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# Mutations of voltage gated sodium channel contribute to pyrethroid resistance in *Panonychus citri*

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**Abstract**

Insecticide resistance in *Panonychus citri* is a major obstacle to mite control in citrus orchards. Pyrethroid insecticides are continually used to control mites in China, although resistance to pyrethroids has evolved in some populations. Here, the resistance to the pyrethroid fenpropathrin was investigated and seven out of eight field-collected populations of *P. citri* exhibited high level of resistance, ranging from 171- to 15,391-fold higher than the susceptible (SS) comparison strain. Three voltage-gated sodium channel (VGSC) mutations were identified in the tested populations: L1031V, F1747L, and F1751I. Amplicon sequencing was used to evaluate the frequency of these mutations in the nineteen field populations. L1031V and F1751I were present in all populations at frequencies of 11.6–82.1% and 0.5–31.8%, respectively, while the F1747L mutation was only present in twelve populations from Chongqing, Sichuan, Guangxi and Yunnan provinces. Introduction of these mutations singly or in combination into transgenic flies significantly increased their resistance to fenpropathrin and these flies also exhibited reduced mortality after exposure to the pyrethroids permethrin and beta-cypermethrin. *P. citri* VGSC homology modeling and ligand docking indicate that F1747 and F1751 form direct binding contacts with pyrethroids, which are lost with mutation, whereas L1031 mutation may diminish pyrethroid effects through an allosteric mechanism. Overall, the results provide molecular markers for monitoring pest resistance to pyrethroids and offer new insights into the basis of pyrethroid actions on sodium channels.

**Key words**: *Panonychus citri*, kdr mutation, pyrethroid resistance, amplicon sequencing, transgenic flies, molecular docking

## Introduction

Voltage-gated sodium channels (VGSCs) are critical components of nearly all animal nervous systems (Banazeer et al., 2022). VGSCs conduct sodium ions across the plasma membrane to initiate and propagate electrical signals that regulate insect behavior (Williams et al., 2022). Pyrethroids target the VGSCs of insects by stabilising the open functional state, resulting in nervous system dysfunction. They are highly efficient and exhibit specific selective toxicity for insects over mammals, leading to their widespread use for terminating or controlling pests or mites (Scott, 2019). However, the extensive use of pyrethroid insecticides has promoted the evolution of resistance, thereby reducing the efficacy of currently used pyrethroids (Pan et al., 2020). The presence and evolution of resistance to pyrethroids has been previously investigated in *Musca domestica* (Scott, 2017, Roca-Acevedo et al., 2023), *Rhipicephalus microplus* (Kumar et al., 2020), *Leptinotarsa decemlineata* (Molnar & Rakosy-Tican, 2021), *Plutella xylostella* (Banazeer et al., 2022), and mosquito vectors (Scott et al., 2015, Smith et al., 2016, Amelia-Yap et al., 2018, Chen et al., 2020).

Pyrethroids stimulate nerve cells to prolonged-activation and consequently leads to paralysis or death, which is known as *knockdown* (Davies et al., 2008). An important resistance mechanism against pyrethroids or DDT is known as 'knockdown resistance' (*kdr*), involving various mutations found in the VGSC of many pests or mites (Rinkevich et al., 2013). More than 61 sodium channel mutations, or combinations of mutations, have been reported to be responsible for, or associated with, pyrethroid resistance in various arthropods (Dong et al., 2014). Recently, several *kdr* mutations conferring pyrethroid resistance have been identified in pest mites. For example, four amino acid substitutions in VGSC (F1528L, L1596P, I1752V, and M1823I) have been determined to be associated with fluvalinate resistance in the varroa mite (*Varroa destructor*) (Wang et al., 2002). Further, the M918T mutation of the domain II region led to high resistance to bifenthrin in *Tetranychus evansi* (Nyoni et al., 2011). In addition, the mutations L1024V, A1215D, and F1538I have been detected in a pyrethroid-resistant strain of *Tetranychus urticae*, with the L1024V and A1215D mutations potentially contributing to fenpropathrin resistance (Kwon et al.,

2010). Moreover, the point mutation F1538I at the IIIS6 site of the sodium channel has also been identified in a fenpropathrin-resistant population of *Panonychus citri* (Ding et al., 2015).

*Panonychus citri* (McGregor) (Acari: Tetranychidae), also known as the citrus red mite, is a dominant pest mite in global citrus orchards. The mites feed on the leaves, fruits, and the fresh shoots of various citrus plants, causing severe damage to the plants (Alavijeh et al., 2020). Defoliation and botchy fruits appear upon profusive mite outbreaks in orchards. Fenpropathrin is a pyrethroid chemical that has been extensively used for mite control in many Chinese citrus orchards (Hu et al., 2010). However, *P. citri* has already developed high resistance to fenpropathrin in several citrus production areas (Pan et al., 2020). Nevertheless, the frequency of VGSC mutations in *P. citri* across these large areas and the contributions of single mutations, or combinations, to pyrethroid resistance have not yet been explored.

Here, resistance to fenpropathrin was investigated among several *P. citri* populations collected from five provinces in China. Three amino acid substitutions of VGSC were identifed in fenpropathrin-resistant populations and the frequencies of these mutations in nineteen *P. citri* field populations were subsequently analyzed. The roles of the VGSC mutations in *P. citri* pyrethroid resistance were further analyzed by constructing transgenic flies carrying these mutations as single mutations or combinations. Further, a homology model of *P. citri* VGSC was generated to investigate differences between wild type (WT) and mutated models that bind to pyrethroids.

## Materials and methods

### Mites

A relatively susceptible strain (SS) of *P. citri* was collected from citrus orchards at the Citrus Research Institute at Southwest University in Chongqing, China (Pan et al., 2020). Mites were maintained at 27 ± 1ºC, 60 ± 5% relative humidity, and with a 16: 8 h light / dark photoperiodic cycle. The SS strain was used as the control for toxicity bioassays. Eight field populations of *P. citri* were collected for bioassays from the Yunnan, Jiangxi, Chongqing, Sichuan, and Guangxi provinces. Sampling location

details are provided in Table S1. Adult female mites were collected for each population from at least 10 trees per citrus orchard. Over 5,000 adult female mites were collected per field population and randomly selected for bioassays. In addition, nineteen field populations were collected for DNA extraction and sequencing to investigate mutation frequency (Table S2). All mites collected from orchards were sampled as described above. Of the collected mites, 100 adult female mites were preserved in ten 1.5 mL centrifuge tubes and then preserved in 100% ethanol at room temperature.

**Bioassay chemicals**

The acaricide chemical 20 g/L EC fenpropathrin (Well-Done Chemical, Hangzhou, China) was used for bioassay. Other chemicals were used to test insecticide susceptibility in transgenic *D. melanogaster*, including permethrin (type I, 95% cis-trans isomer mixture: Macklin Inc, Shanghai, China), beta-cypermethrin (type II, 95%; Xiya Reagent, Shangdong, China), and fenpropathrin (92.9%; Veyong Animal Pharmaceutcal Co., Ltd, Hebei, China).

**Bioassays**

The susceptibility to fenpropathrin in field populations of *P. citri* were assayed using the leaf-dipping method, as described by Yamamoto et al (Yamamoto et al., 1995). Leaf discs of 25 mm diameter were produced from thoroughly washed fresh sweet orange (*Citrus sinensis* (L.) Osbeck) leaves and placed on water-saturated sponges in Petri dishes of 9 cm diameter. A total of 25–30 females were subsequently transferred to each disc with a soft brush. After 2–3 h, the leaf discs with mites were dipped in acaricide solutions containing seven different concentrations for 5 s. Superfluous liquid on the discs was absorbed with absorbent paper to avoid drowning the mites. Leaf discs treated with distilled water were used as control. Sponges were kept wet with enough water to ensure they did not dry. Each evaluated concentration included five replicate assays. All leaf discs were incubated at 25 ± 0.5°C, with 60% relative humidity and a 16: 8 h (L:D) photoperiod. Mite mortality was assessed after 24 h. Drowned mites on the edge of the leaf discs and dead mites due to human factors were not recorded as mortalities, while mites that could not move after light

143 touching with a camel hairbrush were scored as dead.

