Yang, X.; Zhang, Y. J., CYP6DW3 metabolizes imidacloprid to imidacloprid-urea in whitefly (*Bemisia tabaci*). *J. Agric. Food Chem.* **2023,** *71* (5), 2333-2343.

6. Wang, H. D.; Shi, Y.; Wang, L.; Liu, S.; Wu, S. W.; Yang, Y. H.; Feyereisen, R.; Wu, Y. D., CYP6AE gene cluster knockout in *Helicoverpa armigera* reveals role in detoxification of phytochemicals and insecticides. *Nature Commun.* **2018,** *9* (1), 4820.

534 7. Mugenzi, L. M. J.; Tekoh, T. A.; Ibrahim, S. S.; Muhammad, A.; Kouamo, M.; Wondji, M. J.;
535 Irving, H.; Hearn, J.; Wondji, C. S., The duplicated P450s CYP6P9a/b drive carbamates and
536 pyrethroids cross-resistance in the major African malaria vector *Anopheles funestus*. *Plos*
537 *Genet.* **2023,** *19* (3). e1010678.
538 8. Yaghoobi, R.; Khajehali, J.; Alavijeh, E. S.; Nauen, R.; Dermauw, W.; Van Leeuwen, T.,
539 Fenpyroximate resistance in Iranian populations of the European red mite *Panonychus ulmi*
540 (Acari: Tetranychidae). *Exp. Appl. Acarol.* **2021,** *83* (1), 69-79.
541 9. Fiedorczuk, K.; Letts, J. A.; Degliesposti, G.; Kaszuba, K.; Skehel, M.; Sazanov, L. A.,
542 Atomic structure of the entire mammalian mitochondrial complex I. *Nature* **2016,** *538* (7625),
543 406-410.
544 10. Zickermann, V.; Wirth, C.; Nasiri, H.; Siegmund, K.; Schwalbe, H.; Hunte, C.; Brandt, U.,
545 Mechanistic insight from the crystal structure of mitochondrial complex I. *Science* **2015,** *347*
546 (6217), 44-49.
547 11. Duarte, M.; Populo, H.; Videira, A.; Friedrich, T.; Schulte, U., Disruption of iron-sulphur
548 cluster N2 from NADH: ubiquinone oxidoreductase by site-directed mutagenesis. *Biochem. J.*
549 **2002,** *364* (3), 833-839.
550 12. Devine, G. J.; Barber, M.; Denholm, I., Incidence and inheritance of resistance to METI-
551 acaricides in European strains of the two-spotted spider mite (*Tetranychus urticae*) (Acari:
552 Tetranychidae). *Pest. Manag. Sci.* **2001,** *57* (5), 443-448.
553 13. Koo, H. N.; Choi, J.; Shin, E.; Kang, W.; Cho, S. R.; Kim, H.; Park, B.; Kim, G. H.,
554 Susceptibility to acaricides and the frequencies of point mutations in etoxazole- and pyridaben-
555 resistant strains and field populations of the two-spotted spider mite, *Tetranychus urticae* (Acari:

556 Tetranychidae). *Insects*. **2021,** *12* (7), 660.

557 14. Pan, D.; Xia, M. H.; Luo, Q. J.; Liu, X. Y.; Li, C. Z.; Yuan, G. R.; Wang, J. J.; Dou, W.,

558 Resistance of *Panonychus citri* (McGregor) (Acari: Tetranychidae) to pyridaben in China:

559 monitoring and fitness costs. *Pest Manag. Sci.* **2023,** *79* (3), 996-1004.

560 15. Namin, H. H.; Zhurov, V.; Spenler, J.; Grbic, M.; Grbic, V.; Scott, I. M., Resistance to

561 pyridaben in Canadian greenhouse populations of two-spotted spider mites, *Tetranychus*

562 *urticae* (Koch). *Pestic. Biochem. Physiol.* **2020,** *170*.

563 16. Ilias, A.; Vontas, J.; Tsagkarakou, A., Global distribution and origin of target site insecticide

564 resistance mutations in *Tetranychus urticae*. *Insect Biochem. Mol. Biol.* **2014,** *48*, 17-28.

565 17. Snoeck, S.; Kurlovs, A. H.; Bajda, S.; Feyereisen, R.; Greenhalgh, R.; Villacis-Perez, E.;

566 Kosterlitz, O.; Dermauw, W.; Clark, R. M.; Van Leeuwen, T., High-resolution QTL mapping in

567 *Tetranychus urticae* reveals acaricide-specific responses and common target-site resistance

568 after selection by different METI-I acaricides. *Insect Biochem. Mol. Biol.* **2019,** *110*, 19-33.

