An aphid-resistant plant metabolite as a candidate aphicide: Insight into the bioactivity and action mode of betulin against aphids

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ABSTRACT

Pest-resistant plants usually utilize secondary metabolites to cope with insect infestation. Betulin, a key bioactive compound in aphid-resistant wild peach, possesses promising applications in crop protection. Here, betulin, in both greenhouse and field experiments, displayed excellent control efficacy against *Myzus persicae*. RNA-seq, qRT–PCR, and western blotting revealed that betulin significantly inhibited the expression of *MpGABR* (encoding a $GABA_A$ receptor). Besides, RNAi-mediated silencing of *MpGABR* markedly increased aphid sensitivity to betulin. Furthermore, MST (microscale thermophoresis) and voltage-clamp assays indicated that betulin bound to MpGABR (K_d = 2.24 μM) and acted as an inhibitor of MpGABR. Molecular docking, mutagenesis and genome editing suggested that THR228 is a critical and highly conserved site in MpGABR that betulin binds to specifically, causing aphid death. Overall, the activity of betulin depends on specific targeting and inhibition of MpGABR. Elucidating the mechanism of action of this peach-derived insecticide may offer a sustainable green strategy for aphid control.

KEYWORDS: betulin; aphid; $GABA_A$ receptor; insecticide; insecticidal mechanism

1. INTRODUCTION

Over more than 400 million years of coevolution with herbivores, plants have developed a variety of morphological (Soujanya et al. 2023), biochemical (Zhou et al. 2024b), and molecular (Wang et al. 2024) defense strategies against insect herbivore attack. Plant secondary metabolites are highly valuable natural compounds, offering abundant and potent biochemical defense driven by plant-insect coevolution (Jahan et al. 2025a). Interestingly, wild relatives of crops with insect resistance usually accumulate large amounts of biologically active compounds (Alonso-Salces et al. 2022; Wang et al. 2022a). Accordingly, investigating the bioactivities and potential mechanisms of action of compounds derived from wild resistant germplasms may provide effective and sustainable strategies for pest control in crop protection.

Myzus persicae (Sülzer) is one of the most destructive sap-feeding pests worldwide. It is able to settle on more than 400 plant species globally, displaying extraordinary polyphagia and a wide range of hazards (Bass et al. 2014). In addition to ingesting phloem saps and secreting honeydew, *M. persicae* can transmit various plant viruses (Guo et al. 2022), resulting in severe losses in crop yields. To date, the principal control strategy for *M. persicae* has relied on synthetic insecticides, including pyrethroids, organophosphorus compounds, carbamates and neonicotinoids (Jiménez-Jiménez et al. 2019; Martins et al. 2021; Zhang et al. 2023). However, long-term irrational application of these chemical insecticides has led to environmental pollution and promoted the resistance of aphids to insecticides (Troczka et al. 2021; Zhang et al. 2023; Stará et al. 2024). Therefore, it is necessary to develop novel green insecticides as potential alternatives to synthetic insecticides. Fortunately, plant secondary metabolites are a "treasure trove of active compounds" that serve as a rich resource for the development of promising green insecticides.

Our previous study revealed that *Prunus davidiana*, a close wild relative of cultivated peach,

displays strong resistance to *M. persicae* through the accumulation of high levels of betulin (Wang et al. 2022a; Wang et al. 2024). In addition, betulin, a lupane-type triterpene, possesses strong insecticidal activity and is a promising substance for the development of novel aphid-control insecticides. A number of studies have reported that betulin and its derivatives exhibit a wide range of pharmacological activities (Amiri et al. 2020). An immune stimulant, Ir-Bet, was prepared using iridium complex and betulin, which evoked ferritinophagy-enhanced ferroptosis, thereby activating anti-tumor immunity (Lv 2023). The anti-inflammatory effect of betulin has been reported in macrophages at lymphoma site in mice (Szlasa et al. 2023). Betulin has been found to improve hyperlipidemia and insulin resistance and decrease atherosclerotic plaques by inhibiting the maturation of sterol regulatory element-binding protein (Tang et al. 2011). Additionally, betulin and its derivatives have been found to exhibit insecticidal activity against *Plutella xylostella* L. (Huang et al. 2025), *Aedes aegypti* (de Almeida Teles et al. 2024), and *Drosophila melanogaster* (Lee and Min 2024). However, the insecticidal mechanism of betulin against aphids remains unclear.

Gamma-aminobutyric acid (GABA) receptors have been confirmed to be targets of terpenoids that impair neuronal function in insect herbivores (Guo et al. 2023). There are two types of GABA receptors: ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors. The first GABA receptor subunit identified in insects is encoded by *Rdl*, which confers resistance to dieldrin in *Drosophila melanogaster* (Ffrench-Constant et al. 1991). Although a variety of terpenoids play roles as positive allosteric modulators or noncompetitive antagonists of $GABA_A$ receptors (GABRs) (Guo et al. 2023), whether betulin targets the GABR in *M. persicae* needs to be further investigated. GABRs are heteropentameric ligand-gated ion channels in the

85 central nervous system that conduct chloride and bicarbonate ions and are the target of
86 numerous drugs for the treatment of neuropsychiatric disorders (Thompson 2024). GABRs are
87 composed of 5 different types of subunits, each with 4 helical transmembrane domains (TM1-
88 TM4), of which TM2 is located at the center of the pentamer and forms an ion channel (Fan et
89 al. 2024). Previous study has proposed that vertebrate and human GABR genes maintain a
90 broad and conservative gene clustering pattern, while in invertebrates, this pattern is missing,
91 indicating that these gene clusters were formed early in vertebrate evolution and were
92 established after invertebrates diverged. Notably, invertebrates each possess a unique GABR
93 gene pair, which are homologous with human GABR α and β subunits, suggesting that the
94 existing GABR gene cluster evolved from an ancestral α-β subunit gene pair (Tsang et al. 2006).
95 During the coevolution of plants and insects, the duplications and amino acid substitutions in
96 GABR may have been beneficial for adaptation to insecticides and terpenoid compounds (Guo
97 et al. 2023).

98 In this study, betulin was confirmed to have excellent control efficacy against *M. persicae*
99 in both greenhouse and field experiments. RNA-seq, qRT–PCR, and western blotting assays
100 revealed that betulin significantly inhibited the expression of *MpGABR* in aphids. In addition,
101 RNAi-mediated silencing of *MpGABR* markedly increased aphid sensitivity to betulin.
102 Furthermore, microscale thermophoresis (MST) and voltage-clamp assays indicated that
103 betulin can bind to MpGABR (K_d = 2.24 μM) and act as an inhibitor (EC_{50} = 20.66 μM) of
104 MpGABR. Molecular docking analysis suggested that betulin bound to MpGABR via the
105 THR228 amino acid residue. This site is highly conserved across 11 species in the Aphididae
106 family of Hemiptera and may be a critical specific binding site for betulin in MpGABR in aphids.

107 Mutagenesis and genome editing experiments revealed that betulin bound specifically to this
108 amino acid residue in aphids but not in *Drosophila*, resulting in aphid death. This study
109 elucidated the insecticidal mechanism of betulin involving the targeting of MpGABR,
110 providing a sustainable green strategy for aphid control. However, given that betulin may
111 affect a wider range of organisms, it should be used with caution.

112 **2. MATERIALS AND METHODS**

113 **2.1 Test insects**

114 *M. persicae* populations were collected from tobacco (*Nicotiana tabacum* L.) leaves. Wild-
115 type (WT) flies (*D. melanogaster*) were purchased from Fungene Biotechnology Co., Ltd.,
116 Jiangsu, China. The test insects were cultured in a greenhouse (25 °C, 70% relative humidity
117 and 14 h light/10 h dark cycle) in Beibei, Chongqing, China.

118 **2.2 Leaf bioassay**

119 The toxicity of betulin against *M. persicae* was measured using a slip-dip bioassay as
120 previously described (Wang et al. 2022a). Thirty apterous adult *M. persicae* individuals were
121 gently transferred on tobacco (*N. tabacum*) leaves using a fine brush. The tobacco leaves were
122 subsequently immersed in 0.1% (v/v) Tween-80 and 3% (v/v) acetone solution containing
123 betulin or pymetrozine (as a positive control) at 7 doses (0, 0.0625, 0.125, 0.25, 0.5, 1, and 2
124 $mg \cdot mL^{-1}$) for 5 seconds. After drying, the leaves with *M. persicae* were placed on wet filter paper
125 and cultured on a water-soaked sponge for 48 h. Three replicates were tested for each treatment.
126 Furthermore, the LC_{30}, LC_{50} and LC_{70} values of betulin were calculated using log-probit analysis
127 via IBM SPSS Statistics (v.22.0, Chicago, IL, USA) (Zhou et al. 2024b).

128 **2.3 Greenhouse and field bioassays**

129 The control effects of betulin against *M. persicae* in the greenhouse and field were evaluated
130 by a spraying-based method as described previously (Zhou et al. 2023). The commercial
131 insecticide pymetrozine and an aqueous solution containing 0.1% (v/v) Tween-80 and 3% (v/v)
132 acetone were employed as positive and negative controls, respectively. Sprays of 0.1641
133 $mg \cdot mL^{-1}$ (LC_{50}) betulin and 1.0612 $mg \cdot mL^{-1}$ (LC_{50}) pymetrozine (Table S1) were prepared in
134 aqueous solutions including 3% (v/v) acetone and 0.1% (v/v) Tween-80, respectively. Before the
135 control effect assays, each tobacco (*N. tabacum* L.) seedling was pre-inoculated with 60 apterous
136 adults of *M. persicae*. In the greenhouse trial, each treatment included 8 replications, and each
137 replication consisted of 3 tobacco plants growing under controlled conditions as described
138 above. The field trial (Beibei District, Chongqing, China.) was conducted under natural
139 conditions with an average temperature of 20 °C. Each treatment had 8 replications, covering
140 an area of 20 m^2, with 3 protected rows per replication. Furthermore, each tobacco plant was
141 sprayed with 20 mL of the corresponding solution with an electric sprayer. Mortality was
142 recorded at 1, 5, 9, and 14 days after treatment. The control efficacy of betulin against *M. persicae*
143 was calculated according to the methods of Zhou (Zhou et al. 2023).