144 **Detection of candidate target site mutations**

145 Genomic DNA was extracted from approximately 200 adult female mites from
146 different field populations and subjected to PCR amplification. The qualities and
147 concentrations of DNA were assessed with a spectrophotometer (Nanodrop 2000,
148 Thermo Fisher, USA). PCR amplification of *P. citri* VGSC (GenBank: KF646792)
149 regions was conducted, as described for *P. citri* (Ding et al., 2015) and *Tetranychus*
150 *urticae* (Inak et al., 2019, Kwon et al., 2014), using primers shown in Table S3. PCR
151 products were purified using the MiniBEST DNA Fragment Purification Kit (TaKaRa,
152 Dalian, China) and sequenced at the Beijing Genomics Institute (BGI, Beijing,
153 China). Nucleotide or protein sequence alignments were visualized in MEGA (version
154 6.0, USA) and Jalview (version 2.0, USA).

155 **Amplicon sequencing of pooled female adults to detect mutations**

156 *DNA preparation*

157 For each group of *P. citri* adult females from citrus orchards, DNA was extracted
158 from a pool containing 80 adult female mites from each field population and more
159 than eight replicates were prepared. DNA was extracted using a Mollusc DNA Kit
160 (Omega Bio-Tek, USA), with DNA quality and quantity assessed with a
161 spectrophotometer (Nanodrop 2000, Thermo Fisher, USA).

162 *PCR amplification with tagged primers*

163 Six base-pair oligonucleotide barcodes were added to the 5' ends of the forward
164 and reverse primers (Table S4) to enable multiplex sequencing of amplicon pools and
165 subsequent identification of different field samples. PCR mixtures and amplifications
166 were used as described above. PCR products were separated by 1% agarose gel
167 electrophoresis and target DNA fragments were purified using a TaKaRa MiniBEST
168 Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China).

169 *Next-generation sequencing (NGS) of pooled amplicons*

170 PCR products from different populations from each year were pooled for NGS.
171 Amplicon concentrations per population were quantified and PCR products were
172 pooled in equimolar concentrations. Each pool corresponded to a VGSC gene

fragment amplicon group. Sequencing libraries were constructed using TruSeq DNA PCR-Free Kits (Illumina, CA). Pooled amplicons were then sequenced on the Illumina Hiseq2500 platform used paired-end 250 bp sequencing at BIOZERON Biotechnology Ltd. (Shanghai, China).

*DNA read sorting by tags*

Raw sequence quality data was assessed using the FastQC software program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Preprocessing of raw data was conducted with the FASTX_Clipper program, followed by quality filtering with the FASTQ_Quality_Filter. The raw data was subsequently de-duplicated using the FASTX_Collapser program (FASTX-Toolkit, http:/hannonlab.cshl.edu/fastx_toolkit/index.html). Clean data were mapped to the reference genome of *P. citri* (accession number: GWHBAOM00000000) with the BWA (Li & Durbin, 2009) aligner and then filtered using the Seqtk program (https://github.com/lh3/seqtk). Haplotypes were then classified using the FASTX_Barcode_Splitter program based on the unique barcode tags of each population. A threshold value of 1/(2n) was used to filter the data and remove false positive haplotypes from each population, where n is the number of adult females in the pool and represents the theoretical minimal allele frequency for the site (Edwards et al., 2018). Haplotypes were discarded when their frequency was less than the threshold.

**Correlation of resistance level versus allele frequency in field populations of *P. citri***

According to the determined resistance levels in field populations and allele mutation frequencies, the correlation between the frequencies of each mutation (and their combinations) and the resistance level in field populations were analyzed. The resistance ratios of field populations (Table 1) were plotted against the frequency of these mutations (Table 2), and linear regression lines were generated (GraphPad Prism 8.0 software program, GraphPad Software Inc.). When the allele frequencies at each mutation site were conducted in various combinations for correlation analysis, mean parameter values were used (Kwon et al., 2010, Kwon et al., 2004, Kwon et al., 2012,

Chang et al., 2012).

**Transgenic *D. melanogaster* and mortality bioassays**

*Transgenic expression of VGSC (WT and mutated) in Drosophila strains*

To confirm whether mutations of VGSC in *P. citri* conferred pyrethroid resistance, the VGSC genes (6,729 bp) of WT and mutant (L1031V, F1747L and F1751I) genes were expressed in *D. melanogaster* via the Gal4/UAS system from Fungene Biotech (http://www.fungene.tech/) (Beijing, China). WT and mutant genes were synthesized and the ORFs of VGSC genes were subcloned into the expression vector (pJFRC28-10XUAS-IVS-GFP-p10 vector). Clones were microinjected into the germ line of *D. melanogaster* ($W^{1118}$) embryos carrying the attP40 (25C6 of chr.2) docking site on chromosome 2 to generate transgenic lines using the PhiC31 system. The transgenic lines were first crossed with *Bc/CyO* and screened for candidates that were then verified by PCR (Table S5) and sequenced. Successful construction of UAS-VGSC lines (UAS-WT, UAS-L1031V, UAS -F1747L, UAS -F1751I, UAS -L1031V+ F1747L, UAS -L1031V+ F1751I, UAS-F1747L+F1751I, UAS-L1031V+F1747L+F1751I) were balanced by Actin5C-GAL4/*CyO* to drive ubiquitous expression. Gal4/UAS-VGSC lines were finally obtained and PCR was then used to validate VGSC mutation sites (Table S3 and Fig. S1). Flies with the same background as the UAS transgenic lines, but devoid of the driver Actin5C-Gal4 line to overexpress VGSC were used as controls. All fly lines were reared on an artificial diet at 25°C, 65% relative humidity and a 12: 12 h light/dark photoperiod.

*Drosophila contact bioassays*

Bioassays were conducted to assess the susceptibility of transgenic flies to fenpropathrin, as previously described (Ibrahim et al., 2015, Riveron et al., 2013). Insecticide filter papers with five to seven concentrations of fenpropathrin were prepared in distilled water that contained 1% acetone and 0.1% Triton-X 100. The papers were rolled and placed in 5 cc glass vials. Vials were then plugged with cotton soaked in 20% (wt/vol) sucrose, followed by transfer of 25 flies into each vial. The mortality plus knockdown, when their legs cannot move or body back down, was scored after exposure to insecticides for 24 h. Six replicates were used for each

concentration. All tested flies were reared at 25°C, 65% relative humidity, and with a 12: 12 h light/dark photoperiod. Student's *t*-tests were used to compare differences in mortality and knockdown of treatment (Gal4/UAS lines) groups with each control (UAS line) group, in addition to differences between mutant and WT groups.

In addition to the above experiments, the ability of the VGSC mutations to confer resistance to two types of pyrethroids, type I (permethrin) and type II (beta-cypermethrin) was evaluated. Pyrethroid filter papers (permethrin at 50 mg/L and beta-cypermethrin at 5 mg/L) were prepared as described above. The mortality plus knockdown was scored after exposure to insecticides for 1, 2, 3, 6, 12, 24, 36 and 48 h. Six replicates were used for each assay. Student's *t*-tests were used to compare differences in knockdown rate between the experimental (Gal/UAS lines) and control (UAS line) groups, in addition to between mutant and WT groups.

**_P. citri_ VGSC homology modelling and ligand docking**

A homology model of *P. citri* VGSC was generated using the VGSC structure of *Periplaneta americana* (PDB entry 5X0M) as template, as described.(Clarkson et al., 2021) Sequences were aligned using Clustal Omega (Sievers et al., 2011) and the MODELLER program was used to generate 50 homology models (Webb & Sali, 2016). The internal scoring function of MODELLER was used to select 10 models that were then visually inspected and submitted to the VADAR webserver (Willard et al., 2003) to assess stereochemical soundness, allowing selection of the final best model. VGSC mutations were introduced into the homology model, followed by 30 steps of steepest descent and then 50 steps of conjugate gradient energy minimization, using Swiss-PdbViewer software (Guex et al., 1999).