569 18. Alavijeh, E. S.; Khajehali, J.; Snoeck, S.; Panteleri, R.; Ghadamyari, M.; Jonckheere, W.;

570 Bajda, S.; Saalwaechter, C.; Geibel, S.; Douris, V.; Vontas, J.; Van Leeuwen, T.; Dermauw, W.,

571 Molecular and genetic analysis of resistance to METI-I acaricides in Iranian populations of the

572 citrus red mite, *Panonychus citri*. *Pestic. Biochem. Physiol.* **2020,** *164*, 73-84.

573 19. Jones, V. P.; Parrella, M. P., The sublethal effects of insecticides on life table parameters

574 of *Panonychus citri* (Acari: Teranychidae). *Can. Entomol.* **1984,** *116* (7), 1033-1040.

575 20. Ran, C.; Chen, Y.; Wang, J. J., Susceptibility and carboxylesterase activity of five field

576 populations of *Panonychus citri* (McGregor) (Acari: Tetranychidae) to four acaricides. *Int. J.*

577 *Acarol.* **2009,** *35* (2), 115-121.

21. Pan, D.; Luo, Q. J.; O´Reilly, A. O.; Yuan, G.R.; Wang, J. J.; Dou, W., Mutations of voltage-gated sodium channel contribute to pyrethroid resistance in *Panonychus citri*. *Insect Sci*. **2023**. https://doi.org/10.1111/1744-7917.13266.

22. Van Asperen, K., A study of housefly esterases by means of a sensitive colorimetric method. *J. Insect Physiol.* **1962,** *8* (4), 401-416.

23. Wang, L.; Cui, L.; Wang, Q. Q.; Chang, Y. P.; Huang, W. L.; Rui, C. H., Sulfoxaflor resistance in *Aphis gossypii*: resistance mechanism, feeding behavior and life history changes. *J. Pest Sci.* **2022,** *95* (2), 811-825.

24. Hellemans, J.; Mortier, G.; De Paepe, A.; Speleman, F.; Vandesompele, J., qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **2007,** *8* (2), R19.

25. Sun, Q. Z.; Li, X. L.; Shi, Y. F.; Zhang, Y. C.; Chai, W. J.; Chen, R. Y.; Niu, J.; Wang, J. J., GARP: A family of glycine and alanine-rich proteins that helps spider mites feed on plants. *Insect Sci*. **2022**. *0*, 1–15

26. He, Y.; Zhang, J.; Chen, J., Effect of synergists on susceptibility to chlorantraniliprole in field populations of *Chilo suppressalis* (Lepidoptera: Pyralidae). *J. Econ. Entomol.* **2014,** *107* (2), 791-796.

27. Pfeiffer, B. D.; Truman, J. W.; Rubin, G. M., Using translational enhancers to increase transgene expression in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A*. **2012,** *109* (17), 6626-6631.

28. Riveron, J. M.; Irving, H.; Ndula, M.; Barnes, K. G.; Ibrahim, S. S.; Paine, M. J. I.; Wondji, C. S., Directionally selected cytochrome P450 alleles are driving the spread of pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Proc. Natl. Acad. Sci. U. S. A*.

600 **2013,** *110* (1), 252-257.

601 29. Shi, Y.; Sun, S.; Zhang, Y. J.; He, Y. S.; Du, M. H.; ÓReilly, A. O.; Wu, S. W.; Yang, Y. H.;

602 Wu, Y. D., Single amino acid variations drive functional divergence of cytochrome P450s in

603 Helicoverpa species. *Insect Biochem. Mol. Biol.* **2022,** *146*, 103796.

604 30. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T. J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam,

605 H.; Remmert, M.; Söding, J.; Thompson, J. D.; Higgins, D. G., Fast, scalable generation of high-

606 quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol*. **2011,** *7* (1),

607 539.

608 31. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.;

609 Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A.

610 A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.;

611 Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer,

612 T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu, K.; Kohli, P.; Hassabis,

613 D., Highly accurate protein structure prediction with AlphaFold. *Nature* **2021,** *596* (7873), 583-

614 589.

615 32. Mirdita, M.; Schütze, K.; Moriwaki, Y.; Heo, L.; Ovchinnikov, S.; Steinegger, M., ColabFold:

616 making protein folding accessible to all. *Nature Methods* **2022,** *19* (6), 679-682.

617 33. Duarte, A.; Pym, A.; Garrood, W. T.; Troczka, B. J.; Zimmer, C. T.; Davies, T. G. E.; Nauen,

618 R.; O'Reilly, A. O.; Bass, C., P450 gene duplication and divergence led to the evolution of dual

619 novel functions and insecticide cross-resistance in the brown planthopper *Nilaparvata lugens*.

620 *Plos Genet.* **2022,** *18* (6), e1010279.