144 **2.4 RNA-seq assay**

145 After exposure to 0.1641 $mg \cdot mL^{-1}$ (48 h LC_{50}) betulin for 48 h, 100 apterous *M. persicae*
146 adults were sampled for each of 3 replicates. and total RNA was extracted via TRIzol reagent
147 (Tiangen, Beijing, China) to construct mRNA sequencing libraries. RNA-seq analysis was
148 performed on the Ion Proton BGISEQ-500 platform (Beijing Genomic Institute, BGI, China).
149 After the raw reads were filtered, the clean reads were aligned to the *M. persicae* reference
150 genome, which was downloaded from the National Center for Biotechnology Information

151 database, using HISAT2 (v2.1.0) (Kim et al. 2015). The expression levels of genes in each sample
152 were normalized to the fragments per kilobase million mapped reads (FPKM) value. The
153 differentially expressed genes (DEGs) between groups were identified using the fold change
154 factor ($|Log_2(FC)| > 1$). Functional enrichment analysis of DEGs was performed using the Gene
155 Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

156 **2.5 Validation by quantitative real-time PCR (qRT–PCR)**

157 The gene-specific primers used for qRT–PCR, which were designed with Primer Premier
158 5.0, are listed in Table S2. According to the manufacturer's specifications for the Bio-Rad iQ™
159 SYBR® Green Supermix Kit, qRT–PCR was performed with the following conditions: 60 °C for
160 15 s, followed by 48 cycles of 60 °C for 30 s, and 72 °C for 30 s. The *ribosomal protein S18* gene
161 (*RPS18*) was used as the reference gene to normalize the expression levels. The relative
162 expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ approach (Zhou et al.
163 2024b). Three biological replicates were used for each assay.

164 **2.6 Cloning, bioinformatics and phylogenetic analysis**

165 The cDNA of *M. persicae* was synthesized from the extracted total RNA using the
166 PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The coding sequences of *MpGABR*
167 (encoding $GABA_A$ receptor), *MpGABRAP* (encoding $GABA_A$ receptor-associated protein) and
168 *MpGABRB* (encoding $GABA_A$ receptor β subunit) were cloned from *M. persicae* cDNA using
169 the corresponding specific primers (Table S2) and then inserted separately into the pGEM-T
170 Easy vector. The recombinant plasmids were subsequently transferred into *Escherichia coli*
171 DH5α competent cells for sequencing. BioXM (v2.7.1) (http://202.195.246.60/BioXM/) was used
172 to analyze the sequence information of these three genes, including the length, molecular

173 weight, and isoelectric point of the deduced amino acid sequence. MEGA7 was used for
174 multiple amino acid sequence alignment (ClustalW) and phylogenetic analysis (neighbor-
175 joining method with 1000 replicates) of MpGABR.

176 **2.7 RNAi assay**

177 To knock down the target genes (*MpGABR*, *MpGABRAP*, and *MpGABRB*), *MpGABR*-,
178 *MpGABRAP*-, and *MpGABRB*-dsRNA were artificially synthesized using the dsRNA Synthesis
179 Kit (Thermo Scientific, Vilnius, Lithuania, EU). In accordance with a previously described
180 method (Zhou et al. 2024a), fresh tobacco leaves were cut into 3.0 cm-diameter discs and placed
181 in an oven at 50 °C for 3 min. Subsequently, the dried leaf discs were exposed to 10 μL of diethyl
182 pyrocarbonate-treated nuclease-free water (DEPC-water), dsGFP (green fluorescent protein,
183 1000 ng· μL^{-1}, negative control), and *MpGABR*-, *MpGABRAP*-, or *MpGABRB*-dsRNA (1000
184 ng/·μL^{-1}) at 25 °C for 5 h. After the leaf discs had absorbed the solution, they were placed on
185 wet filter paper on a water-soaked sponge. Subsequently, 30 apterous adult *M. persicae*, starved
186 for 24 h, were fed on the treated leaf discs for 48 h. The surviving aphids were collected after
187 exposure for qRT–PCR. Three biological replicates were included for each treatment.

188 **2.8 Prokaryotic expression of *MpGABR* and western blotting**

189 To produce the MpGABR protein *in vitro*, an *Escherichia coli* expression system was
190 constructed (Zhou et al. 2021). Briefly, the coding sequence of *MpGABR* was inserted into the
191 expression vector PET-B2M, obtained from BGI (Shenzhen, China), and transformed into BL21
192 (*DE3*) competent cells. To induce the expression of the MpGABR protein, 2 mM isopropyl *β*-D-
193 1-thiogalactopyranoside (IPTG) was used, and then, the transformed BL21 cells were incubated
194 at 180 rpm and 30 °C for 24 h. Finally, the protein was extracted and purified using Solarbio

195 GST-tag Purification Resin for subsequent assays.

196 The purified MpGABR protein resolved on a 15% SDS–PAGE (sodium dodecyl sulfate–
197 polyacrylamide gel electrophoresis) gel and then transferred to a polyvinylidene fluoride
198 (PVDF) membrane at 20 V for 1 h. Moreover, 1 × TBST (Tris-Borate-Sodium Tween-20)
199 containing 5% fat-free milk powder was used to block the PVDF membrane. Then, the PVDF
200 membrane was incubated with an antibody against MpGABR (polyclonal rabbit antibody,
201 provided by GeneCreate Biotechnology) at 25 °C for 1 h. Subsequently, the membrane was
202 further incubated with the corresponding horseradish peroxidase (HRP)-conjugated goat anti-
203 rabbit immunoglobulin G (IgG) antibody at 25 °C for 1 h and then washed three times with
204 TBST. Finally, the bands on the membrane were visualized using a Chemi Doc MP Imaging
205 System (Bio-Rad) after incubation with a chemiluminescence reagent (ECL, Bio-Rad). GAPDH
206 was used as a reference protein.

207 **2.9 Molecular docking**

208 In accordance with a previously reported method (Zhou et al. 2023), the protein structure
209 and function of MpGABR were predicted via the I-TASSER server (Iterative Threading
210 ASSEmbly Refinement). AutoDockTools software (v1.5.7) was used to conduct molecular
211 docking. Furthermore, PyMOL (v3.1) and Discovery Studio (v4.5) were used to evaluate the
212 molecular docking model.

213 **2.10 Site-directed mutagenesis**

214 The coding sequence of *MpGABR* was inserted into the PET-B2M vector and used as the
215 template for site-directed mutagenesis. In accordance with the manufacturer's specifications,
216 the Fast MultiSite Mutagenesis Kit (TransGen, Beijing, China) was used to generate the

217 multisite mutations in MpGABR (Ma et al. 2020). Then, the MpGABR mutants, with the
218 mutations of R224A (ARG224 replaced by ALA), A226T (ALA226 replaced by THR), F227Y
219 (PHE227 replaced by TYR), or T228R (THR228 replaced by ARG), were amplified, and the
220 amplicons were used to replace the *Bam*HI-*Sma*I fragment in the recombinant MpGABR
221 plasmid.

222 **2.11 Method for *Drosophila* genome editing**

223 To generate *Drosophila* mutants bearing the DmGABR^{R122T} mutation, homologous to
224 THR228 in MpGABR, the Clustered Regularly Interspaced Short Palindromic Repeats-Cas9
225 (CRISPR/Cas9) genomic editing strategy was used. First, CRISPR Optimal Target Finder
226 (http://targetfinder.flycrispr.neuro.brown.edu/) was used to generate short guide RNA
227 (sgRNA) targets without off-target hits. Then, the Precision gRNA Synthesis Kit (Thermo
228 Scientific) was used to synthesize the sgRNA. The 228 bp homologous arm in R122T, for
229 homology-directed repair, was subsequently employed to synthetize single-stranded DNA
230 (ssDNA) bearing the desired mutation (DmGABR^{R122T}). Subsequently, 1 nL of a mixture
231 containing 160 ng· μL^{-1} ssDNA, 300 ng· μL^{-1} both sgRNAs, and 90 ng· μL^{-1} Cas9 protein was
232 injected into *Drosophila* embryos via the Drummond Nanoject III (Broomall, PA, USA) system.
233 CRISPR/Cas9-induced mutations were verified by amplifying and sequencing genomic DNA
234 flanking the genome editing target site. Finally, according to the traditional topical application
235 and bioassay approach described previously (Zhou et al. 2023), the acute insect toxicity of
236 betulin in genome-modified or nonmodified (WT) 3-day-old adult flies was determined. The
237 oligonucleotide sequences used for PCR, the sgRNA synthesis template and the fragment of
238 donor DNA used for homology-directed repair (HDR) are listed in Table S3.

239 **2.12 Microscale thermophoresis (MST) assay**

240 The binding affinity of MpGABR for betulin was measured via a NanoTemper
241 Technologies Monolith Kit (MO, Munich, Germany) according to the manufacturer's
242 specifications. Briefly, MpGABR was fluorescently labeled via a protein labeling kit (RED-NHS
243 2nd generation, Monolith™) and then mixed with sixteen concentrations (from 5 nM to 60 mM)
244 of betulin. The mixtures were subsequently loaded into NanoTemper capillaries for MST
245 measurements with 40% LED and 60% MST power at 25 °C. MO Control NanoTemper
246 Technologies GmbH software (v2.3) and MO Affinity Analysis software (v2.3, NanoTemper
247 Technologies GmbH) were used to acquire and analyze the recorded data, respectively. Three
248 replicates were set for each concentration. The dissociation constant (K_d), indicating the binding
249 affinity, was calculated using data from three replicates for each experiment.

250 **2.13 Electrophysiological recording**

251 In accordance with previously described methods (Chen et al. 2019), oocytes isolated from
252 mature healthy *Xenopus* females were digested at 25 °C for 1 h to remove the follicular layer.
253 Individual oocytes were selected to express *MpGABR* and incubated at 18 °C with 1× Ringer's
254 solution (96 mM NaCl, 5 mM $MgCl_2$, 5 mM HEPES, 2 mM KCl, and 0.8 mM $CaCl_2$)
255 supplemented with 550 mg·mL^{-1} sodium pyruvate, 100 mg·mL^{-1} streptomycin, 50 mg·mL^{-1}
256 tetracycline, and 5% dialyzed horse serum. After 3 days of incubation, the oocytes were used
257 for electrophysiological recording. GABA was dissolved in 1 × Ringer's solution to prepare 100
258 μM GABA solution. Subsequently, the 100 μM GABA solutions containing different
259 concentrations of betulin (0, 5, 10, 20, 40, 80, 160, 320 μM) were used to perfuse the oocytes. The
260 whole-cell currents were recorded via a two-electrode voltage-clamp. The data were collected

261 and analyzed using Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., Union
262 City, CA, USA), respectively (Xu et al. 2020).

263 **2.14 Statistical analysis**

264 Statistical analysis was performed using SPSS (v22). Statistical significance was
265 determined using one-way analysis of variance (ANOVA) for differences among groups. The
266 replicate numbers are indicated by the black dots in the figures.