A 3-dimensional structure file for fenpropathrin, permethrin or beta-cypermethrin was generated using MarvinSketch (v19.22) of the ChemAxon suite (http://www.chemaxon.com). These files were then prepared using the AutoDockTools program (v.1.5.7; Molecular Graphics Laboratory, Scripps Research Institute, La Jolla, CA, USA) to define rotatable bonds and merge nonpolar hydrogens. The Autodock Vina (Trott & Olson, 2010) program was used for docking the ligands within a grid of 20 x 20 x 20 points of 1 Å spacing that was centered on

the PyR1 binding pocket (O'Reilly et al., 2006a, Du et al., 2015) of the WT VGSC model. Figures were produced using Pymol 2.2.3 (DeLano Scientific, San Carlos, CA, USA).

**Statistical analyses**

PoloPlus v.2.0 (LeOra Sofware 2008) was used to calculate the median lethal concentration ($LC_{50}$) and 95% confidence limits (95% CL) for bioassays of *P. citri* and transgenic flies. Student's *t*-tests were used to test differences between experimental and control groups and also between mutant and WT groups. The GraphPad Prism 8.0 software program (GraphPad Software Inc.) was used for data analysis and plot generation.

**Results**

**Resistance to fenpropathrin**

The fenpropathrin resistance of *P. citri* collected from four field sampling sites varied (Table 1). The $LC_{50}$ values of fenpropathrin against the *P. citri* field populations from Yuxi (YX-YN), Ganzhou (GZ-JX), Liangping (LP-CQ), Anyue (AY-SC), and Nanning (NN-GX) ranged from 45.5 to 4,093.9 mg/L in 2021. The WZ-CQ population was more susceptible to fenpropathrin than the NN-GX, Tongnan (TN-CQ), Wanzhou (WZ-CQ) and Guilin (GL-GX) populations collected in 2022. Except for the LP-CQ, TN-CQ, and WZ-CQ populations, very high levels of resistance (i.e., >3,000-fold resistance levels) were detected in field populations compared to the SS strain.

**VGSC mutations in *P. citri* populations**

To detect potential VGSC mutations in *P. citri* populations of southwest China, the presence of two potential mutation sites (L1031 and F1751) of *P. citri* VGSC gene (the amino acid positions are numbered based on the *P. citri* VGSC protein sequence) were investigated that corresponded to the L1024 and F1538 mutations of *T. urticae* (Inak et al., 2019). Three mutation types were observed for *P. citri*, with the L1031V amino acid change at segment 6 of domain II (DII S6) in addition to the F1747L and F1751I mutations at segment 6 of domain III (DIII S6) (Fig. 1) in the resistant populations of YX-YN, AY-SC, NN-GX, and GL-GX.

**Frequency of the L1031V, F1747L, and F1751I mutations in field populations**

The mutations in over 14,000 mites were evaluated using amplicon sequencing of genes encoded by individuals from nineteen different sites across four provinces (Table 2). The frequency of mutations varied from 11.6 to 82.1%, 0 to 34.8%, and 0.5 to 31.8% for L1031V, F1747L and F1751I, respectively. The L1031V mutation was widespread across sample sites, with highest frequency of 82.1% in the GL2-GX population. The F1751I mutation was most prevalent at the Liangping locality in 2022, occurring in 31.8% of genotypes. Moreover, the F1751I mutation was widespread among the nineteen samples. A new mutation (F1747L) that had not previously been reported in pest mites was also identified in twelve samples, with the highest frequency (34.8%) observed in the RL-YN samples.

**Correlation of fenpropathrin resistance level and VGSC mutation frequency in *P. citri* field populations**

When the mutation frequencies were plotted against the fenpropathrin resistance levels in *P. citri*, the L1031V, F1747L, and F1751I mutations were lowly correlated with the fenpropathrin resistance level ($r^2$ = 0.1166, 0.1262 and 0.0092, respectively) (Fig. S2). In addition, low correlations were also observed between the frequencies of mutation combinations and the resistance levels ($r^2$ = 0.0649, 0.1052 and 0.0632 for L1031V+F1747L, L1031V+F1751I and L1031V+F1747L+F1751L, respectively) (Fig. S2). Nevertheless, the upward tendency of resistance ratio was recorded when the allele frequency increased.

**Importance of the VGSC mutation in pyrethroid resistance using transgenic expression in *Drosophila melanogaster***

Bioassays with fenpropathrin revealed that flies with the VGSC mutations L1031V, F1747L, and F1751I were resistant to fenpropathrin (Table 3). However, different contributions of VGSC mutations to fenpropathrin resistance were observed for transgenic flies. Varying $LC_{50}$ values of 4.17, 2.97, and 5.63 mg/L were identified in the Gal4/UAS-mutant (L1031V, F1747L, and F1751I) strains. Compared to the Gal4/UAS-WT strain, the resistance of the Gal4/UAS-mutant (L1031V, F1747L, and F1751I) strains were significantly higher (2.71-, 1.93-, and 3.66-fold higher,

respectively), with no overlap at the 95% CL level. Combinations of VGSC mutations in transgenic flies further increased resistance to fenpropathrin compared to single mutations (Table 3). Indeed, the highest resistance (4.55-fold compared to the Gal4/UAS-WT lines) was detected when all three sites were mutated. Similar results were observed in the UAS-VGSC lines (Table S6).

Bioassays with permethrin (type I pyrethroid) and beta-cypermethrin (type II pyrethroid) were also performed to assess their contributions to pyrethroid resistance in transgenic lines (Fig. 2). Flies with VGSC mutations exhibited lower mortality and knockdown rates compared to WT flies. The Gal4/UAS-mutant (L1031V, F1747L, F1751I, L1031V+F1747L, L1031V+F1751L, and L1031V+F1747L+F1751I) lines exhibited significantly lower mortality and knockdown rates (76, 81, 73.6, 64, 51, and 50%, respectively) to permethrin compared to the Gal4/UAS-WT line (94%) at 48 h ($p < 0.001$) (Fig. 2). No significant differences were observed between flies with overexpressed VGSC genes (Gal4/UAS lines) compared to control (UAS line) flies (Fig. S3). Similar results were observed with beta-cypermethrin bioassays, with significantly reduced mortalities and knockdown rates in Gal4/UAS-mutant lines (70, 79, 70.4, 68, 60, and 56%, respectively) compared with Gal4/UAS-WT flies (90%) after exposure for 48 h ($p < 0.001$) (Fig. 2). For Gal4/UAS and UAS lines exposed to beta-cypermethrin, flies with VGSC-WT and VGSC-L1031V+F1747L genes exhibited significant differences in mortalities and knockdowns at exposure times of 24 and 36 h (Fig. S4).

These results indicate that mutations of *P. citri* VGSC genes confer resistance to pyrethroids, but with varying degrees of resistance. The F1751I mutation conferred the highest resistance to pyrethroids, followed by the L1031V and F1747L mutations.

**Molecular modeling of the VGSC mutations**

A homology model of *P. citri* VGSC was generated in order to assess how mutations could affect pyrethroid binding. Although L1031 is located on the DII S6 while F1747 and F1751 are located on the DIII S6 (Fig. 1A), in the 3-dimensional model (Fig. 3A, B), these three residues are positioned in close spatial proximity with <13 Å distance between their α-carbons. Their side chains shape the lower end of the

lipid-accessible cavity that forms pyrethroid binding site PyR1 (O'Reilly et al., 2006a, Du et al., 2015).

A docking prediction for permethrin with an estimated free energy of binding ($\Delta G_b$) of -8.15 kcal/mol positioned the pyrethroid to occupy the length of the binding cavity (Fig. 3A and Fig. S5B, E). The phenyoxy group of permethrin makes direct binding interactions with F1747 and F1751 side chains, suggesting stacking between the three aromatic rings is a major binding interaction. In contrast, the L1031 side-chain is too far (>4.5 Å) to directly interact with permethrin but it does contact the F1751 side chain and pack against the DIII S6 helix, thus contributing to shaping the binding site. Similar docking poses, estimated binding energies and interactions with F1747 and F1751 side chains were found with fenpropathrin ($\Delta G_b$ of -8.0 kcal/mol) and beta-cypermethrin $\Delta G_b$) of -6.78 kcal/mol (Fig. S5).