621 34. Guex, N.; Diemand, A.; Peitsch, M. C., Protein modelling for all. *Trends Biochem. Sci.* **1999,**

622 *24* (9), 364-367.

623 35. Irwin, J. J.; Tang, K. G.; Young, J.; Dandarchuluun, C.; Wong, B. R.; Khurelbaatar, M.;

624 Moroz, Y. S.; Mayfield, J.; Sayle, R. A., ZINC20—A free ultralarge-scale chemical database for

625 ligand discovery. *J. Chem. Inf. Model.* **2020,** *60* (12), 6065-6073.

626 36. Trott, O.; Olson, A. J., AutoDock Vina: Improving the speed and accuracy of docking with

627 a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010,** *31*

628 (2), 455-461.

629 37. Perry, T.; Batterham, P.; Daborn, P. J., The biology of insecticidal activity and resistance.

630 *Insect Biochem. Mol. Biol.* **2011,** *41* (7), 411-422.

631 38. Kim, Y. J.; Lee, S. H.; Lee, S. W.; Ahn, Y. J., Fenpyroximate resistance in *Tetranychus*

632 *urticae* (Acari : Tetranychidae): cross-resistance and biochemical resistance mechanisms. *Pest*

633 *Manag.t Sci.* **2004,** *60* (10), 1001-1006.

634 39. Feng, K. Y.; Ou, S. Y.; Zhang, P.; Wen, X.; Shi, L.; Yang, Y. W.; Hu, Y.; Zhang, Y. C.; Shen,

635 G. M.; Xu, Z. F.; He, L., The cytochrome P450 *CYP389C16* contributes to the cross-resistance

636 between cyflumetofen and pyridaben in *Tetranychus cinnabarinus* (Boisduval). *Pest Manag.*

637 *Sci.* **2020,** *76* (2), 665-675.

638 40. Khalighi, M.; Dermauw, W.; Wybouw, N.; Bajda, S.; Osakabe, M.; Tirry, L.; Van Leeuwen,

639 T., Molecular analysis of cyenopyrafen resistance in the two-spotted spider mite *Tetranychus*

640 *urticae*. *Pest Manag. Sci.* **2016,** *72* (1), 103-112.

641 41. Kim, E.; Ahn, K. S.; Gil-Hah, K.; 유정수; 서동규; 한종빈, Inheritance and cross resistance

642 of bifenazate resistance in two-spotted spider mite, *Tetranychus urticae*. *Korean J. Appl.*

643 *Entomol.* **2005,** *44* (2), 151-156.

644 42. Feyereisen, R., Insect CYP genes and P450 enzymes. *Insect Mol. Biol. Biochem.* **2012**;
645 236-316.
646 43. Liu, N.; Li, M.; Gong, Y.; Liu, F.; Li, T., Cytochrome P450s – their expression, regulation,
647 and role in insecticide resistance. *Pestic. Biochem. Physiol.* **2015,** *120*, 77-81.
648 44. Ding, T. B.; Niu, J. Z.; Yang, L. H.; Zhang, K.; Dou, W.; Wang, J. J., Transcription profiling
649 of two cytochrome P450 genes potentially involved in acaricide metabolism in citrus red mite
650 *Panonychus citri*. *Pestic. Biochem. Physiol.* **2013,** *106* (1-2), 28-37.
651 45. Perry, T.; Batterham, P., Harnessing model organisms to study insecticide resistance. *Curr.*
652 *Opin. Insect Sci.* **2018,** *27*, 61-67.
653 46. Li, X. X.; Li, R.; Zhu, B.; Gao, X. W.; Liang, P., Overexpression of cytochrome P450
654 *CYP6BG1* may contribute to chlorantraniliprole resistance in *Plutella xylostella* (L.). *Pest Manag.*
655 *Sci.* **2018,** *74* (6), 1386-1393.
656 47. Wang, L. X.; Tao, S.; Zhang, Y.; Jia, Y. L.; Wu, S. F.; Gao, C. F., Mechanism of metabolic
657 resistance to pymetrozine in Nilaparvata lugens: over-expression of cytochrome P450
658 CYP6CS1 confers pymetrozine resistance. *Pest Manag. Sci.* **2021,** *77* (9), 4128-4137.
659 48. Zeng, B.; Liu, Y. T.; Feng, Z. R.; Chen, F. R.; Wu, S. F.; Bass, C.; Gao, C. F., The
660 overexpression of cytochrome P450 genes confers buprofezin resistance in the brown
661 planthopper, *Nilaparvata lugens* (Stål). *Pest Manag. Sci.* **2023,** *79* (1), 125-133.
662 49. Guengerich, F. P.; Waterman, M. R.; Egli, M., Recent structural insights into cytochrome
663 P450 function. *Trends. Pharmacol. Sci.* **2016,** *37* (8), 625-640.
664 50. Pan, L.; Wen, Z.; Baudry, J.; Berenbaum, M. R.; Schuler, M. A., Identification of variable
665 amino acids in the SRS1 region of CYP6B1 modulating furanocoumarin metabolism. *Arch.*