267 **3. RESULTS**

268 **3.1 Control efficacy of betulin against aphids**

269 Bioassays revealed that the LC_{50} values of betulin and pymetrozine against *M. persicae* at
270 48 h were 0.1641 and 1.0612 mg·mL^{-1}, respectively (Table S1). To assess the control efficacy of
271 betulin, tobacco plants infected with *M. persicae* were exposed to betulin (0.1641 mg·mL^{-1}) and
272 pymetrozine (1.0612 mg·mL^{-1}, as a positive control) in both the greenhouse and field. In the
273 greenhouse test (Fig. 1A-D), at 14 days after treatment, *M. persicae* in the CK group (negative
274 control) reproduced rapidly, and tobacco leaves turned yellow and wrinkled, whereas tobacco
275 leaves in the betulin and pymetrozine treatment groups were barely affected by the aphids (Fig.
276 1A-B). The control efficacy of betulin against *M. persicae* was significantly greater than that of
277 pymetrozine at 1 day after treatment ($P < 0.01$), while there were no significant differences
278 between the control efficacies of betulin and pymetrozine at 5, 9, or 14 days after treatment (Fig.
279 1E). After 14 days of treatment, the control efficacies of betulin and pymetrozine reached 91.54%
280 and 95.15%, respectively. Throughout the field test (Fig. 1F), no difference in the control efficacy
281 was observed between betulin and pymetrozine, with values of 87.79% and 90.14%,
282 respectively, after 14 days of treatment (Fig. 1G). These results suggest that betulin has

283 immense potential for development as a commercial aphid insecticide like pymetrozine.

284 **3.2 Expression patterns of genes in aphids after treatment with betulin**

285 To investigate the expression patterns of genes in *M. persicae* after exposure to betulin,
286 RNA-seq was performed on *M. persicae* with 0.1641 mg·mL^{-1} betulin treatment for 48 h and
287 without (CK) (Fig. 2A). Principal coordinate analysis (PCA) revealed that the expressed genes
288 were strongly clustered in the CK group and betulin group (Fig. 2B). Compared with those in
289 the CK group, there were 130 up- and 41 downregulated significant differentially expressed
290 genes (DEGs) in *M. persicae* after betulin treatment (Fig. 2C and D, Table S4). To validate the
291 RNA–seq results, fifteen DEGs were randomly selected with different expression levels. The
292 relative expression trends of the fifteen DEGs determined via qRT–PCR were similar to those
293 detected via RNA–seq, supporting the reliability of the RNA–seq data (Fig. 2E). To further
294 analyze the functions of the DEGs, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of
295 Genes and Genomes) enrichment analyses were performed. GO term enrichment
296 demonstrated that the DEGs were enriched mainly in the $GABA_A$ receptor activity term (Fig.
297 2F). Interestingly, KEGG enrichment also found that the DEGs were enriched in the GABAergic
298 signaling related pathway and GABAergic synapse terms (Fig. 2G). Additionally, the
299 expression of DEGs related to GABR, including *MpGABR* (encoding $GABA_A$ receptor),
300 *MpGABRAP* (encoding $GABA_A$ receptor-associated protein) and *MpGABRB* (encoding $GABA_A$
301 receptor β subunit), in the betulin group was significantly lower than that in the CK group (Fig.
302 2H). In particular, the $\log_2$(fold change) value of *MpGABR* was the lowest, at -3.8 (Table S4).
303 These results suggested that *MpGABR* may be a candidate target for betulin against *M. persicae.*

304 **3.3 *MpGABR* expression was inhibited by betulin**

305 Analysis of the sequence information revealed that the MpGABR, MpGABRAP and
306 MpGABRB proteins have 694, 118, and 250 amino acids, respectively, with calculated molecular
307 weights of 77.20, 14.08, and 28.01 kDa and isoelectric points of 9.85, 9.63, and 7.20, respectively
308 (Table S5). Additionally, structure prediction indicated that MpGABR, MpGABRAP and
309 MpGABRB contain 4, 0, and 1 transmembrane helical domain, respectively (Fig. 3A), implying
310 that only MpGABR has a complete transmembrane structure.

311 To further verify the inhibitory effect of betulin on GABR-related gene expression, the
312 relative expression of the three GABR-related genes was detected in *M. persicae* exposed to the
313 LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h. The relative expression of all three genes decreased
314 gradually as the concentration of betulin increased (Fig. 3B-D). After exposure to the LC_{30} of
315 betulin for 48 h, the relative expression of *MpGABR*, *MpGABRAP* and *MpGABRB* decreased by
316 82.91%, 10.53%, and 11.49%, respectively. These results indicated that *MpGABR* was the most
317 sensitive to betulin. To further investigate the effect of betulin on the MpGABR protein, *M.*
318 *persicae* was exposed to betulin concentrations of LC_{30}, LC_{50}, and LC_{70} for 48 h. As the
319 concentration of betulin increased, the MpGABR protein content in *M. persicae* gradually
320 decreased (Fig. 3E and F). Compared with that in the CK group, the MpGABR protein content
321 in *M. persicae* exposed to betulin concentrations of LC_{30}, LC_{50}, and LC_{70} decreased by 33.68%,
322 44.89%, and 76.89%, respectively.

323 Furthermore, after these three genes were silenced via RNAi (Fig. 4D), the expression
324 levels of *MpGABR*, *MpGABRAP* and *MpGABRB* in *M. persicae* significantly decreased by 64.57%,
325 63.28%, and 67.33%, respectively, compared with those in the control groups (DEPC-treated
326 water and dsGFP) ($P < 0.0001$, Fig. 4A-C). After exposure to the LC_{50} of betulin for 48 h, the

327 mortality of *M. persicae* with *MpGABR* silenced markedly increased ($P < 0.001$) by 30.44%,
328 compared with that in the control groups, whereas the mortalities of *M. persicae* in the
329 *MpGABRAP*- and *MpGABRB*-silenced groups did not significantly differ from that in the
330 control groups (Fig. 4E). Additionally, western blotting analysis revealed that MpGABR
331 protein expression significantly decreased by 69.91% after RNAi ($P < 0.0001$, Fig. S1A, B). These
332 results further implied that *MpGABR* might be a target for betulin against *M. persicae*.

333 **3.4 Phylogenetic analysis of GABRs in insects**

334 Furthermore, phylogenetic analysis of GABRs in insects was performed using the amino
335 acid sequences of 70 GARB proteins from Hemiptera, Diptera, Lepidoptera, Thysanoptera,
336 Hymenoptera, and Coleoptera. MpGABR (XP_022173711.1) was found to be genetically closely
337 related to CAI6365831.1 from *Macrosiphum euphorbiae*, XP 008183008.2 from *Acyrthosiphon pisum*,
338 and XP 060864885.1 from *Metopolophium dirhodum* (Fig. 5 and Table S6), indicating that
339 MpGABR may be functionally analogous to CAI6365831.1, XP 008183008.2, and XP 060864885.1.

340 **3.5 Interaction between MpGABR and betulin**

341 The WT (wild type) MpGABR was expressed in *Escherichia coli* (Fig. 6A). The binding
342 affinity of betulin with WT MpGABR was further measured using MST. The K_d value was 2.24
343 μM (Fig. 6B), suggesting that betulin could bind to MpGABR. Additionally, voltage-clamp-
344 based electrophysiological recording demonstrated that MpGABR strongly responded to
345 betulin (Fig. 6C). In the presence of only GABA, a fast inward current was generated. As the
346 concentration of added betulin increased, the current and channel activity of MpGABR
347 decreased correspondingly, with an EC_{50} value of 20.66 μM (Fig. 6C and D). These results
348 indicated that betulin acted as an inhibitor of MpGABR.

349 **3.6 Key sites for the binding of betulin to MpGABR**

350 To further study the binding mode of betulin in the active pocket of MpGABR, molecular
351 docking was performed to explore the structure–function relationships between betulin and
352 MpGABR. The docking results revealed that the predicted binding energy between betulin and
353 MpGABR was −6.38 kcal·mol^{-1} (Table S7), verifying that betulin could act as a specific ligand
354 for MpGABR. The three-dimensional binding pattern of betulin with MpGABR indicated that
355 the four key amino acid residues (ARG224, ALA226, PHE227 and THR228) interacted with
356 betulin in the MpGABR binding pocket (Fig. 7A). Among the four key amino acid residues,
357 ALA226 and THR228 interacted with betulin via hydrogen bonding in the MpGABR binding
358 pocket (Table S7). Betulin generated nonconventional H-bonds (C…H) with ALA226 (3.31 Å)
359 and conventional H-bonds (C-O H) with THR228 (2.16 Å). Furthermore, sequence alignment
360 of the key amino acid residues of GABR in different species from Hemiptera, Diptera,
361 Lepidoptera, Hymenoptera, Thysanoptera, and Coleoptera indicated that, among the four
362 residues, only THR228 was conserved across 11 species in the Aphididae family of Hemiptera
363 (Fig. 7B), implying that THR228 may be an essential specific site for the action of betulin on
364 MpGABR in aphids. Mutants of aphid MpGABR were subsequently constructed, including
365 R224A (ARG224 replaced by ALA), A226T (ALA226 replaced by THR), F227Y (PHE227
366 replaced by TYR, similar to the site in *Drosophila*), or T228R (THR228 replaced by ARG, similar
367 to the site in *Drosophila*) (Fig. 7C). The binding affinity of betulin with these mutants was
368 measured using MST. The results revealed that the K_d values of R224A, A226T, F227Y, T228R,
369 and WT were 2.31, 2.27, 2.25, 5321, and 2.24 μM, respectively (Fig. 7D and E, Table S8). This
370 result suggested that the mutation at the THR228 site, with the highest K_d value, markedly

371 reduced the binding ability of betulin to MpGABR, implying that the THR228 site may be an
372 essential specific site for the binding of betulin to MpGABR.