The loss of an aromatic ring with either the F1747 or F1751 mutation could reduce aromatic-aromatic binding interactions with a pyrethroid phenyoxy group, which is common to permethrin, fenpropathrin and beta-cypermethrin. Furthermore, F1747L and F1751I mutations each introduce a branched hydrocarbon side-chain that reshapes the binding cavity in that region (Fig. 3B-E), which may sterically hinder pyrethroid binding. In contrast, L1031V is not predicted to affect pyrethroid binding directly but the mutation to a smaller side chain may affect packing between DII and DIII S6 helices, thereby conferring resistance by allosterically modifying the pyrethroid binding site.

**Discussion**

In this study, different degrees of resistance to the pyrethroid fenpropathrin were identified among mites in most citrus orchards of Southern China. The most common mechanism of pyrethroid resistance has arisen from substitution of certain amino acids in pest VGSCs (Dong et al., 2014). Indeed, many such mutations associated with pyrethroid resistance have been described in diverse arthropod species, including substitution of M918T/L/V/I, L925I/V, T929I/C/V/N, L932F, L1014F/H/S/W, F1534C, and F1538I (numbering based on *Musca domestica* VGSC) (Scott, 2019, Dong et al., 2014). Limited mutations of VGSCs have been identified in pest mite

populations with pyrethroid resistance, with the few being L925V/I (Koc et al., 2021, Almecija et al., 2022), L1024V, A1215D, and F1538I (numbering according to *M. domestica*) (Simma et al., 2020, Zhang et al., 2022, Ding et al., 2015). The L1031V (L1024), F1747L (F1534), and F1751I (F1538) mutations were detected in field-collected populations of *P. citri* in this study. However, the L925 and A1215 mutations of VGSCs were not identified in these *P. citri* populations. In addition, the F1747L (F1534) mutation identified in pest mites of this study represent the first identification of this mutation in mites. The F1747L (F1534) mutation of the IIIS6 site of VGSC was also identified in DDT/permethrin-resistant *Aedes aegypti* and was closely associated with a resistant phenotype (Yanola et al., 2011).

The frequencies of point mutations in *P. citri* that were related to pyrethroid resistance were evaluated with amplicon sequencing, as previously conducted for *H. armigera* (Jin et al., 2018). The L1031V and F1751I mutations exhibited frequencies of 11.6–82.1% and 0.5–31.8%, respectively, in field populations collected from nineteen citrus producing sites, while the F1747L mutation occurred in ten field sites and had a frequency of 1.1–34.8%. The frequency of F1538I in VGSCs of *T. urticae* field populations ranged from 10–100% (Zhang et al., 2022). The high frequency of the F1747I (F1538I) mutation observed in the current study is consistent with other studies of *P. citri* (Ding et al., 2015), *T. urticae* (Riga et al., 2017), *Panonychus ulmi* (Rameshgar et al., 2019), *Dermanyssus gallinae* (Katsavou et al., 2020), and *Tetranychus cinnabarinus* (Xu et al., 2013). The L1031V (L1024) mutation has also been detected in *T. urticae* (Kwon et al., 2010, Simma et al., 2020) and *P. ulmi* (Rameshgar et al., 2019) field populations, where it conferred pyrethroid resistance. When the relations were analyzed between the frequencies of each mutation (and their combinations) and the resistance level in field populations, no positive relations were observed between the VGSC mutation frequency and the resistance levels of *P. citri* field populations to fenpropathrin. The diverse acaricides application usually led to the complex resistance evolution within a complex genetic background in mites. This phenomenon was more likely caused by some factors, for instance, the increased detoxification enzymes activities, cross-resistance occurrence. Previous studies

413 indicated that carboxylesterases genes were directly involved in fenpropthrin
414 resistance of the spider mites, *Tetranychus cinnabarinus* (Boisduval) (Wei et al.,
415 2019) and *P. citri* (Shen et al., 2016). What's more, glutathione S-transferase and
416 cytochrome P450 monooxygenases might contribute to fenpropathrin resistance in
417 mites as well (Shen et al., 2014, Liao et al., 2018, Shi et al., 2016). The bioassay
418 showed that moderate cross-resistance to fenpropathrin occurred when *Tetranychus*
419 *urticae* (Koch) developed extremely resistance to fenpyroximate (Kim et al., 2004).
420 Similarly, a significant reduced susceptibility to fenpropathrin was observed after an
421 extremely abamectin resistance was developed in *T. urticae* (Sato et al., 2005). More
422 importantly, resistance monitoring indicated that field populations of *P. citri* had
423 developed different degrees of resistance to all tested acaricides (Pan et al., 2020). All
424 these potential factors led to the current negative correlation between fenpropathrin
425 resistance and VGSC mutation frequency in *P. citri*. Thus, more evidences need to be
426 supplied by other functional verification, like the bioassay in transgenic flies after
427 VGSC mutation introduction.
428 Therefore, we introduced the VGSC mutations L1031V, F1747L, and F1751I
429 into the model organism *Drosophila melanogaster* through CRISPR/Cas9 genome
430 editing in order to functionally validate the ability of these mutations to confer
431 insecticide resistance *in vivo*. Bioassays revealed significantly increased resistance (in
432 the order of F1751I > L1031V > F1747L) to pyrethroids (both types I and II) in
433 transgenic flies carrying VGSC mutations compared to control flies (VGSC-WT). The
434 F1747L (F1534L) mutation conferred lower resistance to types I and II pyrethroids. In
435 contrast to previous observation, the F1534L mutation did not affect channel
436 resistance to pyrethroids in *Aedes albopictus* (Gao et al., 2018, Xu et al., 2016). In a
437 previous study, the F1534L mutation significantly reduced channel sensitivity to Type
438 I pyrethroids, but not to two Type II pyrethroids (Yan et al., 2020), contrasting with
439 our results. It is possible that different insect genomic backgrounds may underly the
440 differences in sensitivity in bioassays among different studies, possibly via the
441 presence of additional mutations or nucleotide sequence polymorphisms (Yan et al.,
442 2020). The relationships between VGSC mutations and pyrethroid resistance

consequently require further studies, such as cryo-electron microscopy investigation.

*In vivo* functional validation has been investigated for L1024V, revealing channel sensitivity in oocytes and changes in channel sensitivity to pyrethroids (Du et al., 2015). A previous study also observed that the F1538I mutation occurred in the WZ population of *P. citri* (Ding et al., 2015). Previous identification of mutations in VGSCs have suggested that F1538I conferred strong resistance to pyrethroids in *T. urticae* (Tsagkarakou et al., 2009) and *T. cinnabarinus* (Feng et al., 2011). The bioassays in this study further confirmed that the F1538I mutation of VGSCs plays an important role in the strong resistance to pyrethroids. Combinations of VGSC mutations (e.g., L1031V+F1751I and L1031V+F1747L+F1751I) were also observed in several *P. citri* field populations in this study. The L1031V+F1747L+F1751I mutation combination (equivalent to L1024V+F1534L+F1538I) was first detected in highly fenpropathrin-resistant populations of *P. citri*. A similar combination (L1024V+F1538I) was also detected in resistant *P. ulmi* populations and was shown to confer pyrethroid resistance (Rameshgar et al., 2019). An additional mutation combination (A1215D+F1538I and L1024V+A1215D) was identified in *T. urticae* VGSCs among different resistant strains or populations (Kwon et al., 2010, Xu et al., 2018). When these mutation combinations (L1031V+F1751I and L1031V+F1747L+F1751I) were introduced to transgenic flies, significant pyrethroid resistance was observed compared to control flies. Thus, mutations in combination can further increase resistance to pyrethroids. After these mutations were introduced into the *Drosophila*, although a small change in resistance was recorded, with the highest resistance of only 4.55-fold, it is reliable based on a recent report in *T. urticae*. Selection with abamectin for generations resulted in a resistance ratio of 105 in the mite. Subsequently, the introduction of glutamate-gated chloride channel I321T into transgenic flies through CRISPR/Cas9 genome editing led to only a 2.66-fold resistance to abamectin (Xue et al., 2021).

