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 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

38 **1. INTRODUCTION**

39 Over more than 400 million years of coevolution with herbivores, plants have developed
40 a variety of morphological,[1] biochemical,[2] and molecular[3] defense strategies against insect
41 herbivore attack. Plant secondary metabolites are highly valuable natural compounds,
42 offering abundant and potent biochemical defense driven by plant-insect coevolution.[4]
43 Interestingly, wild relatives of crops with insect resistance usually accumulate large amounts
44 of biologically active compounds.[5, 6] Accordingly, investigating the bioactivities and potential
45 mechanisms of action of compounds derived from wild resistant germplasms may provide
46 effective and sustainable strategies for pest control in crop protection.

47 *Myzus persicae* (Sülzer) is one of the most destructive sap-feeding pests worldwide. It is
48 able to settle on more than 400 plant species globally, displaying extraordinary polyphagia
49 and a wide range of hazards.[7] In addition to ingesting phloem saps and secreting honeydew,
50 *M. persicae* can transmit various plant viruses,[8] resulting in severe losses in crop yields. To
51 date, the principal control strategy for *M. persicae* has relied on synthetic insecticides,
52 including pyrethroids, organophosphorus compounds, carbamates and neonicotinoids.[9-11]
53 However, long-term irrational application of these chemical insecticides has led to
54 environmental pollution and promoted the resistance of aphids to insecticides.[11-13] Therefore,
55 it is necessary to develop novel green insecticides as potential alternatives to synthetic
56 insecticides. Fortunately, plant secondary metabolites are a "treasure trove of active
57 compounds" that serve as a rich resource for the development of promising green
58 insecticides.

59 Our previous study revealed that *Prunus davidiana*, a close wild relative of cultivated
60 peach, displays strong resistance to *M. persicae* through the accumulation of high levels of

61 betulin.[3, 5] In addition, betulin, a lupane-type triterpene, possesses strong insecticidal activity
62 and is a promising substance for the development of novel aphid-control insecticides. A
63 number of studies have reported that betulin and its derivatives exhibit a wide range of
64 pharmacological activities,[14] which have been used to treat various diseases, such as cancer,[15]
65 glioblastoma,[16] inflammation[17] and hyperlipidemia.[18] However, the insecticidal mechanism of
66 betulin against aphids remains unclear.

67 Gamma-aminobutyric acid (GABA) receptors have been confirmed to be targets of
68 terpenoids that impair neuronal function in insect herbivores.[19] In mammals, there are two
69 types of GABA receptors: ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors. $GABA_A$
70 receptors (GABRs) are heteropentameric ligand-gated ion channels in the central nervous
71 system that conduct chloride and bicarbonate ions and are the target of numerous drugs for
72 the treatment of neuropsychiatric disorders.[20] GABRs are composed of 5 different types of
73 subunits, each with 4 helical transmembrane domains (TM1-TM4), of which TM2 is located at
74 the center of the pentamer and forms an ion channel.[21] However, the structure and assembly
75 of insect GABRs have not been fully elucidated. The first GABA receptor subunit identified in
76 insects is encoded by *Rdl*, which confers resistance to dieldrin in *Drosophila melanogaster*.[22]
77 Although a variety of terpenoids play roles as positive allosteric modulators or
78 noncompetitive antagonists at GABRs,[19] whether betulin targets the GABR in *M. persicae*
79 needs to be further investigated.

80 In this study, betulin was confirmed to have excellent control efficacy against *M. persicae*
81 in both greenhouse and field experiments. RNA-seq, qRT–PCR, and western blotting assays
82 revealed that betulin significantly inhibited the expression of *MpGABR* in aphids. In addition,

83 RNAi-mediated silencing of *MpGABR* markedly increased aphid sensitivity to betulin.
84 Furthermore, microscale thermophoresis (MST) and voltage-clamp assays indicated that
85 betulin can bind to MpGABR (K_d = 2.24 μM) and act as an inhibitor (EC_{50} = 20.66 μM) of
86 MpGABR. Molecular docking analysis suggested that betulin bound to MpGABR via the
87 THR228 amino acid residue. This site is highly conserved in the family Hemiptera and may
88 be a critical specific binding site for betulin in MpGABR in aphids. Mutagenesis and genome
89 editing experiments revealed that betulin bound specifically to this amino acid residue in
90 aphids but not in *Drosophila*, resulting in aphid death. This study elucidated the insecticidal
91 mechanism of betulin involving the targeting of MpGABR, providing a sustainable green
92 strategy for aphid control.