373 **3.7 Betulin binds specifically to MpGABR via THR228**

374 To further prove that THR228 is the specific binding site for betulin in MpGABR, the WT
375 and mutant (R122T, equivalent to THR228 in MpGABR) *Drosophila* DmGABR proteins were
376 expressed in *E. coli* and extracted (Fig. 8A and D). The binding affinities of betulin and
377 pymetrozine with $DmGABR^{WT}$ and $DmGABR^{R122T}$ were evaluated using MST. As shown in Fig.
378 8B, betulin was able to bind to $DmGABR^{R122T}$ (K_d = 342.4 μM) but not to $DmGABR^{WT}$. However,
379 pymetrozine exhibited no binding affinity for $DmGABR^{R122T}$ or $DmGABR^{WT}$ (Fig. 8C).
380 Moreover, to generate *Drosophila* mutant individuals carrying $DmGABR^{R122T}$, the CRISPR/Cas9
381 genomic editing strategy was used. After exposure to different concentrations of betulin, the
382 mortality rate of $DmGABR^{R122T}$ *Drosophila* was significantly greater than that of $DmGABR^{WT}$
383 *Drosophila* (Fig. 8E). In addition, the LD_{50} value of betulin against $DmGABR^{R122T}$ *Drosophila* at 72
384 h was 27.19 $\mu g \cdot fly^{-1}$, whereas the LD_{50} value of betulin against $DmGABR^{WT}$ *Drosophila* could not
385 be calculated because the lethal concentration exceeded 1000 $\mu g \cdot fly^{-1}$ (Table S9). Additionally,
386 there was no significant difference in mortality between $DmGABR^{WT}$ and $DmGABR^{R122T}$
387 *Drosophila* after exposure to pymetrozine (Fig. 8F). The LD_{50} values of pymetrozine against
388 $DmGABR^{WT}$ and $DmGABR^{R122T}$ *Drosophila* at 72 h were 0.23 and 0.24 $\mu g \cdot fly^{-1}$, respectively (Table
389 S9). Moreover, THR228 is located at the neurotransmitter-gated ion-channel ligand-binding
390 domain in MpGABR (Fig. S2), implying that betulin may play a role as a competitive antagonist
391 of MpGABR. Taken together, these findings suggested that betulin bound specifically to
392 MpGABR via THR228, resulting in the death of *M. persicae*.

393 **4. DISCUSSION**

394 Although chemical pesticides are effective in pest control, the long-term unreasonable
395 application of these substances has led the emergence of resistant pests, environmental
396 deterioration, and deleterious effects on nontarget organisms, including humans, raising
397 widespread and intense concerns (Devi et al. 2022). These challenges have made it urgent for
398 the development of alternative strategies for pest control. Recently, the utilization of plant
399 secondary metabolites as insecticides has become increasingly popular as an eco-friendly and
400 biocontrol approach (Ling et al. 2022; Ayilara et al. 2023; Jahan et al. 2025b). Our previous study
401 revealed that *P. davidiana*, a wild relative of cultivated peach, strongly resists *M. persicae* by
402 accumulating high contents of betulin (Wang et al. 2022a; Wang et al. 2024). In addition, betulin,
403 a lupane-type triterpene, possesses potent insecticidal activity and is a promising substance for
404 the development of novel insecticides for aphid control. In this study, the insecticidal effect of
405 betulin was further evaluated by comparing the control efficacy of betulin with that of
406 pymetrozine against aphids in greenhouses and fields (Fig. 1A-G). These results indicate that
407 betulin has a similar control effect to pymetrozine and has immense potential for development
408 as a plant-derived insecticide.

409 Terpenes are a diverse group of plant secondary metabolites that can increase the
410 resistance of plants to insect herbivores through direct (Wang et al. 2025b) and indirect (Wang
411 et al. 2025a) defense mechanisms. In direct defense against herbivores, triterpenes play
412 important roles in diverse biological activities, including antiparasitic, insecticidal, and
413 antifeedant activities (Tian et al. 2021; Ivana et al. 2023). Azadirachtin, a tetracyclic triterpenoid
414 compound isolated from the Indian neem tree (*Azadirachta indica*), is one of the most prominent

415 commercial biopesticides, exhibiting strong insect antifeedant properties as well as growth-
416 and reproduction-regulating effects (Dawkar et al. 2019; Bae et al. 2022). Besides, triterpene
417 glycoside compounds play crucial roles in the defense of tobacco (*N. attenuata*) against tobacco
418 hornworm (*Manduca sexta*) larvae (Yang et al. 2023). Although betulin exhibits various
419 pharmacological activities (Amiri et al. 2020; Li et al. 2022; Yan 2022; Lv 2023), reports on its
420 insecticidal activity are limited. Encouragingly, the effects of betulin and its derivatives on pests
421 have attracted increasing attention. Betulinic acid and its derivatives showed larvicidal activity
422 against *Aedes aegypti* larvae (da Silva et al. 2016). Betulin-cinnamic acid-related hybrid
423 compound 5b exhibited strong aphicidal activity, and compound 2l could destroy the
424 ultrastructure of midgut cells and significantly inhibit the activity of α-amylase in
425 diamondback moth (*Plutella xylostella* L.) larvae (Huang et al.). Our previous studies also
426 indicated that betulin possesses potent insecticidal activity and is a key endogenous secondary
427 metabolite related to the defense of peach against *M. persicae* (Wang et al. 2022a). Elucidating
428 the insecticidal mechanism of betulin against aphids will provide a basis for the development
429 of novel aphicides and sustainable strategies for aphid control.

430 GABA receptors have been confirmed to be targets of terpenoids that impair insect
431 neuronal function in herbivores (Guo et al. 2023). GABRs are heteropentameric ligand-gated
432 ion channels in the central nervous system that conduct chloride and bicarbonate ions. These
433 receptors are targets of numerous drugs for the treatment of neuropsychiatric disorders
434 (Thompson 2024). A variety of terpenoids act as positive allosteric modulators or
435 noncompetitive antagonists of GABRs, such as diterpenoids (isopimaric acid and miltirone),
436 sesquiterpenoids (picrotoxin, bilobalide and ginkgolides) and monoterpenoids (α-thujone and

437 thymol) (Guo et al. 2023). In this study, both GO and KEGG enrichment analyses revealed that
438 the DEGs identified by RNA-seq were enriched in GABAergic signaling-related pathways (Fig.
439 2F and G). Additionally, the expression of the DEGs related to GABRs, particularly *MpGABR*
440 (Table S4), in the betulin group was significantly lower than that in the CK group (Fig. 2H).
441 Besides, the relative expression of *MpGABR*, *MpGABRAP* and *MpGABRB* decreased gradually
442 after *M. persicae* was exposed to the LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h (Fig. 3B-D). Among
443 them, *MpGABR* was the most sensitive to betulin, and its expression was reduced by 82.91%
444 after exposure to the LC_{30} of betulin for 48 h. Furthermore, compared with the control group,
445 the *M. persicae* group with *MpGABR* silenced by RNAi presented a significant increase in
446 mortality ($P < 0.001$), by 30.44%, after 48 h of exposure to the LC_{50} of betulin (Fig. 4E).
447 Collectively, these results suggest that betulin may have insecticidal effects on aphids by
448 inhibiting *MpGABR* expression. The regulation of gene expression is sophisticated and delicate
449 (Pope and Medzhitov 2018). The regulatory network controlling *GABR* expression remains
450 unclear. In adult rats, epileptic seizures have been reported to increase the levels of brain-
451 derived neurotrophic factor (BDNF), which in turn prompted the transcription factors CREB
452 and ICER to reduce the gene expression of the GABR α1 subunit (Lund et al. 2008). In *Drosophila*,
453 it has been demonstrated that WIDE AWAKE, which regulated the onset of sleep, interacted
454 with the GABR and upregulated its expression level (Liu et al. 2014). In *Drosophila* brain,
455 circular RNA circ_sxc was found to inhibit the expression of miR-87-3p in the brain through
456 sponge adsorption, thereby regulating the expression of neurotransmitter receptor ligand
457 proteins, including GABR, and ensuring the normal function of synaptic signal transmission in
458 brain neurons (Li et al. 2024). However, it remains unclear how betulin reduces *MpGABR*

459 expression, and further research is needed.

460 Additionally, betulin has been reported to be able to bind to GABRs (Manayi et al. 2016).
461 We further investigated the interaction of betulin with the MpGABR protein. The MST assay
462 revealed that betulin was able to bind to MpGABR (K_d = 2.24 μM) (Fig. 6B), which is consistent
463 with previous findings showing that betulin binds to GABA receptors in mouse brains *in vitro*
464 (Muceniece et al. 2008). Voltage-clamp-based electrophysiological recordings indicated that
465 betulin acted as an inhibitor (EC_{50} = 20.66 μM) for MpGABR (Fig. 6C and D). Subsequent
466 molecular docking analysis suggested that four key amino acid residues (ARG224, ALA226,
467 PHE227 and THR228) interact with betulin in the MpGABR binding pocket (Fig. 7A), among
468 which merely ALA226 and THR228 interact with betulin via hydrogen bonding (Table S7). The
469 results of the sequence alignment revealed that only THR228 was conserved across 11 species
470 in the Aphididae family of Hemiptera (Fig. 7B). Furthermore, evaluation of the ability of betulin
471 to bind to MpGABR mutants with mutations at those four sites revealed that the ability of
472 betulin to bind to T228R was significantly weaker than its ability to bind to the WT (Fig. 7D and
473 E, Table S8), indicating that THR228 is an essential specific site for the binding of betulin to
474 MpGABR. Moreover, to further prove that THR228 is the specific binding site for betulin in
475 MpGABR, the binding affinities of betulin with the WT and mutant (R122T, equivalent to
476 THR228 in MpGABR) *Drosophila* DmGABR proteins were assessed using MST. The results
477 showed that betulin was able to bind to $DmGABR^{R122T}$ (K_d = 342.4 μM) but not $DmGABR^{WT}$ (Fig.
478 8B). Additionally, after exposure to different concentrations of betulin, the mortality rate of
479 $DmGABR^{R122T}$ *Drosophila* was significantly greater than that of $DmGABR^{WT}$ *Drosophila* (Fig. 8E
480 and Table S9). Similarly, a previous study indicated that the R122G amino acid site substitution,

481 generated via RNA editing, affects the sensitivity of *Drosophila* to fipronil (Es-Salah et al. 2008).
482 Together, these findings suggest that betulin binds specifically to MpGABR via THR228, acting
483 as an inhibitor of MpGABR and causing aphid death. Studies on key amino acids that are
484 crucial for GABR function has primarily focused on transmembrane regions. For instance,
485 based on the mutational research and *Drosophila* GABR modeling approach, multiple key
486 amino acids have been identified as insecticide targets in the transmembrane domain (Nakao
487 and Banba 2021). Guo et al. proposed that amino acid substitutions in transmembrane domain
488 2 contribute to terpenoid insensitivity during plant-insect coevolution (Guo et al. 2023).
489 However, these studies have neglected the extracellular domain. Our study signified that
490 betulin targets the THR228 site in the extracellular domain of MpGABR, which is conserved
491 only in the Aphididae family. Therefore, betulin is speculated to be a specific insecticidal
492 substance evolved by plants in response to aphid infestation. Besides, further verification is
493 needed to determine whether betulin is toxic to other insect species.