Our modelling indicates that all three resistance-associated mutations are brought into close proximity in the folded protein in a region of PyR1 that is also lined by the DII S4-S5 linker and DII S5 (O'Reilly et al., 2006b). Our previous electrophysiology

study demonstrated that resistance-associated mutations in this region greatly diminish the effect of pyrethroids with a phenyoxy group have but not fenfluthrin, which lacks the terminal phenyl ring, nor DDT (Usherwood et al., 2007). Numerous modelling studies have predicted that a pyrethroid's phenyoxy group binds in this lower portion of PyR1 (O'Reilly et al., 2006b, Usherwood et al., 2007, O'Reilly et al., 2014, Wu et al., 2017) and here we propose aromatic-aromatic interactions between F1747 and F1751 side-chains and a pyrethroid's terminal phenyl ring as a specific binding interaction. If F1747L and F1751I mutations confer resistance through our proposed steric hinderance mechanism with a pyrethroid's phenyoxy group, then use of pyrethroids lacking a terminal phenyl ring may overcome this resistance. Alternatively, our previous modelling predicts that miticidal analogues of DDT bind in the upper portion of PyR1 (O'Reilly et al., 2014) and so may be too distant from F1747 and F1751 to be affected by their mutations. Assessing alternatives to the currently-used pyrethroids to overcome resistance in mutant *P.citri* populations will therefore require field trials and further experimentation.

In conclusion, high resistance to fenpropathrin was detected in populations of *P. citri* collected from the field. Amplicon sequencing revealed the frequencies of the mutations L1031V, F1747L and F1751I of the *P. citri VGSC* gene, with L1031V and F1751I occurring in all evaluated populations at frequencies of 11.6–82.1% and 0.5–31.8%, respectively. The action of pyrethroids (types I and II) was also demonstrated in transgenic flies. Compared to VGSC-WT flies, significant decreases in sensitivity were observed in VGSC-mutant flies to varying degrees. Modeling of the *P. citri* VGSC suggested that pyrethroids are located at PyR1 and that interactions were disturbed after the substitution of the residues at the L1031, F1747, and F1751 sites. Consequently, the interaction to pyrethroids in *P. citri* was directly caused by the substitution of certain residues that affect interactions with pyrethroids.

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## Figure legends

**Fig. 1. A. Schematic showing the *P. citri* voltage gated sodium channel (VGSC). Alignment of amino acid sequences in the IIS6 (B) and IIIS6 (C) regions indicating the alignment positions of the mutations L1031V, F1747L, and F1751I.**

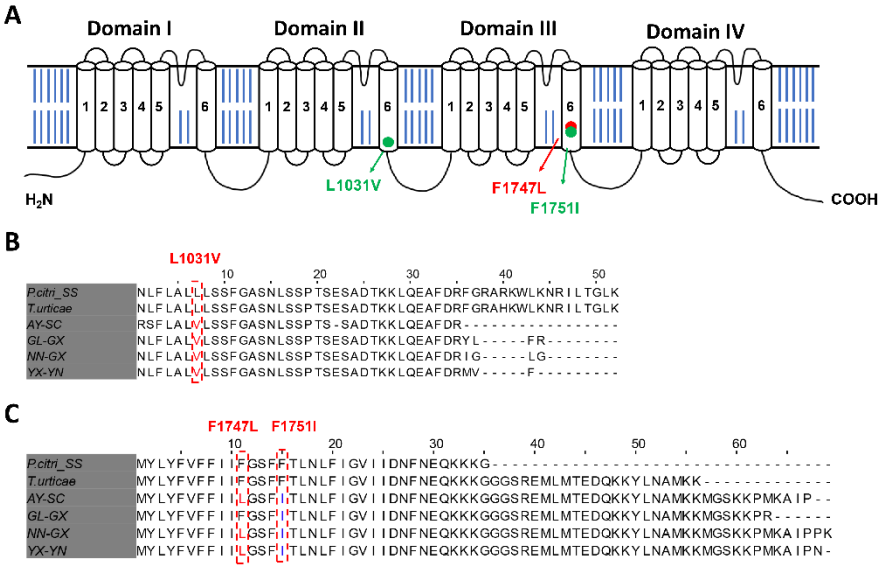

**Fig. 2**. **Pyrethroid resistance bioassays of transgenic flies.** Data are means ± S.E.M. Statistically significantly differences are indicated as: ***: $p < 0.001$. "*" was only showed at most close to WT-lines for 48 h and the rest were omitted with a significant difference too.

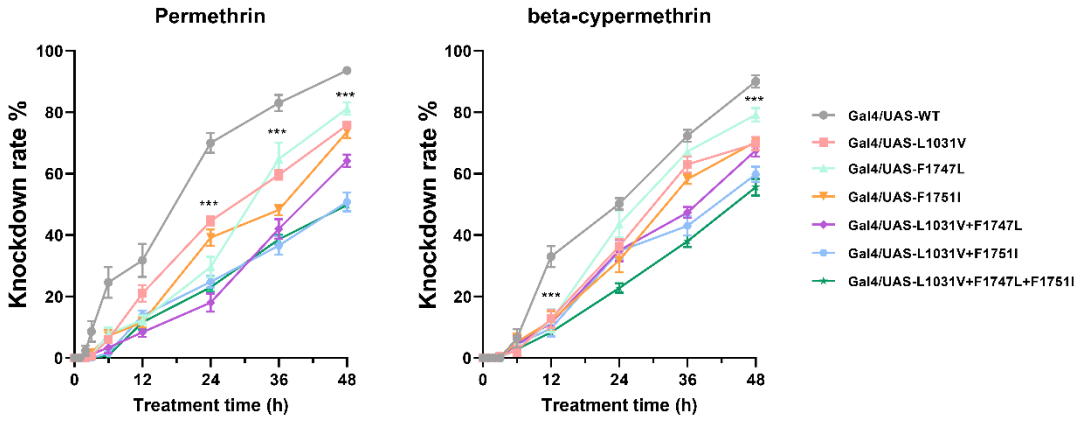

**Fig. 3**. **Homology model of the *P. citri* sodium channel showing residues at position 1031 (brown), 1747 (green) and 1751 (orange)** (**A**) A docking prediction for permethrin shown in yellow space-fill within the lipid-accessible pyrethroid binding site. (**B**-**C**) The wildtype channel is shown in ribbon (**B**) or surface representation (**C**). (**D**-**E**) the triple-mutant channel is shown in ribbon (**D**) or surface representation (**E**).