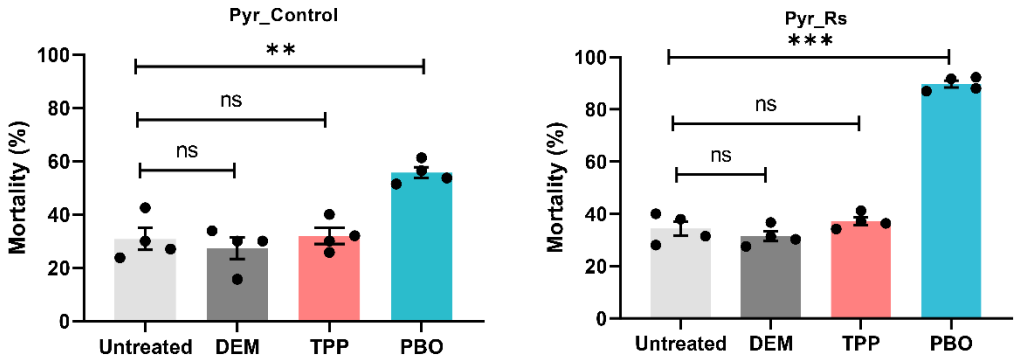
666 *Biochem. Biophys.* **2004,** *422* (1), 31-41.

667 51. Schuler, M. A.; Berenbaum, M. R., Structure and function of cytochrome P450S in insect

668 adaptation to natural and synthetic toxins: insights gained from molecular modeling. *J. Chem.*

669 *Ecol.* **2013,** *39* (9), 1232-1245.

670



671

672 **Fig. 1. Susceptibility of Pyr_Control and Pyr_Rs strains to LC_{30} pyridaben**

673 **after treatment with three enzyme inhibitors.** Data are means ± S.E.M.

674 Asterisks indicate statistically significant difference: ***: $P < 0.001$, ns indicates

675 no significance. Four dots mean four biological replicates in each condition.

676 DEM refers to diethyl maleate; TPP refers to S, S, S tributyl-phosphorotrithioate;

677 PBO refers to piperonylbutoxide.

678

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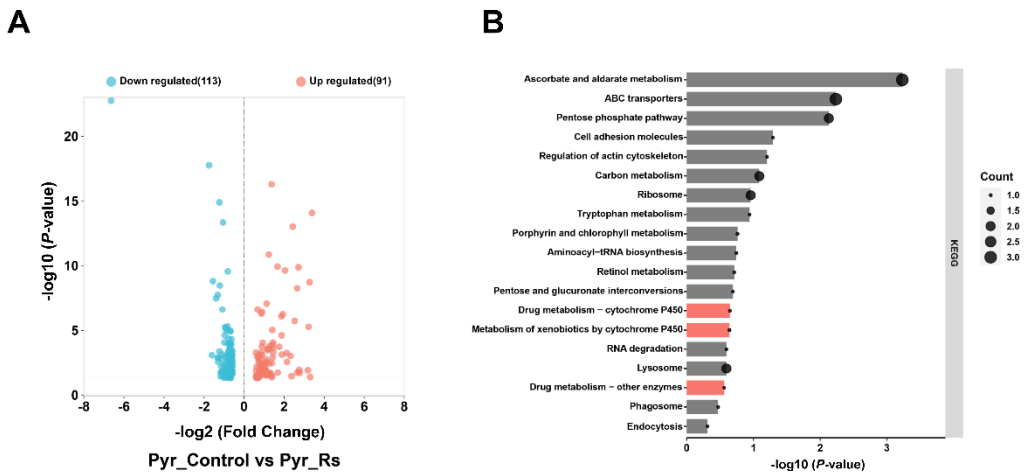