494 GABRs belong to the cysteine loop (Cys)-loop superfamily of neurotransmitter receptors
495 and are essential heteropentameric ligand-gated ion channels in the central nervous system.
496 After GABA binds to the extracellular Cys-loop of a GABR (Ashby et al. 2012), the GABR is
497 activated and opens up its central pore to allow chloride ions to pass through, thereby
498 hyperpolarizing neurons and attenuating excitatory neurotransmission (Tremblay et al. 2016).
499 In insects, GABRs play important roles in circadian rhythms (Schellinger et al. 2022), sleep
500 (Chaturvedi et al. 2022), movement (Eick et al. 2022), and olfactory memory (Yamagata et al.
501 2021). Additionally, GABR is a critical target for a variety of insecticides and ectoparasiticides.
502 Insecticides targeting GABRs are categorized into noncompetitive antagonists (NCAs) and

503 competitive antagonists (CAs) according to their different binding sites. NCAs block chloride
504 channels in nerve cells by interacting with amino acid residues of the GABA-gated chloride
505 channel, causing a conformational change in the receptor, which interferes with the normal
506 function of the central nervous system and ultimately leads to insect death (Nakao and Banba
507 2021; Guo et al. 2023). A series of first- and second-generation NCA insecticides have been
508 successfully developed. Picrotoxinin, isolated from *Anamirta cocculus* fruit, is the oldest natural
509 NCA and is toxic to houseflies (Tong et al. 2023). Among the first-generation NCAs,
510 polychlorocycloalkanes, including dieldrin and lindane, were commonly used as pesticides in
511 the middle and late 20th centuries (Hainzl et al. 1998; Tanaka 2019). As second-generation
512 NCAs, fipronil and its derivatives are commercially available phenylpyrazole insecticides that
513 have been widely used for agricultural pest control (Sheng et al. 2018; Li et al. 2021).
514 Additionally, CAs bind to orthogonal binding sites, such as the GABA recognition site in the
515 extracellular region, competitively inhibiting the binding of GABA to its receptor, leading to
516 toxic effects in insects. Given the lack of interference from existing insect resistance mechanisms,
517 CAs hold promise for the development of efficient new insecticides. Novel 1, 6-dihydro-6-
518 iminopyridazine-derived insecticides, as CAs, have insecticidal properties against common
519 cutworms and houseflies (Liu et al. 2022). Besides, nootkatone, a sesquiterpenoid, also acts as
520 a CA to induce insect mortality (Norris et al. 2022). Fluralaner, an isooxazoline ectoparasiticide,
521 inhibits both parasites and *Aedes aegypti* by acting as a CA (Wang et al. 2022b; Asahi et al. 2023).
522 Our results revealed that the binding site (THR228) for betulin in MpGABR was located in the
523 extracellular neurotransmitter-gated ion-channel ligand-binding domain (Fig. S2), implying
524 that betulin acts as a CA of MpGABR. Although the mechanism by which betulin competes

525 with GABA for binding to MpGABR requires further experimental validation, our work may
526 have provided a novel target for developing insecticides. In this study, betulin, on the one hand,
527 inhibited the expression of *MpGABR* and, on the other hand, specifically bound to MpGABR
528 through THR228, acting as an inhibitor of MpGABR and causing aphid death (Fig. 9).

529 The development of bioinsecticides should not only focus on the toxic effects of active
530 substance on target organisms, but also on their influence on the ecosystem (Haddi et al. 2020).
531 Although our results indicate that betulin has specific toxicity to aphids, previous studies have
532 reported that betulin and its derivatives had effects on *Plutella xylostella* L. (Huang et al. 2025),
533 *Aedes aegypti* (de Almeida Teles et al. 2024), and *Drosophila melanogaster* (Lee and Min 2024).
534 Therefore, further research is needed to determine whether there are other insecticidal
535 mechanisms or off target effects of betulin. Additionally, betulin exhibits a wide range of
536 pharmacological activities (Amiri et al. 2020), which have been used to treat various diseases,
537 such as cancer (Lv 2023), glioblastoma (Li et al. 2022), inflammation (Szlasa et al. 2023) and
538 hyperlipidemia (Tang et al. 2011). Before applying betulin in the field, it is necessary to fully
539 verify and consider whether betulin has any impact on farmers' health. Furthermore, will
540 betulin cause residue or diffusion during the field application process? Will long-term
541 application promote the evolution of resistance to aphids or other insects? These issues also
542 need further experimental verification. In summary, before any field application, further
543 research on the environmental behavior, degradation process, and safety of betulin is needed.

544 **5. CONCLUSION**

545 Betulin, a key metabolite in the aphid-resistant wild peach *P. davidiana*, possesses potent
546 aphicidal effects on *M. persicae.* This study confirmed that betulin exhibited excellent control

547 efficacy against *M. persicae* in both greenhouse and field experiments. RNA-seq, qRT–PCR and
548 western blotting assays revealed that betulin significantly inhibited the expression of *MpGABR*
549 in aphids. In addition, RNAi-mediated silencing of *MpGABR* markedly increased the
550 sensitivity of aphids to betulin. Moreover, MST and voltage-clamp-based electrophysiological
551 recording assays indicated that betulin was able to bind to MpGABR (K_d = 2.24 μM) and acted
552 as an inhibitor (EC_{50} = 20.66 μM) of MpGABR. Molecular docking analyses suggested that the
553 amino acid residue THR228, which is highly conserved across 11 species in the Aphididae
554 family of Hemiptera, might be a critical specific binding site for betulin in MpGABR.
555 Mutagenesis and genome editing assays revealed that betulin bound specifically to this amino
556 acid residue in aphids but not in *Drosophila*, resulting in aphid death. Collectively, the results
557 suggest that the aphicidal effects of betulin on aphids occur in a two-pronged manner: on the
558 one hand, betulin inhibits *MpGABR* expression; on the other hand, it specifically binds to
559 MpGABR via THR228 and acts as an inhibitor of MpGABR. Elucidating the insecticidal
560 mechanism of betulin against aphids will provide a basis for the development of novel
561 insecticides and sustainable strategies for aphid control.

562 **ASSOCIATED CONTENT**

563 **Supporting Information**

564 Protein expression of MpGABR after RNAi (Figure S1); Betulin binding site on the domain of
565 MpGABR (Figure S2); LC_{50} values of betulin and pymetrozine against *M. persicae* at 48 h (Table
566 S1); Primers used for cloning, RNAi, heterologous expression, and qRT-PCR (Table S2);
567 Oligonucleotide sequences for PCR, the sgRNA synthesis template and the fragment of donor
568 DNA for homology-directed repair (HDR) (Table S3); Screening of genes significantly

differentially expressed in *M. persicae* in response to betulin at 48 h post treatment (Table S4); Complete sequence information for the $GABA_A$ receptor gene of *M. persicae* (Table S5); Sequences and relevant information for phylogenetic analysis of $GABA_A$ receptor (Table S6); Binding energy and nonbonding interactions between betulin and $GABA_A$ receptor (Table S7); Parameters of dose-response curves from microscale thermophoresis experiments (Table S8); LD_{50} values of betulin and pymetrozine against *D. melanogaster* at 72 h (Table S9).

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Notes

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614 **ABBREVIATIONS**

615 GABA, Gamma-aminobutyric acid; GABR, $GABA_A$ receptor; GABRB, $GABA_A$ receptor β
616 subunit; GABRAP, $GABA_A$ receptor associated protein; MST, microscale thermophoresis; PCA,
617 principal coordinates analysis; DEGs, differentially expressed genes; GO, Gene Ontology;
618 KEGG, Kyoto Encyclopedia of Genes and Genomes; K_d, dissociation constant; RDL, resistance
619 to dieldrin; NCA, noncompetitive antagonists; CA, competitive antagonists.

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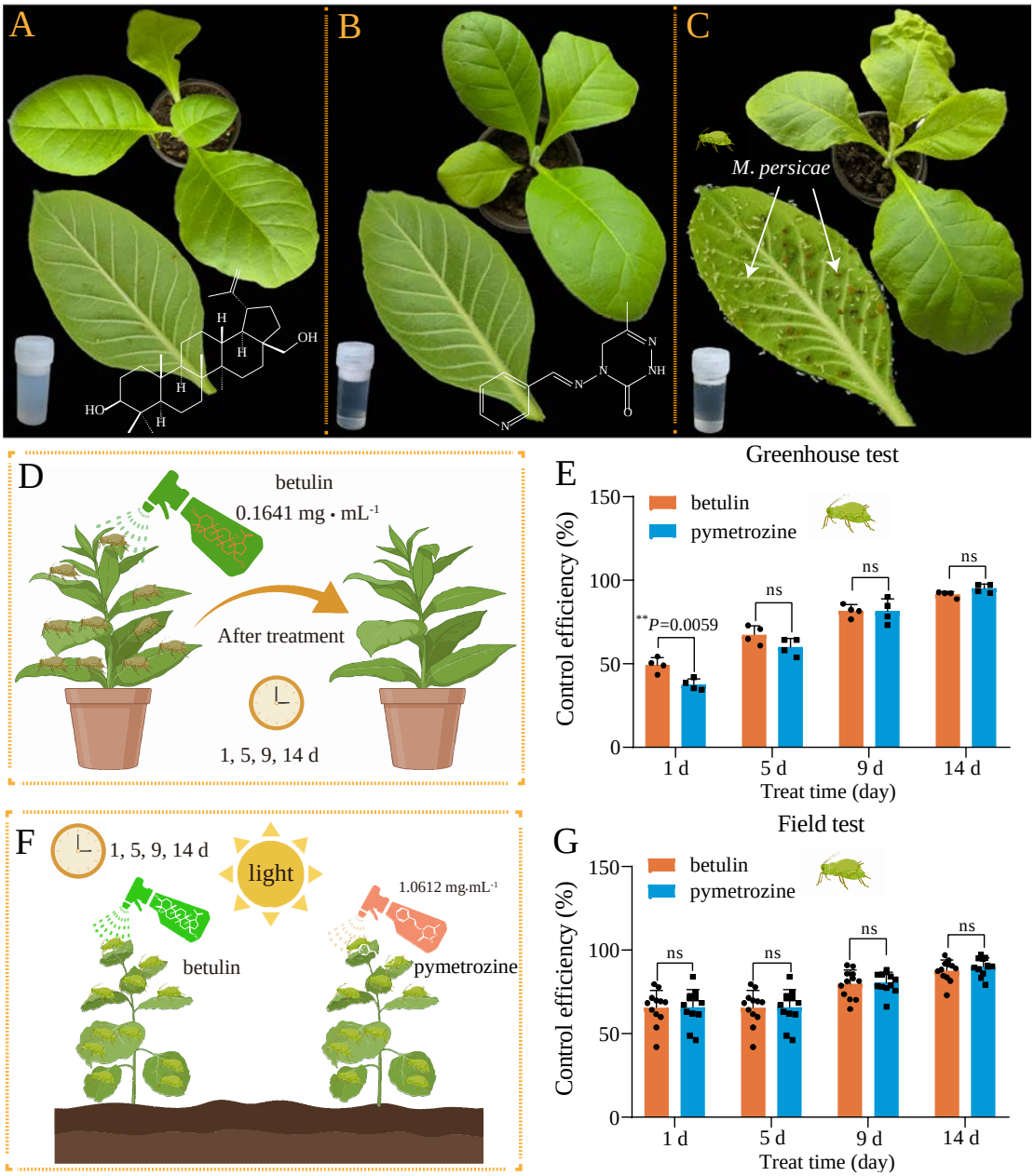


Figure 1. The aphicidal activity of plant-derived derivative betulin against *M. persicae.* (A-G) Representative images of the control effects of betulin (A, 0.1641 mg·mL^{-1}, 48 h LC_{50} value of betulin), pymetrozine (B, 1.0612 mg·mL^{-1}, a positive control), and CK (C, a negative control) against *M. persicae* in greenhouse (A-E) and field (F, G) tests after treatment for 14 d. The data are shown as the mean ± SD from twelve independent experiments. ** $P < 0.01$. ns, not significant.