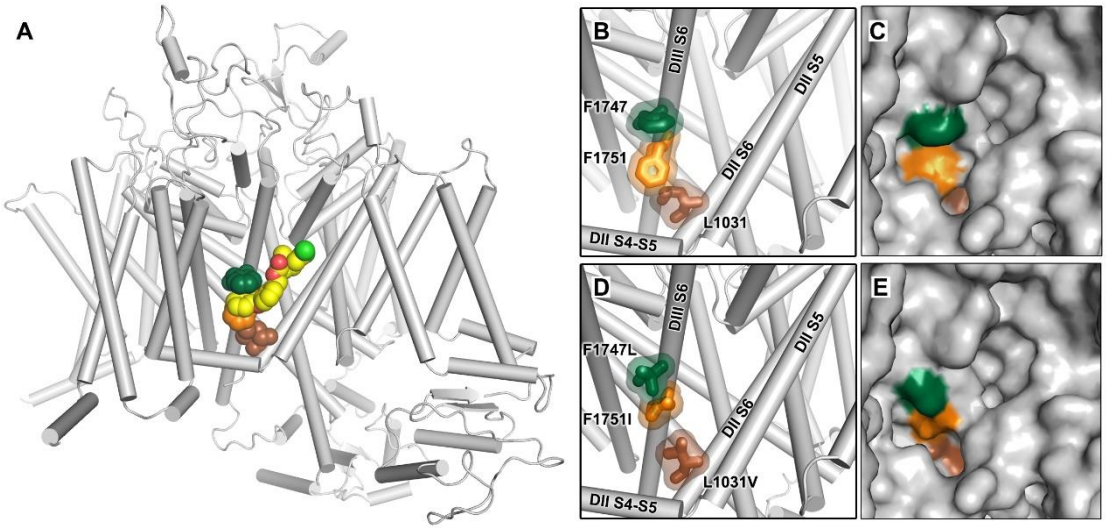

**Table 1**. Toxicity of fenpropathrin in eight field populations of *Panonychus citri* sampled from 2021 to 2022.

| Population | Year | N | Slope (±SE) | $LC_{50}$ (mg/L) | 95% CL[a] | $\chi^2$ (df) | RR[b] |
|---|---|---|---|---|---|---|---|
| SS | - | 481 | 1.026 ± 0.011 | 0.266 | 0.196-0.371 | 4.371 (3) | 1 |
| YX-YN | 2021 | 595 | 1.502 ± 0.119 | 1300.287 | 989.122-1795.749 | 6.8534 (4) | 4888.30 |
| GZ-JX | 2021 | 932 | 2.851 ± 0.157 | 854.885 | 541.823-1328.939 | 14.590 (5) | 3213.85 |
| LP-CQ | 2021 | 635 | 3.107 ± 0.217 | 45.468 | 29.600-65.761 | 14.490 (3) | 170.93 |
| AY-SC | 2021 | 578 | 4.380 ± 0.558 | 4093.913 | 3690.024-4496.152 | 1.700 (5) | 15390.65 |
| NN-GX | 2021 | 521 | 2.253 ± 0.194 | 1668.069 | 1021.821-2894.240 | 10.396 (3) | 6270.93 |
| NN-GX | 2022 | 540 | 3.498 ± 0.443 | 2771.812 | 29.600-65.761 | 1.9 (4) | 10420.35 |
| TN-CQ | 2022 | 568 | 1..242 ± 0.117 | 191.580 | 155.559-240.830 | 3.757 (3) | 760.23 |
| WZ-CQ | 2022 | 550 | 1.107 ± 0.150 | 3.217 | 2.562-4.249 | 1.760 (3) | 12.09 |
| GL-GX | 2022 | 480 | 2.452 ± 0.211 | 1395.170 | 2470.044-3046.003 | 4.7919 (3) | 5245.00 |

[a]CL, confidence limits.

[b]RR, resistance ratio = $LC_{50}$ of the field population / $LC_{50}$ of the susceptible strain.

YX-YN, collected from Yuxi, Yunnan province (E103°7′20″, N24°12′15″); GZ-JX: Ganzhou, Jiangxi province (E114°52′4.44″, N25°47′21.48″); LP-CQ; Liangping, Chongqing (E107°39′37.02″, N30°36′48.70″); AY-SC: Anyue, Sichuan province (E105°18′36″, N30°8′32″); NN-GX: Nanning, Guangxi province (E108°31′48″, N23°14′49.2″); TN-CQ: Tongnan, Chongqing city (E105°46′2″, N30°4′47″); WZ-CQ: Wanzhou, Chongqing city (E108°30′4″, N30°56′3″); GL-GX: Guilin, Guangxi province (E110°17′14″, N25°28′33″).

**Table 2**. Frequency of the L1031V, F1747L and F1751I mutations in in VGSCs sequenced from pools of field-captured *P. citri* from southwest China.

| Population | Date | Mutation site and frequency (Mean ± SEM) (%) | | |
|---|---|---|---|---|
| | | **L1031V** | **F1747L** | **F1751I** |
| TN1-CQ | 2021, 3 | 43.8 ± 0.9 | 11.3 ± 0.9 | 13.2 ± 0.4 |
| TN2-CQ | 2022, 4 | 51.0 ± 1.7 | 10.6 ± 0.4 | 14.6 ± 1.2 |
| LP1-CQ | 2021, 4 | 44.8 ± 1.4 | 13.3 ± 0.5 | 31.8 ± 0.4 |
| LP2-CQ | 2022, 6 | 68.7 ± 1.1 | 0 | 14.1 ± 0.4 |
| WZ-CQ | 2021, 4 | 26.7 ± 1.4 | 1.20 ± 0.1 | 0.50 ± 0.1 |
| LZ-SC | 2021, 3 | 45.6 ± 0.5 | 0 | 19.6 ± 0.7 |
| ZZ-SC | 2021, 4 | 46.1 ± 0.7 | 0 | 17.8 ± 0.6 |
| YB-SC | 2021, 6 | 40.0 ± 1.9 | 0 | 21.0 ± 0.2 |
| NJ-SC | 2021, 5 | 41.3 ± 1.7 | 33.7 ± 0.5 | 13.2 ± 0.4 |
| LS-SC | 2021, 5 | 45.6 ± 0.2 | 15.2 ± 1.2 | 20.8 ± 0.4 |
| AY-SC | 2021, 7 | 49.6 ± 0.5 | 0 | 19.5 ± 0.4 |
| NN1-GX | 2021, 9 | 40.9 ± 1.8 | 15.7 ± 0.3 | 27.8 ± 1.2 |
| NN2-GX | 2022, 7 | 74.6 ± 0.4 | 1.10 ± 0.2 | 25.8 ± 0.7 |
| GL1-GX | 2021, 5 | 48.1 ± 1.3 | 0 | 18.1 ± 0.3 |
| GL2-GX | 2022, 7 | 82.1 ± 0.3 | 1.50 ± 0.2 | 16.4 ± 0.6 |
| YX-YN | 2021, 5 | 11.6 ± 0.7 | 19.2 ± 0.9 | 4.60 ± 0.3 |
| WSZ-YN | 2021, 10 | 43.5 ± 0.8 | 7.70 ± 1.0 | 14.7 ± 0.1 |
| GZ-JX | 2021, 5 | 24.4 ± 0.10 | 3.4 ± 0.26 | 45.3 ± 0.42 |
| RL-YN | 2021, 11 | 27.4 ± 0.4 | 34.8 ± 1.3 | 19.3 ± 0.3 |

TN1/2-CQ, collected from Tongnan, Chongqing city (E105°45′11″, N30°9′9″; E105°46′2″, N30°4′47″); LP1/2-CQ: Liangping, Chongqing city (E107°39′37.02″, N30°36′48.70″; E107°42′6″, N30°36′47″); WZ-CQ: Wangzhou, Chongqing city (E108°41′49.92″, N28°53′56.4″); LZ-SC: Luzhou, Sichuan province (E105°34′8.4″, N28°53′56.4″); ZZ-SC: Zizhong, Sichuan province (E104°54′58″, N29°41′31″); YB-SC: Yibing, Sichuan province (E104°25′27″, N28°37′54); NJ-SC: Neijiang, Sichuan province (N29°2′40″, E105°1′1″); LS-SC: Leshang, Sichuan province (N29°2′40″, E103°34′51.80″); AY-SC: Anyue, Sichuan province (E105°18′36″, N30°8′32″); NN1/2-GX: Nanning, Guangxi province (E108°31′48″, N23°14′49.2″); GL1/2-GX: Guilin, Guangxi province (E110°27′25.78″, N25°19′20.28″; E110°17′14″, N25°28′33″); YX-YN: Yuxi, Yunnan province (E103°7′20″, N24°12′15″); WSZ-YN: Wenshanzhou, Yunnan province (E103°56′22″, N23°10′15″); GZ-JX: Ganzhou, Jiangxi province (E114°52′4.44″, N25°47′21.48″); RL-YN: Ruili, Yunnan province (E97°52′12″, N24°1′9″).

**Table 3**. Bioassays of fenpropathrin resistance in transgenic flies.

| **Strains** | **N** | **Slope (±SE)** | **$LC_{50}$（95%CL）[a] (mg/L)** | **$\chi^2$ (df)** | **RR[b]** |
|---|---|---|---|---|---|
| Gal4/UAS -WT | 286 | 1.272 ± 0.243 | 1.54 (1.06-2.31) | 0.143 (4) | 1 |
| Gal4/UAS - L1031V | 270 | 1.689 ± 0.234 | 4.17 (3.35-5.31) | 0.417 (3) | 2.71 |
| Gal4/UAS - F1747L | 289 | 2.440 ± 0.289 | 2.97 (2.47-3.56) | 0.102 (3) | 1.93 |
| Gal4/UAS - F1751I | 278 | 2.125 ± 0.403 | 5.63 (4.57-7.11) | 0.175 (4) | 3.66 |
| Gal4/UAS - L1031V+ F1747L | 282 | 0.991 ± 0.171 | 4.73 (3.17-8.22) | 1.774 (3) | 3.08 |
| Gal4/UAS - L1031V+ F1751I | 288 | 1.526 ± 0.244 | 5.82 (4.51-8.20) | 1.373 (4) | 3.78 |
| Gal4/UAS - L1031V+ F1747L+ F1751I | 288 | 2.637 ± 0.295 | 6.99 (4.98-8.15) | 1.232 (2) | 4.55 |

[a]CL, confidence limits.