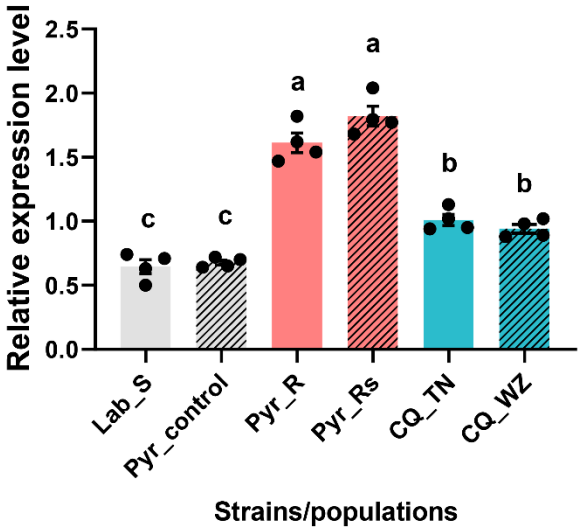
680

681 **Fig. 2. Comparative transcriptome analysis between Pyr_Control and**

682 **Pyr_Rs strains. A.** Differentially expressed genes between Pyr_Control and

683 Pyr_Rs strains. **B**. KEGG pathway function annotation of up-regulated genes.

684



685

686 **Fig. 3. Transcript levels of *CYP4CL2* in different strains/populations.** Data
687 are means ± S.E.M. Different letters indicated a significant difference among
688 strains/populations. (ANOVA, Tukey, $P < 0.05$). Four dots mean four biological
689 replicates in each strain or population. Lab_S, the susceptible strain was raised
690 in lab from 2016. Pyr_R, a pyridaben-resistant population collected from a citrus
691 orchard at Nanning, Guangxi Province, China in 2019. Pyr_Control and Pyr_Rs,
692 two laboratory strains were recombination inbred lines obtained from
693 hybridization between Lab_S and Pyr_R within pyridaben selection. CQ_TN
694 and CQ_WZ, two field populations were collected from citrus orchards in
695 Tongnan, Chongqing municipality and Wanzhou, Chongqing municipality.

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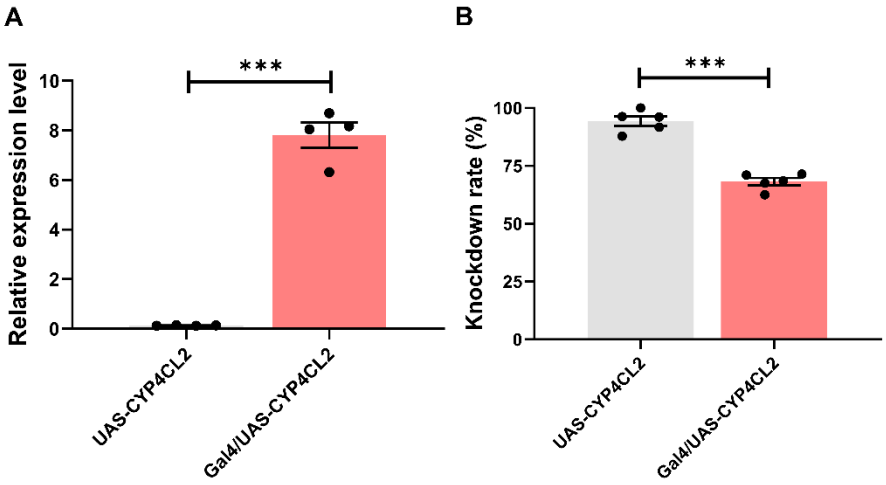