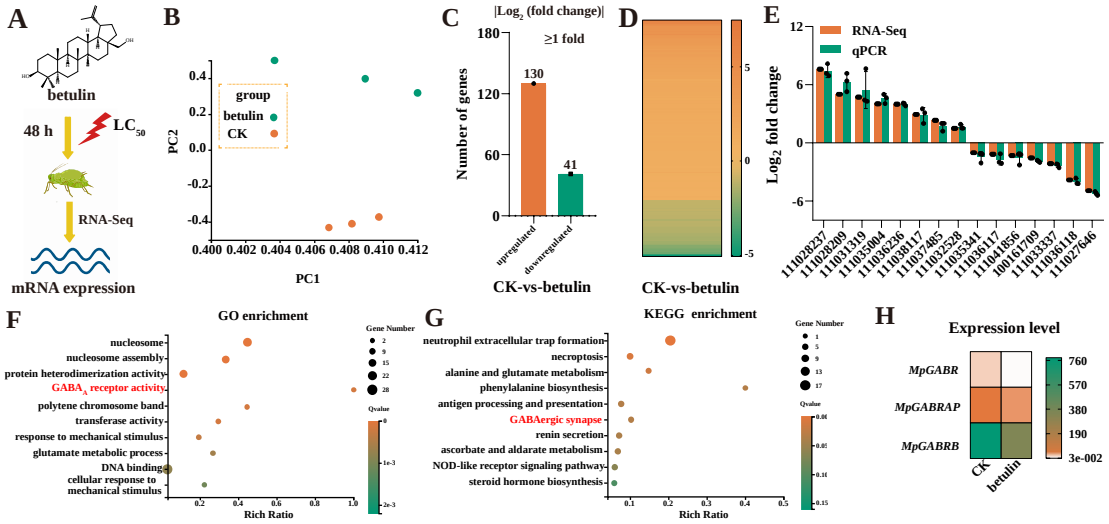
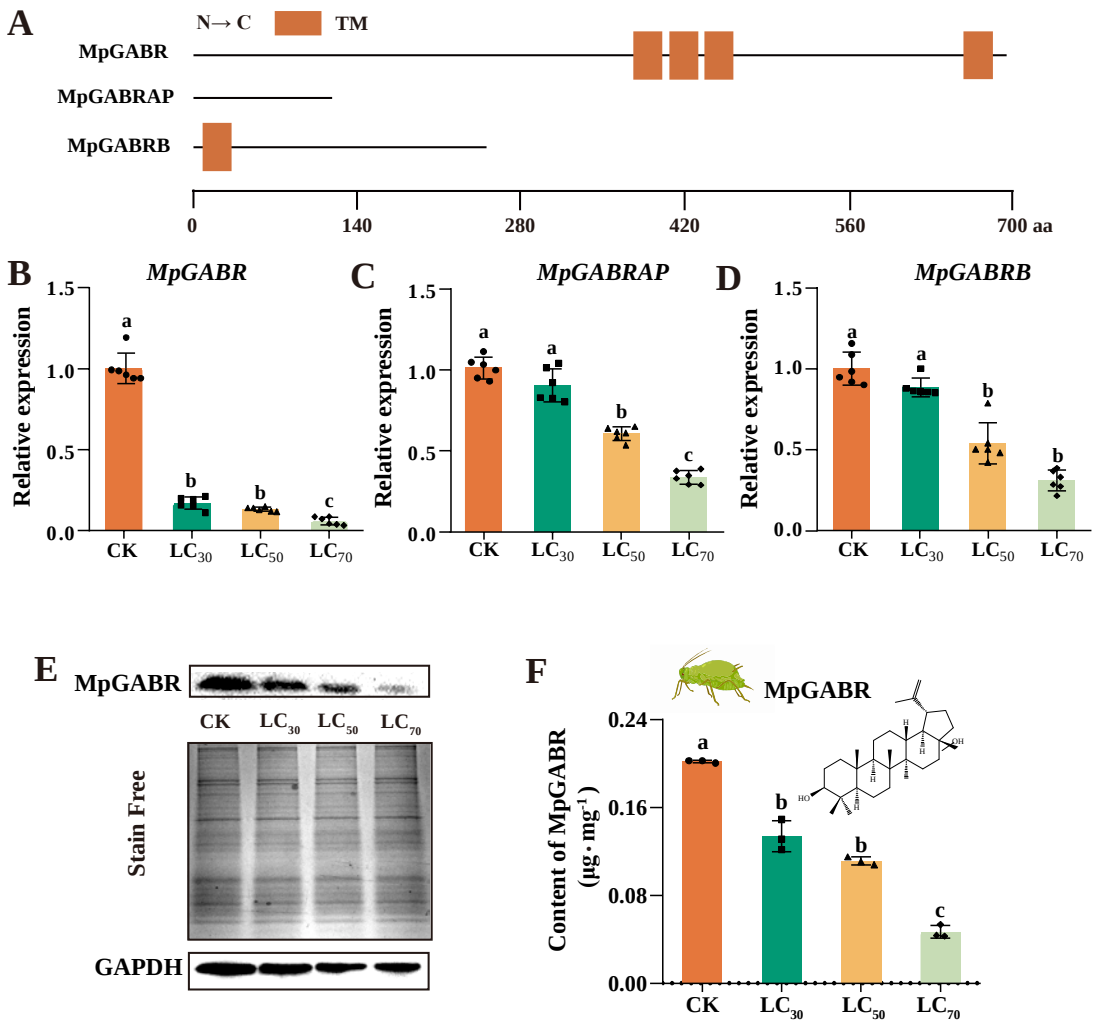


Figure 2. RNA-Seq analysis revealed candidate targets for betulin against *M. persicae*. (A) Diagram of the candidate targets for betulin against aphids as determined by RNA-Seq. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). (B) Principal coordinates analysis (PCA) analysis differentially expressed genes in RNA-Seq. (C-D) Distribution (C) and heatmap (D) of the significantly differentially expressed genes (DEGs) in *M. persicae*. (E) qPCR validation of 15 DEGs identified by RNA-Seq. (F-G) Top 10 enriched GO (F) and KEGG (G) pathways of the DEGs. The "rich ratio" was defined as the ratio of the number of DEGs enriched in the pathway to the total number of genes enriched in the same pathway. (H) Heatmap of the expression level of the GABR genes identified by RNA-Seq. *MpGABR*, encoding $GABA_A$ receptor; *MpGABRAP*, encoding $GABA_A$ receptor associated protein; *MpGABRB*, encoding $GABA_A$ receptor β subunit.



847

848 **Figure 3.** *MpGABR* expression in aphids was significantly inhibited by treatment with betulin. (A)

849 Schematic drawing of MpGABR, MpGABRAP and MpGABRB. TM, transmembrane helices. (B-D) qPCR

850 expression analysis of *MpGABR* (B), *MpGABRAP* (C) and *MpGABRB* (D) transcripts in *M. persicae* exposed

851 to LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h. The bars represent the average (± SD). Different letters above

852 the error bars indicate significant difference (ANOVA, Tukey's test, $P < 0.05$). (E, F) Western blotting

853 analysis of MpGABR protein after betulin treatment for 48 h at three different concentrations (LC_{30}, LC_{50}

854 and LC_{70}). The bars represent the average (± SD). Different letters above the error bars indicate significant

855 difference (ANOVA, Tukey's test, $P < 0.05$).

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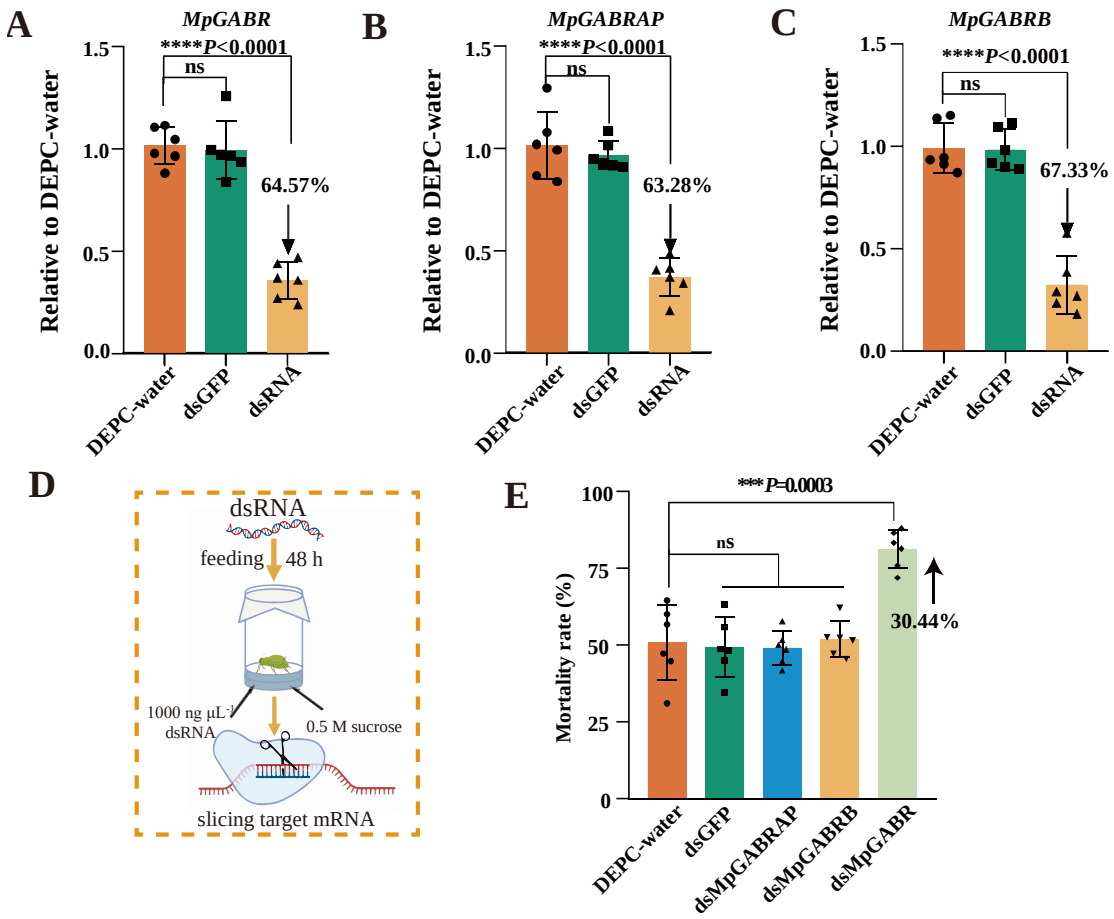