[b]RR, resistance ratio = $LC_{50}$ of the mutant lines / $LC_{50}$ of the WT lines.

## Appendix A. Supplementary material

**Table S1**. **Locations, origins, and geographic information for *Panonychus citri* populations collected for bioassays.**

| Population | Location | Collection date | Host plant | Longitude and latitude | |
|---|---|---|---|---|---|
| YX-YN | Yuxi, Yunan | 2021, 5 | *Citrus reticulata 'Unshiu'* | E103°7′20″ | N24°12′15″ |
| GZ-JX | Ganzhou, Jiangxi | 2021, 5 | *Citrus limon* | E114°52′4.44″ | N25°47′21.48″ |
| LP-CQ | Liangping, Chongqing | 2021, 6 | *Citrus maxima (Burm.) Merr.cv. Liangping Yu* | E107°39′37.02″ | N30°36′48.70″ |
| AY-SC | Anyue, Sichuan | 2021, 7 | *Citrus limon* | E105°18′36″ | N30°8′32″ |
| NN-GX | Nanning, Guangxi | 2021, 9<br>2022, 7 | *Fertile orange* | E108°31′48″ | N23°14′49.2″ |
| TN-CQ | Tongnan, Chongqing | 2022, 4 | *Citrus limon* | E105°46′2″ | N30°4′47″ |
| WZ-CQ | Wanzhou, Chongqing | 2022, 6 | *Citrus sinensis* | E108°30′4″ | N30°56′3″ |
| GL-GX | Guilin, Guangxi | 2022, 7 | *Citrus reticulata* | E110°17′14″ | N25°28′33″ |

**Table S2**. **Locations, origins, and geographic information for *Panonychus citri* populations collected for mutation frequency analysis.**

| Population | Collection location | Collection date | Host plant | Longitude and latitude | |
|---|---|---|---|---|---|
| TN1-CQ | Tongnan, Chongqing | 2021, 3 | *Citrus limon* (L.) Burm. F. | E105°45′11″ | N30°9′9″ |
| TN2-CQ | Tongnan, Chongqing | 2022, 4 | *Citrus limon* (L.) Burm. F. | E105°46′2″ | N30°4′47″ |
| LP1-CQ | Liangping, Chongqing | 2021, 4 | *Citrus maxima* (Burm.) Merr.cv. Liangping Yu | E107°39′37.02″ | N30°36′48.70″ |
| LP2-CQ | Liangping, Chongqing | 2022, 6 | *Citrus reticulata Banco* | E107°42′6″ | N30°36′47″ |
| WZ-CQ | Wangzhou, Chongqing | 2021, 4 | *Citrus reticulata 'Banco'* | E108°41′49.92″ | N28°53′56.4″ |
| LZ-SC | Luzhou, Sichuan | 2021, 3 | *Citrus sinensis* | E105°34′8.4″ | N28°53′56.4″ |
| ZZ-SC | Zizhong, Sichuan | 2021, 4 | *Citrus sinensis* | E104°54′58″ | N29°41′31″ |
| YB-SC | Yibing, Sichuan | 2021, 6 | *Citrus reticulata' Ponkan* | E104°25′27″ | N28°37′54 |
| NJ-SC | Neijiang, Sichuan | 2021, 5 | *Citrus kanper* | N29°2′40″ | E105°1′1″ |
| LS-SC | Leshang, Sichuan | 2021, 5 | *Citrus reticulata 'Banco'* | N29°2′40″ | E103°34′51.80″ |
| AY-SC | Anyue, Sichuan | 2021, 7 | *Citrus limon* (L.) Burm. F. | E105°18′36″ | N30°8′32″ |
| NN1-GX | Nanning, Guangxi | 2021, 9 | *Fertile orange* | E108°31′48″ | N23°14′49.2″ |
| NN2-GX | Nanning, Guangxi | 2022, 7 | *Fertile orange* | E108°31′48″ | N23°14′49.2″ |
| GL1-GX | Guilin, Guangxi | 2021, 5 | *Citrus reticulata 'Banco'* | E110°27′25.78″ | N25°19′20.28″ |
| GL2-GX | Guilin, Guangxi | 2022, 7 | *Citrus reticulata 'Banco'* | E110°17′14″ | N25°28′33″ |
| YX-YN | Yuxi, yunnan | 2021, 5 | *Citrus reticulata 'Unshiu'* | E103°7′20″ | N24°12′15″ |
| WS-YN | Wenshanzhou, yunnan | 2021, 10 | *Tribute Citru* | E103°56′22″ | N23°10′15″ |
| GZ-JX | Ganzhou, Jiangxi | 2021, 5 | *Citrus limon (L.) Burm. F.* | E114°52′4.44″ | N25°47′21.48″ |
| RL-YN | Ruili, yunnan | 2021, 11 | *Citrus limon* (L.) Burm. F. | E97°52′12″ | N24°1′9″ |

**Table S3**. **PCR primers used to amplify VGSC fragments and detect mutants.**

| Primer name | Direction | Sequence (5'-3') | Products (bp) |
|---|---|---|---|
| *Pc*VGSC_L1031V | Forward | CGTGTTCTTTGTGGTGAATG | 236 |
| | Reverse | TTCTGAGCTCTACCGAAACG | |
| *Pc*VGSC_F1747L | Forward | GCAACATTCAAGGGTTGGAC | 271 |
| | Reverse | GGAATCGCTTTCATCGGCTT | |
| *Pc*VGSC_F1751I | Forward | GCAACATTCAAGGGTTGGAC | 271 |
| | Reverse | GGAATCGCTTTCATCGGCTT | |

**Table S4**. **Barcodes used in PCR amplification of VGSCs of *P. citri* populations to enable multiplex sequencing.**

| Population | Primer tag |
|---|---|
| TN1-CQ | CGATGT |
| TN2-CQ | |
| LP1-CQ | GGCTAC |
| LP2-CQ | |
| WZ-CQ | ATCACG |
| LZ-SC | TTAGGC |
| ZZ-SC | TGACCA |
| YB-SC | CAGATC |
| NJ-SC | ACTTGA |
| LS-SC | GATCAG |
| AY-SC | TGACCA |
| NN1-GX | ACAGTG |
| NN2-GX | |
| GL1-GX | GGCTAC |
| GL2-GX | |
| YX-YN | CGATGT |
| WSZ-YN | TTAGGC |
| RL-YN | ACAGTG |

**Table S5**. **PCR primers used to validate VGSC gene integration in transgenic fly constructs.**

| Transgenic lines | Direction | Sequence (5'-3') |
|---|---|---|
| UAS-VGSC-pm-WT | Forward | CAAGACTATCTGTGATCAAC |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm-L1031V | Forward | AGAGATACCCCGTTGGAATTT |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm-F1747L | Forward | CAAGACTATCTGTGATCAAC |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm-F1751I | Forward | AGAGATACCCCGTTGGAATTT |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm- L1031V+ F1747L | Forward | AGAGATACCCCGTTGGAATTT |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm- L1031V+ F1751I | Forward | AGAGATACCCCGTTGGAATTT |
| | P10-R | GCCACTAGCTCGCTATACACT |
| UAS-VGSC-pm- L1031V+ F1747L+ F1751I | Forward | AGAGATACCCCGTTGGAATTT |
| | P10-R | GCCACTAGCTCGCTATACACT |

**Table S6. Bioassays of transgenic fly resistance to pyrethroids.**

| Strains | N | Slope (±SE) | $LC_{50}$（95%Cl）[a] (mg/L) | $\chi^2$ | RR[b] |
|---|---|---|---|---|---|
| UAS - WT | 280 | 1.823 ± 0.271 | 1.64 (1.25-2.24) | 0.080 | 1 |
| UAS - L1031V | 265 | 1.817 ± 0.235 | 3.42 (2.75-4.23) | 0.415 | 2.08 |
| UAS - F1747L | 276 | 2.052 ± 0.283 | 3.60 (2.78-4.59) | 0.503 | 2.19 |
| UAS - F1751I | 287 | 1.833 ± 0.251 | 4.45 (3.59-5.67) | 0.268 | 2.71 |
| UAS - L1031V+ F1747L | 290 | 1.109 ± 0.179 | 5.81 (4.01-9.72 | 1.267 | 3.53 |
| UAS - L1031V+ F1751I | 287 | 1.776 ± 0.253 | 5.26 (4.21-6.91) | 1.673 | 3.20 |
| UAS - L1031V+ F1747L+ F1751I | 270 | 2.128 ± 0.386 | 5.73 (4.68-7.18) | 0.282 | 3.48 |