698

699 **Fig. 4. Transcript levels of *CYP4CL2* in transgenic *Drosophila***

700 ***melanogaster* and pyridaben resistance of transgenic lines. A.** Relative

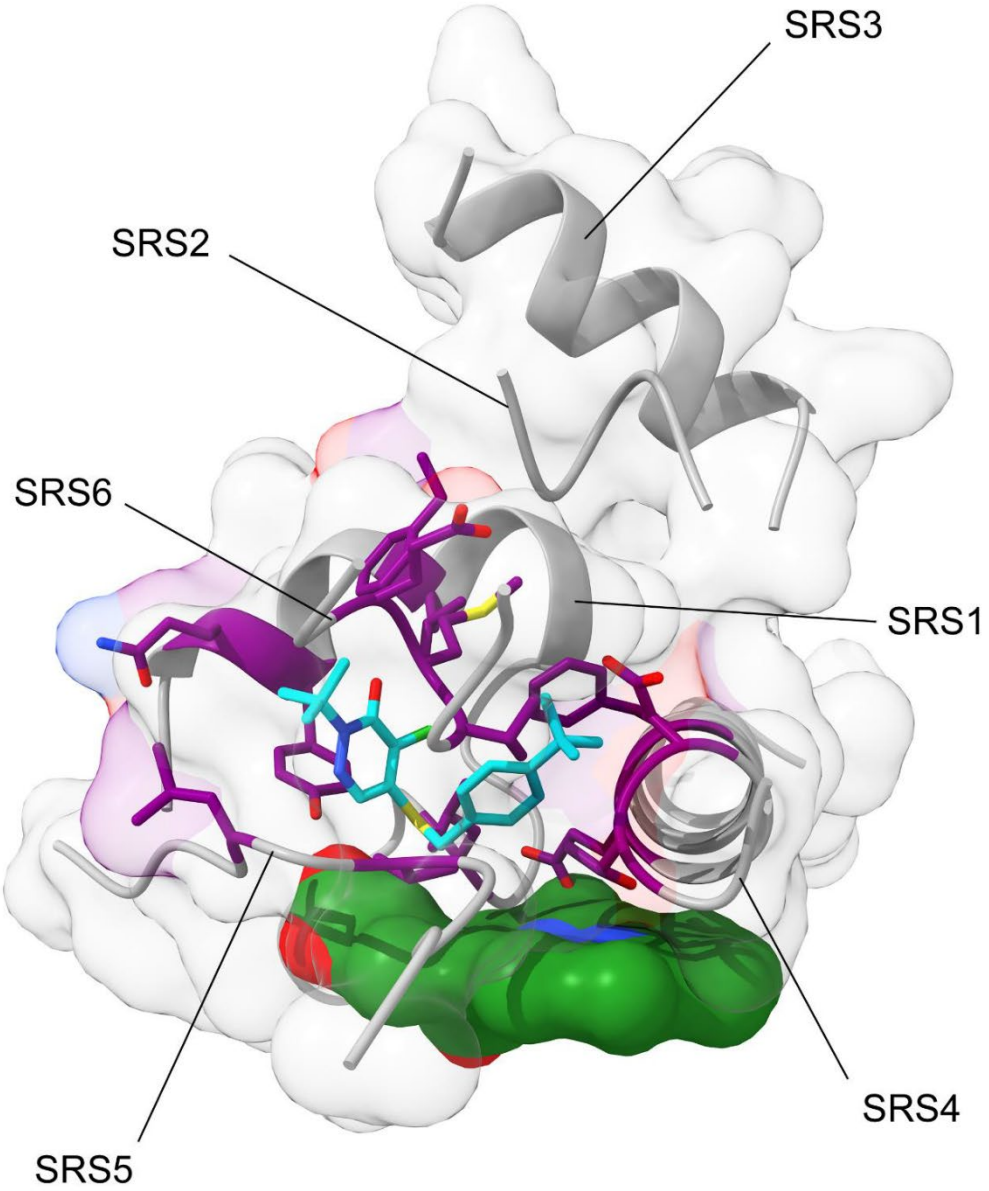
701 transcript levels of *CYP4CL2* in UAS-CYP4CL2 and Gal4/UAS-CYP4CL2 lines.

702 **B**. Mortality of transgenic lines after treatment with pyridaben. UAS-CYP4CL2

703 dots are shown on light grey bar and Gal4/UAS-CYP4CL2 dots are shown on

704 pink bar. Data are means ± S.E.M. Asterisks indicate significant difference (***:

705 $P < 0.001$). Four dots mean four biological replicates in each line.



706

707 **Fig. 5. Docking prediction for pyridaben in an Alphafold2-generated**

708 **CYP4CL2 model**. SRS1–6 regions surrounding the active site of CYP4CL2 are

709 shown as ribbons with semi-transparent surfaces. Heme co-factor is shown in

710 green space-fill and pyridaben is shown as cyan sticks. Amino acids <4.0 Å

711 from docked pyridaben are shown as purple sticks.

712

Table 1. Bioassays of different acaricides with various modes of action against Pyr_Control and Pyr_Rs strains.

Groups	Acaricides	Strain	N	Slope (±SE)	LC_{50} (mg/L)95%CL[a]	χ^2 (df)	RRs[b]
Mitochondrial complex II electron transport inhibitors	Cyflumetofen	Pyr_control	426	1.546±0.164	6.917 (3.863 – 18.216)	13.972 (4)	1
		Pyr_Rs	440	1.601±0.157	5.926 (3.404 – 12.566)	14.573 (4)	0.857
	Cyenopyrafen	Pyr_control	509	2.356±0.189	0.560 (0.479 – 0.649)	4.473 (5)	1
		Pyr_Rs	571	2.225±0.183	1.838 (1.575 – 2.130)	2.178 (4)	3.282
Glutamate-gated chloride channel (GluCl) allosteric modulators	Abamectin	Pyr_control	334	1.248±0.117	0.032 (0.006 – 0.081)	19.772 (4)	1
		Pyr_Rs	764	1.718±0.164	0.077 (0.052 – 0.116)	7.625 (4)	2.406
Sodium channel modulators	Fenpropathrin	Pyr_control	507	4.053±0.419	27.508 (22.478 – 33.181)	4.480 (4)	1
		Pyr_Rs	390	2.041±0.199	35.511 (29.669 – 43.286)	3.167 (4)	1.291
Inhibitors of mitochondrial ATP synthase	Propargite	Pyr_control	433	1.474±0.150	40.131 (32.599 – 50.304)	0.167 (4)	1
		Pyr_Rs	454	1.438±0.165	79.161 (48.178 – 186.089)	9.680 (4)	1.973
Mitochondrial complex III electron transport inhibitors – Qo site	Bifenazate	Pyr_control	501	1.860±0.166	28.468 (20.344 – 38.450)	5.596 (4)	1
		Pyr_Rs	569	2.098±0.191	136.275 (105.658 – 174.956)	5.437 (4)	4.787
Mitochondrial	Fenpyroximate	Pyr_control	637	1.904±0.153	14.889 (11.216 – 18.932)	4.764 (4)	1