Figure 4. Silencing the expression of *MpGABR*, *MpGABRAP* and *MpGABRB* via RNAi. (A-C) qPCR expression analysis of *MpGABR* (A), *MpGABRAP* (B) and *MpGABRB* (C) after RNAi at 48 h post-dsRNA feeding relative to the expression levels after DEPC-water treatment. (D) Schematic drawing of the RNAi assay in *M. persicae*. (E) Mortality of aphids exposed to the LC_{50} of betulin for 48 h after RNAi. An asterisk (*) on the error bar indicates a significant difference between the treatment and group CK according to *t* tests, ***$P < 0.001$, ****$P < 0.0001$. ns, not significant.

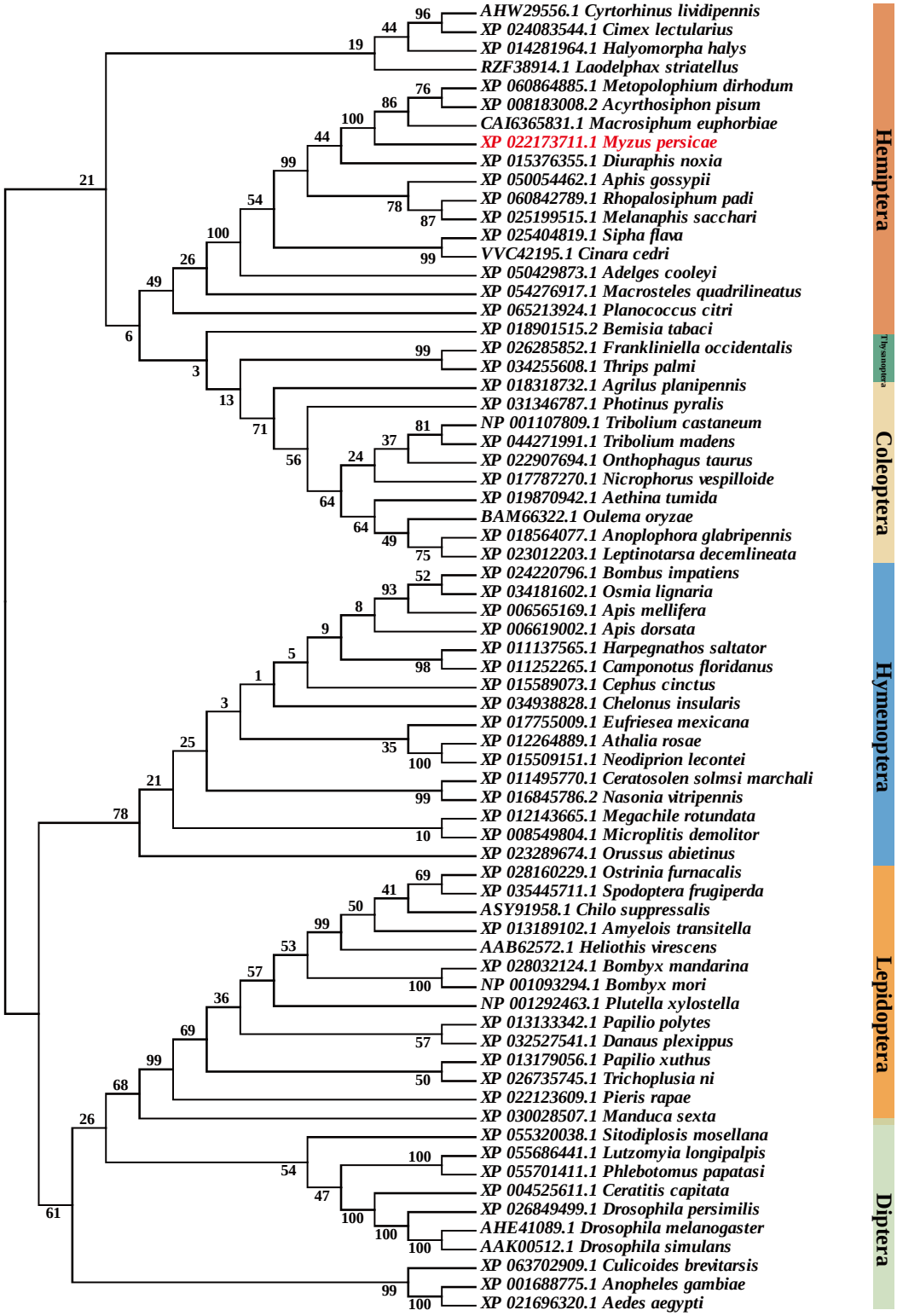


Figure 5. Phylogenetic analysis of GABR in insects. Neighbor-joining method with 1000 replicates based phylogenetic tree of GABR from six orders: Hemiptera, Diptera, Lepidoptera, Thysanoptera, Hymenoptera, and Coleoptera. Protein sequence alignment was performed using ClustalW in MEGA 7. The sequences used for constructing the tree were listed in Table S6.

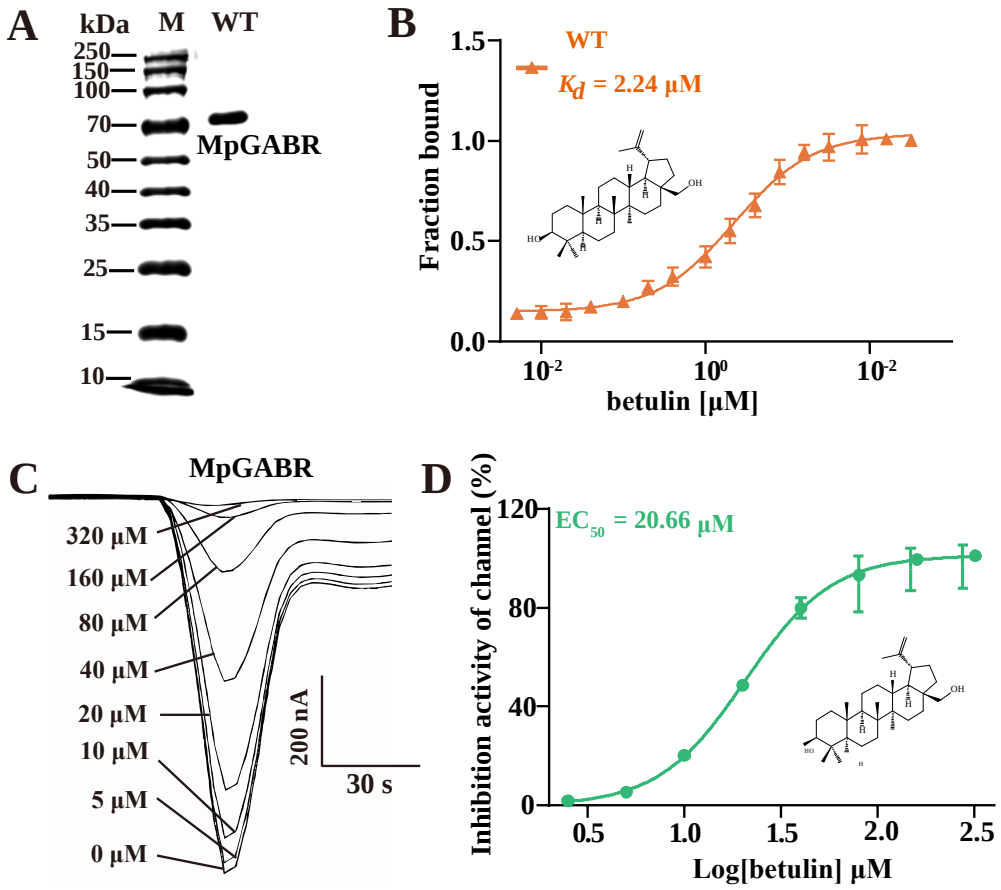
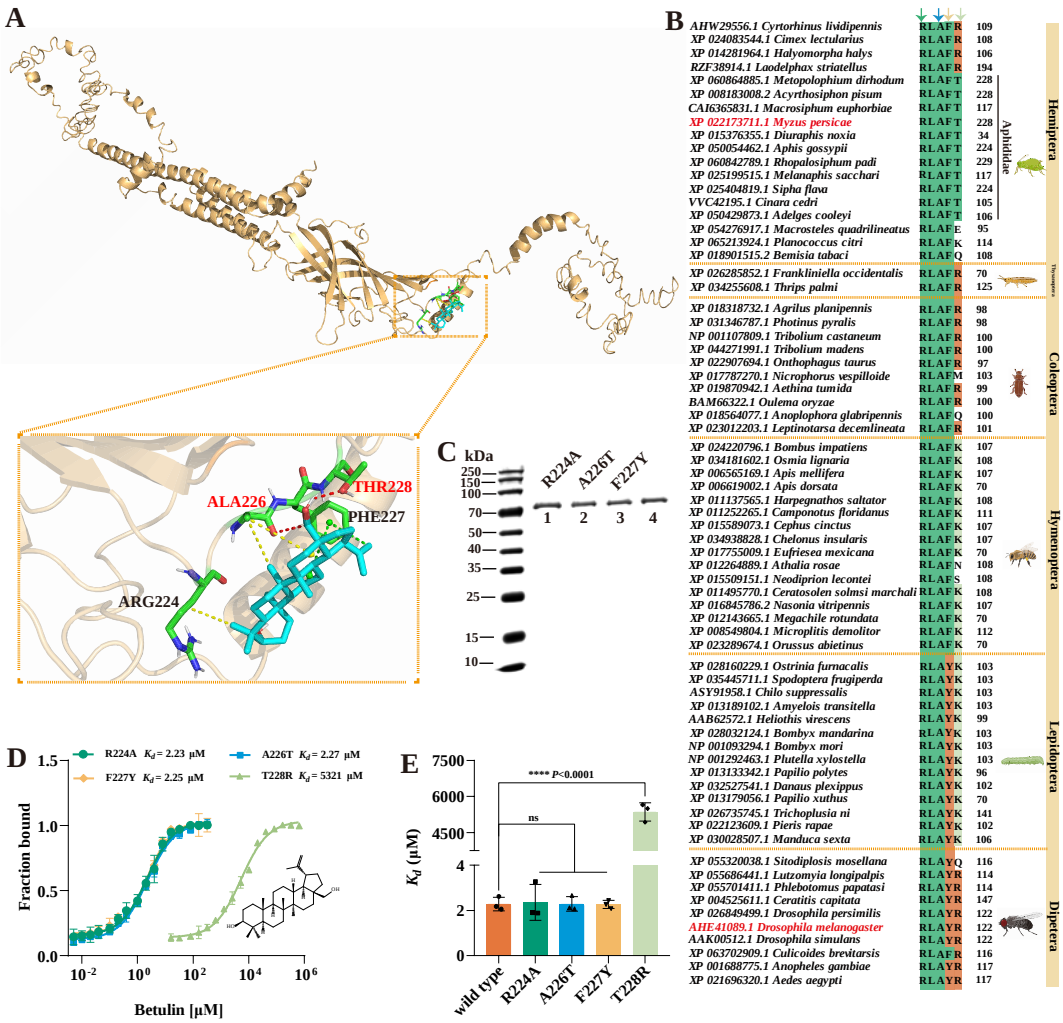