**Fig. S1. Alignment of amino acid sequences in the IIS6 (A) and IIIS6 (B) regions of VGSCs to verify the mutations L1031V, F1747L, and F1751I in transgenic flies.**

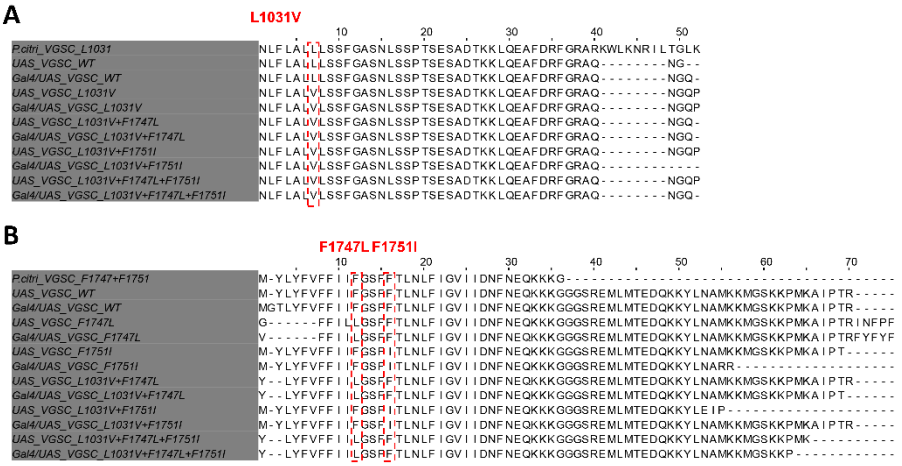

**Fig. S2**. **Correlation between the fenpropathrin resistance level and the frequency of VGSC mutations in the *P. citri* field populations**. Resistance ratios of field populations to fenpropathrin were plotted against the frequency of VGSC mutations with single or combinations. Regression equation and correlation coefficient were included on the graphs.

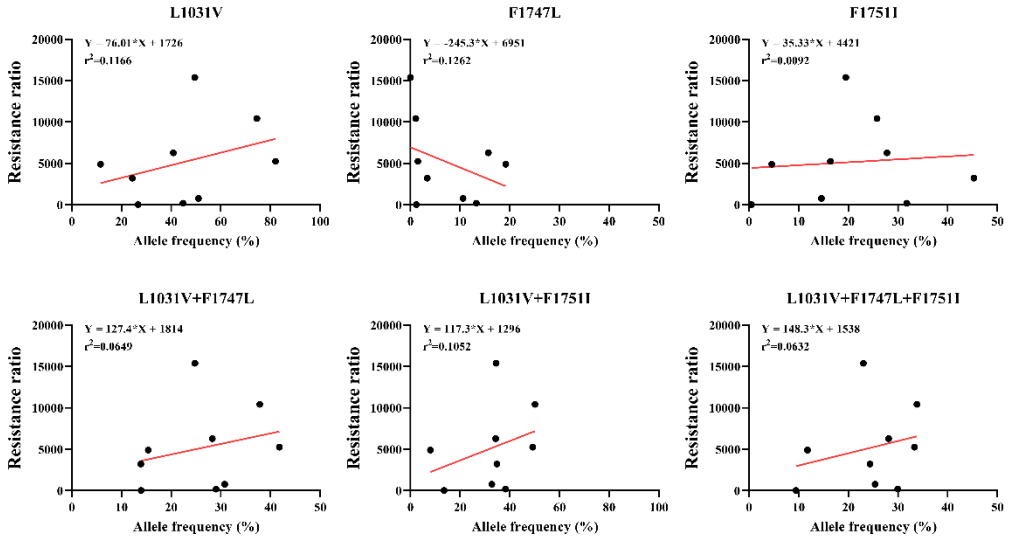

**Fig. S3**. **Bioassays of permethrin resistance in transgenic flies**. Data are means ± S.E.M. Statistically significantly differences are indicated as: *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$.

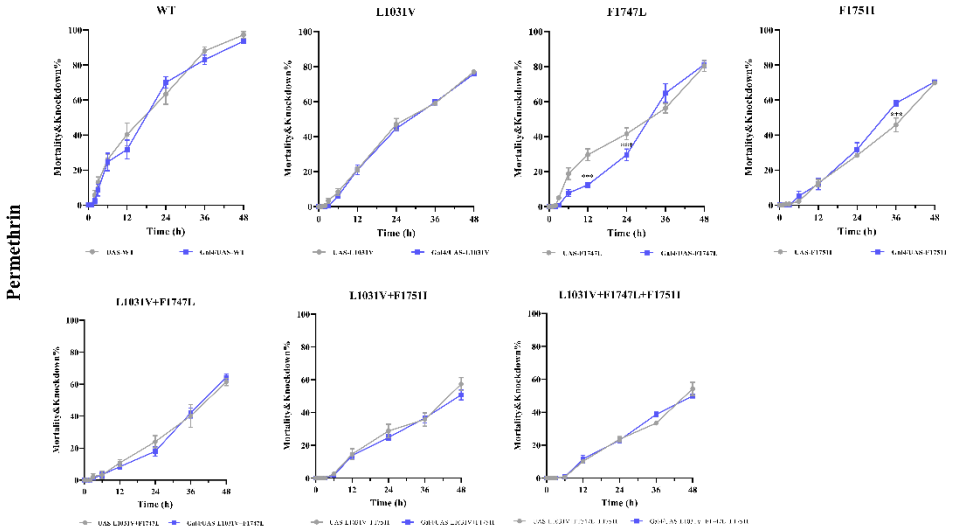

**Fig. S4**. **Bioassays of beta-cypermethrin resistance in transgenic flies**. Data are means ± S.E.M. Statistically significantly differences are indicated as: *: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$.

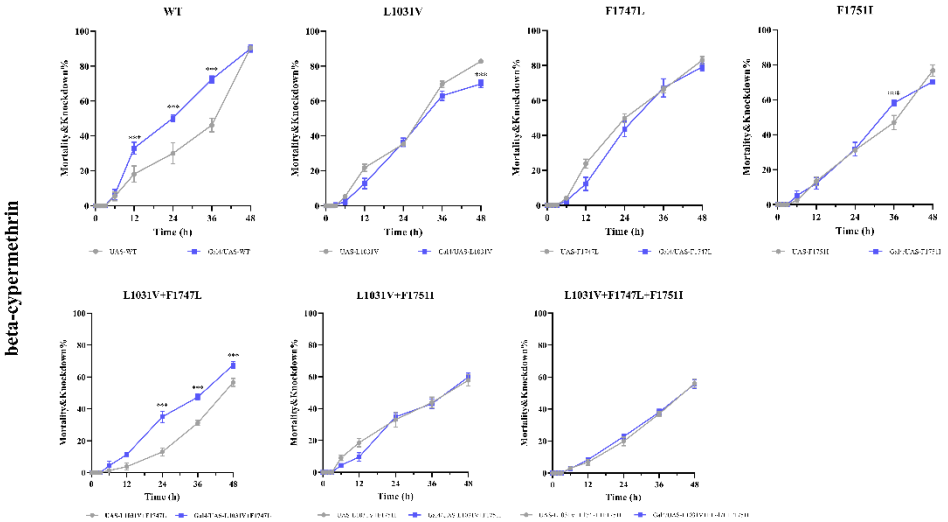

**Fig. S5**. **Pyrethroid docking predictions.** Predictions for (**A**) permethrin, (**B**) fenpropathrin, and (**C**) beta-cypermethrin are shown as yellow sticks and residues within 6 Å of docked ligands are shown as grey sticks or are colored green (residue 1747), orange (1751) or brown (1031). Bottom panels (**D**-**F**) show the same docking prediction for each pyrethroid as corresponding top panels, but with the channel rendered in surface view.