complex I electron		Pyr_Rs	608	2.663±0.270	943.212 (639.422 – 1409.787)	15.059 (4)	63.349
transport inhibitors	Tolfenpyrad	Pyr_control	443	1.533±0.160	289.230 (187.633 – 425.892)	5.796 (4)	1
		Pyr_Rs	497	1.556±0.198	2104.924 (1313.934 – 5072.115)	10.550 (4)	7.278
	Tebufenpyrad	Pyr_control	440	2.298±0.189	145.347(114.310 –188.029)	5.177 (4)	1
		Pyr_Rs	529	2.949±0.252	225.965(191.511 – 272.152)	5.230 (4)	1.555
	Fenazaquin	Pyr_control	520	2.263±0.203	30.894(26.389 – 36.428)	1.970 (4)	1
		Pyr_Rs	699	2.405±0.176	22.415(19.792 – 25.400)	2.998 (4)	0.726

[a]CL, confidence limits.

[b]RR, resistance ratio = LC_{50} of Pyr_Rs / LC_{50} of Pyr_control.

Table 2. **CarEs, GST, and P450 activities in Pyr_Control and Pyr_Rs strains.**

Detoxification enzymes	Strain	Activity of detoxification enzymes	R/S	*P*-value[a]
GSTs	Pyr_control	0.233 ± 0.018	0.910	0.130
(μmol/mg pro/min)	Pyr_Rs	0.212 ± 0.014		
CarEs	Pyr_control	0.141 ± 0.004	1.014	0.390
(nmol/mg pro/min)	Pyr_Rs	0.143 ± 0.002		
P450s	Pyr_control	0.169 ± 0.001	1.550	0.006
(nmol/mg pro/min)	Pyr_Rs	0.262 ± 0.057		

[a]*P*-value, the significance of the detoxification enzymes activity between Pyr_Control and Pyr_Rs were analyzed by Student's *t*-test using GraphPad 8.0 statistical software.

Table 3 Susceptibility to pyridaben after silencing *CYP4CL2* expression in laboratory strains and field populations of *P. citri*.

Origins	Mite	Treatments	Total number	Slope±SE	LC_{50} (95% CL) mg/L[a]	χ2 (df)	LCR_{50} (95% CL)[b]
Laboratory	Pyr_Control	ds*GFP*	502	2.717 ± 0.250	2.535 (2.261-2.901)	1.046 (5)	-
		ds*CYP4CL2*	431	2.964 ± 0.296	1.185 (1.039-1.322)	1.865 (4)	2.140 (1.799-2.545)
	Pyr_Rs	ds*GFP*	562	2.183 ± 0.210	1276.617(1134.408-1452.943)	4.445 (5)	-
		ds*CYP4CL2*	456	3.241 ± 0.331	637.595 (555.586-711.531)	3.513 (4)	2.002 (1.682-2.384)
Field	CQ_TN	ds*GFP*	381	2.082 ± 0.472	174.709 (134.830-261.562)	1.029 (4)	-
		ds*CYP4CL2*	459	1.596 ± 0.556	91.028 (25.559-129.269)	1.794 (3)	1.919 (1.076-3.424)
	CQ_WZ	ds*GFP*	517	1.589 ± 0.184	215.237 (180.123-269.557)	1.399 (6)	-
		ds*CYP4CL2*	527	1.402 ± 0.188	93.766 (73.103-113.524)	3.108 (5)	2.295 (1.712-3.076)

[a]CL, confidence limits.

[b]LCR_{50}, lethal concentration ratio at LC_{50}. If the 95% CL of LCR_{50} >1, it indicated that ds*CYP4CL2* significantly reduced the resistance against pyridaben in *P. citri*.

Pyr_Control and Pyr_Rs, two laboratory strains were recombination inbred lines obtained from hybridization and pyridaben selection.

CQ_TN and CQ_WZ, two field populations were collected from citrus orchards in Tongnan, Chongqing municipality and Wanzhou, Chongqing municipality.