Figure 6. The interaction of betulin with MpGABR protein. (A) Expression of recombinant wild type-aphid MpGABR (1, WT) protein by *Escherichia coli*. (B) Quantification of the binding affinity of betulin with wild type-aphid MpGABR using MST. Current responses (C) and inhibition activity (D) of MpGABR induced by different concentration of betulin in the presence of GABA. 0 μM MpGABR indicates the presence of only GABA.



875 **Figure 7.** Molecular docking, binding site and inhibitory effect of betulin on MpGABR. (A) Best
876 conformations of betulin docked to the binding pocket of MpGABR in aphids. An enlarged view of the
877 betulin binding sites in MpGABR is indicated by a dashed frame. Potential Pi–alkyl (green), Alkyl (yellow),
878 and hydrogen bond (red) interactions are indicated by dashed lines. (B) Sequence alignment of the key
879 amino acids bound to betulin. The conserved residues among the different species in GABR are shown in
880 orange, cyan, and light green. The numbers next to the amino acid indicates the site of the last residue of
881 the key amino acids. (C) Expression of the recombinant mutation type-aphid MpGABR by *Escherichia coli*.
882 Lane 1: R224A, Lane 2: A226T, Lane 3: F227Y, Lane 4: T228R. (D, E) Quantification of the binding affinity
883 of betulin with wild-type and mutant type-aphid MpGABR using MST. The bars represent the average (±
884 SD) values. * indicates a significant difference (Student's *t* test, ****$P < 0.0001$).

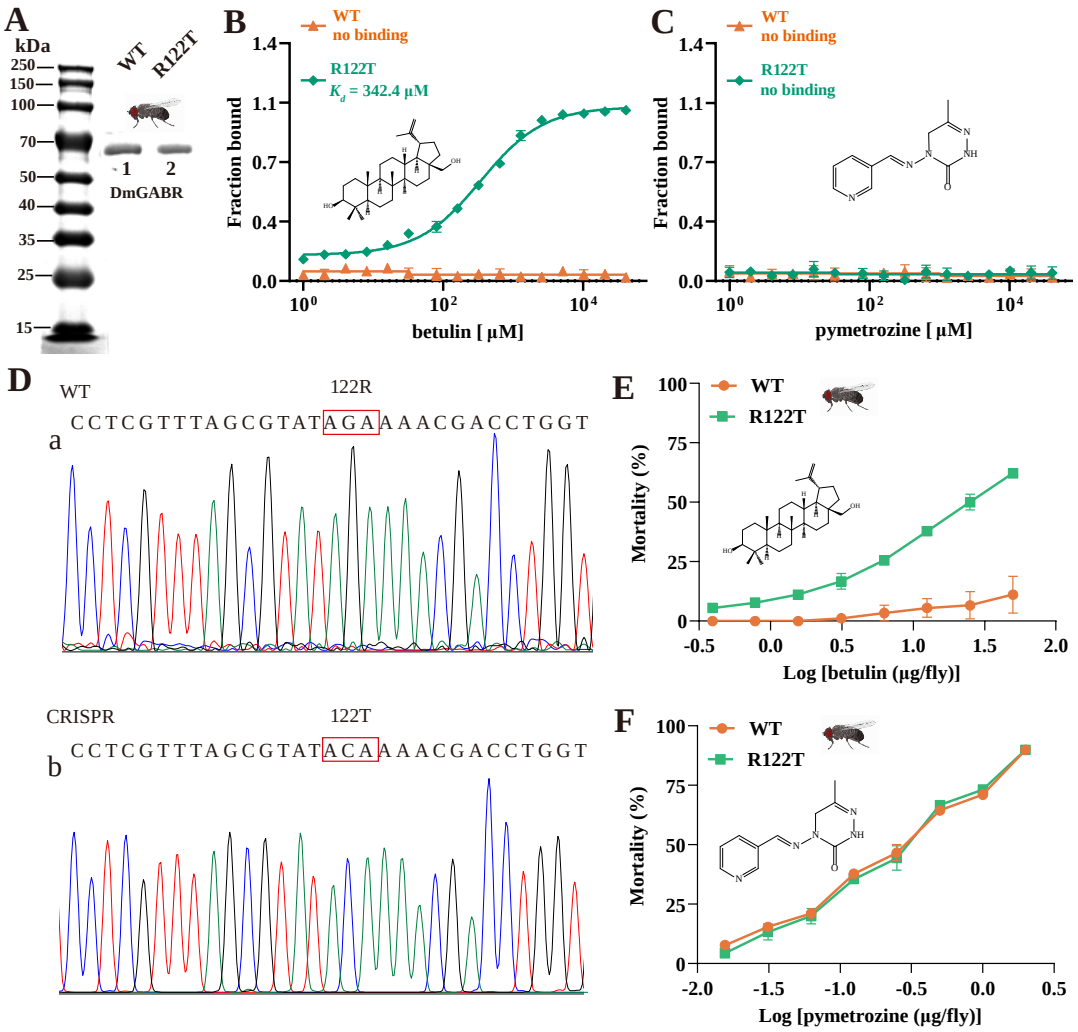


Figure 8. Gene editing in *Drosophila* validated the species-specific binding site of betulin. (A) Expression of the recombinant wild type (WT) and mutation type (R122T) of DmGABR (*Drosophila melanogaster*) by *E. coli*. Lane 1: wild type, Lane 2: R122T. (B, C) Quantification of the binding affinity of betulin (B) and pymetrozine (C) with WT and R122T of DmGABR using MST. (D) Sanger sequencing of the *DmGABR* gene in flies. Direct sequencing chromatograms of PCR products amplified from a fragment of gDNA flanking the WT (a), and introduced R122T (b) mutant flies. (E-F) Toxicity curves of betulin (E), and pymetrozine (F) against WT, and the DmGABR^{R122T} of the Cas9 *Drosophila*.

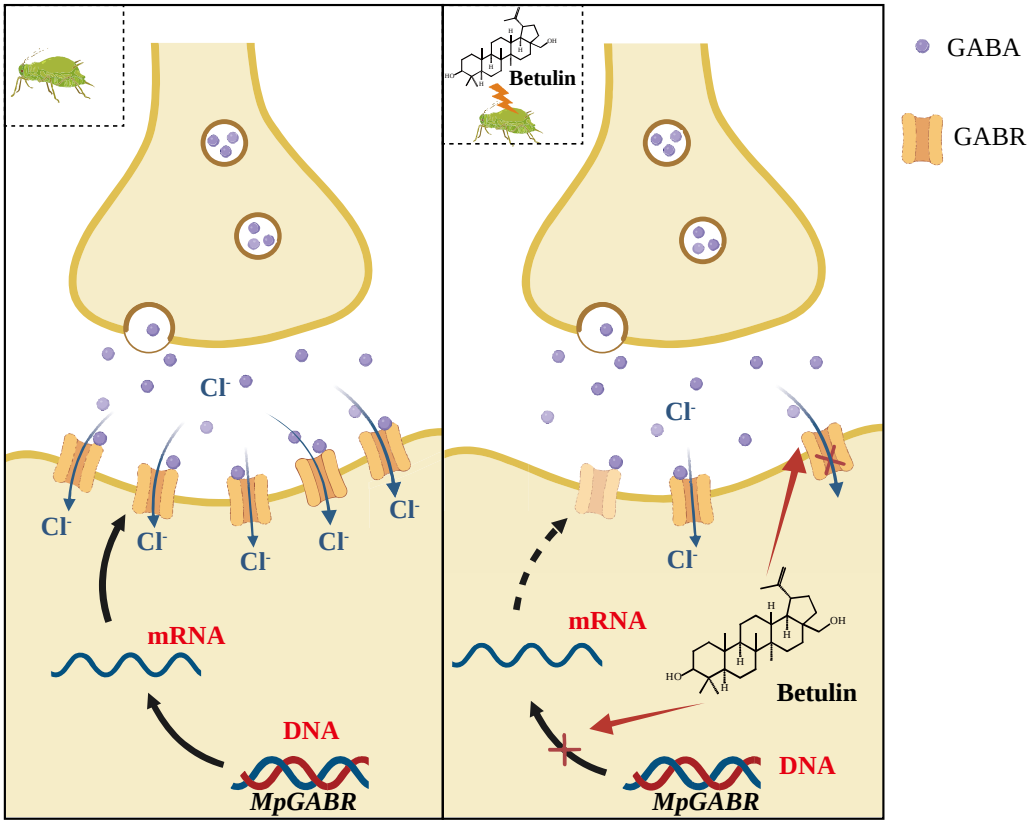


Figure 9. Proposed model for the mechanism of action against *Myzus persicae* by targeting $GABA_A$ receptors (GABR). After exposure to betulin, the expression of *MpGABR* was inhibited the level of MpGABR protein is decreased, resulting in a decrease in the channel of chloride ion influx. Besides, betulin directly and specifically binds to the amino acid residue THR228 of MpGABR, thereby disabling it.