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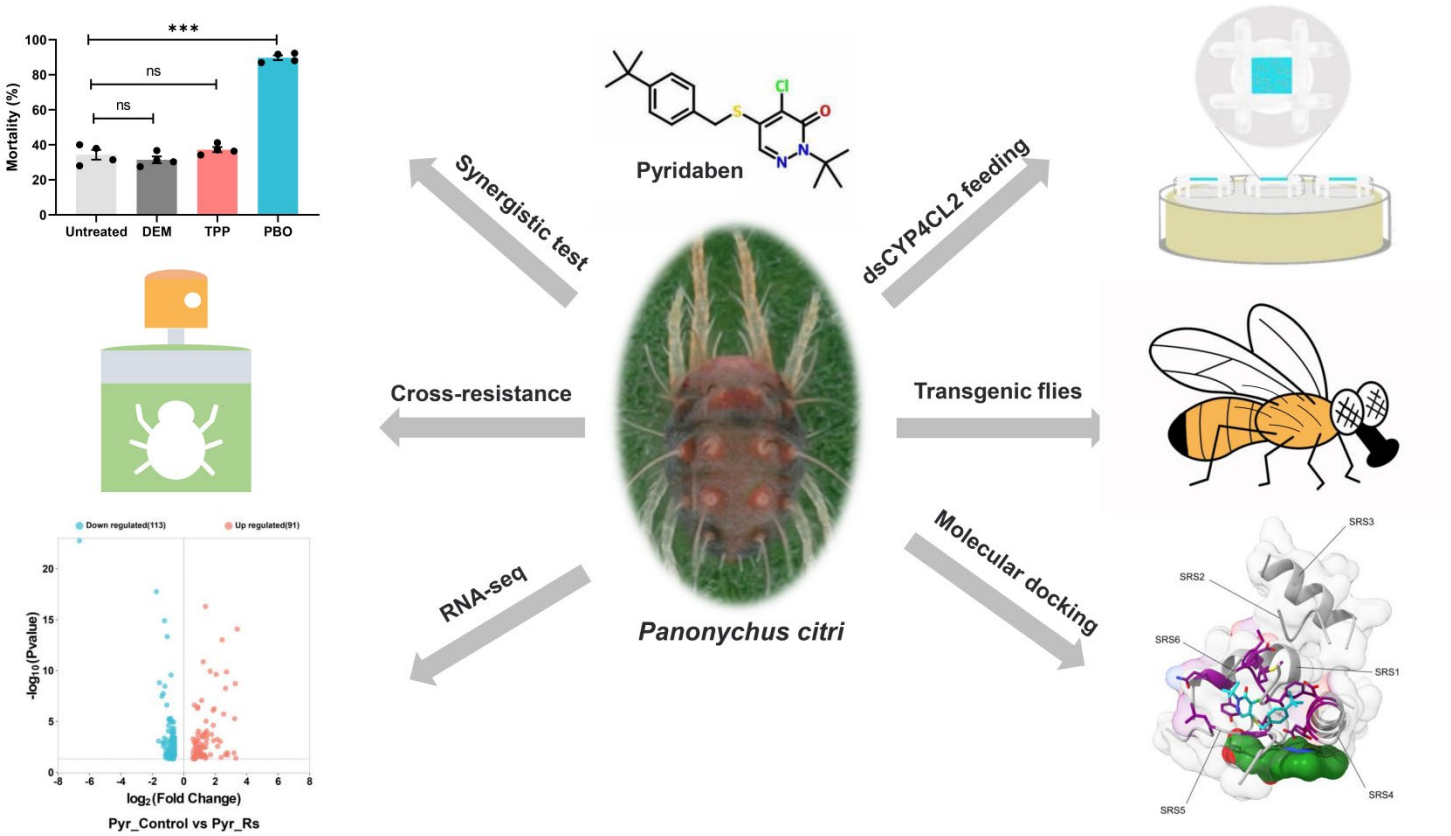

Based on the segregated pyridaben resistant and susceptible strains, this study systematically investigated the cross-resistance to eleven acaricides in *Panonychus citri* when the pyridaben resistance developed. We comparatively analyzed the detoxification enzymes activity (P450, CarEs and GSTs) in resistant and susceptible strains, and confirmed the effects of three synergists (DEM,

TPP and PBO) on the susceptibility of the mite to pyridaben. A upregulated P450 gene (*CYP4CL2*) was identified in resistant strain via RNA-seq. Furtherly, function verification of RNA interference-mediated and overexpression in transgenic flies demonstrated the important role of *CYP4CL2* in pyridaben resistance development. Molecular docking provided more evidences in the binding pattern between pyridaben and CYP4CL2.