93 **2. MATERIALS AND METHODS**

94 **2.1 Test insects**

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

99 **2.2 Bioassay**

100 The toxicity of betulin against *M. persicae* was measured using a slip-dip bioassay as
101 previously described.[5] Thirty apterous adult *M. persicae* individuals were gently transferred
102 on tobacco (*N. tabacum*) leaves using a fine brush. The tobacco leaves were subsequently
103 immersed in 0.1% (v/v) Tween-80 and 3% (v/v) acetone solution containing betulin or
104 pymetrozine (as a positive control) at 6 doses (0, 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg· mL^{-1}) for

105 5 seconds. After drying, the leaves with *M. persicae* were placed on wet filter paper and
106 cultured on a water-soaked sponge for 48 h. Three replicates were tested for each treatment.
107 Furthermore, the LC_{30}, LC_{50} and LC_{70} values of betulin were calculated using log-probit
108 analysis via IBM SPSS Statistics (v.22.0, Chicago, IL, USA).[2]

109 **2.3 Control effect of betulin against *M. persicae***

110 The control effects of betulin against *M. persicae* in the greenhouse and field were
111 evaluated by a spraying-based method as described previously.[23] The commercial insecticide
112 pymetrozine and an aqueous solution containing 3% (v/v) acetone and 0.1% (v/v) Tween-80
113 were employed as positive and negative controls, respectively. Sprays of 0.1641 $mg \cdot mL^{-1}$ (LC_{50})
114 betulin and 1.0612 $mg \cdot mL^{-1}$ (LC_{50}) pymetrozine (Table S1) were prepared in aqueous solutions
115 including 3% (v/v) acetone and 0.1% (v/v) Tween-80, respectively. Before the control effect
116 assays, each tobacco (*N. tabacum* L.) seedling was pre-inoculated with 60 apterous adults of *M.*
117 *persicae*. In the greenhouse trial, each treatment included 8 replications, and each replication
118 consisted of 3 tobacco plants growing under controlled conditions as described above. The
119 field trial (Beibei District, Chongqing, China.) was conducted under natural conditions with
120 an average temperature of 20 °C. Each treatment had 8 replications, covering an area of 20 m^2,
121 with 3 protected rows per replication. Furthermore, each tobacco plant was sprayed with 20
122 mL of the corresponding solution with an electric sprayer. Mortality was recorded after 1, 5, 9,
123 and 14 days of treatment. The control efficacy of betulin against *M. persicae* was calculated
124 according to the methods of Zhou.[23]

125 **2.4 RNA-seq assay**

126 After exposure to 0.1641 $mg \cdot mL^{-1}$ (48 h LC_{50}) betulin for 48 h, 100 wingless *M. persicae*

127 adults were sampled, and total RNA was extracted via TRIzol reagent (Tiangen, Beijing,
128 China) to construct mRNA sequencing libraries. RNA-seq analysis was performed on the Ion
129 Proton BGISEQ-500 platform (Beijing Genomic Institute, BGI, China). After the raw reads
130 were filtered, the clean reads were aligned to the *M. persicae* reference genome, which was
131 downloaded from the National Center for Biotechnology Information database, using
132 HISAT2 (v2.1.0).[24] The expression levels of genes in each sample were normalized to the
133 fragments per kilobase million mapped reads (FPKM) value. The differentially expressed
134 genes (DEGs) between groups were identified using the fold change factor ($|Log_2(FC)| > 1$).
135 Functional enrichment analysis of DEGs was performed using the Gene Ontology (GO) and
136 Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

137 **2.5 Validation by quantitative real-time PCR (qRT–PCR)**

138 The gene-specific primers used for qRT–PCR, which were designed with Primer 5.0, are
139 listed in Table S2. According to the manufacturer's specifications for the Bio-Rad iQ™ SYBR®
140 Green Supermix Kit, qRT–PCR was performed with the following conditions: 60 °C for 15 s,
141 followed by 48 cycles of 60 °C for 30 s, and 72 °C for 30 s. The *ribosomal protein S18* gene
142 (*RPS18*) was used as the reference gene to normalize the expression levels. The relative
143 expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ approach.[2] Three
144 biological replicates were used for each assay.

145 **2.6 Cloning, bioinformatics and phylogenetic analysis**

146 The cDNA of *M. persicae* was synthesized from the extracted total RNA using the
147 PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The coding sequences of
148 *MpGABR* (encoding $GABA_A$ receptor), *MpGABRAP* (encoding $GABA_A$ receptor-associated

149 protein) and *MpGABRB* (encoding $GABA_A$ receptor β subunit) were cloned from *M. persicae*
150 cDNA using the corresponding specific primers (Table S2) and then inserted separately into
151 the pGEM-T Easy vector. The recombinant plasmids were subsequently transferred into
152 *Escherichia coli* DH5α competent cells for sequencing. BioXM (v2.7.1)
153 (http://202.195.246.60/BioXM/) was used to analyze the sequence information of these three
154 genes, including the length, molecular weight, and isoelectric point of the deduced amino
155 acid sequence. MEGA7 was used for multiple amino acid sequence alignment (ClustalW) and
156 phylogenetic analysis (neighbor-joining method with 1000 replicates) of MpGABR.

157 **2.5 *In vitro* RNAi assay**

158 To knock down the target genes (*MpGABR*, *MpGABRAP*, and *MpGABRB*), *MpGABR*-,
159 *MpGABRAP*-, and *MpGABRB*-dsRNA were artificially synthesized using the dsRNA
160 Synthesis Kit (Thermo Scientific, Vilnius, Lithuania, EU). In accordance with a previously
161 described method,[25] fresh tobacco leaves were cut into 3.0 cm-diameter discs and placed in an
162 oven at 50 °C for 3 min. Subsequently, the dried leaf discs were exposed to 10 μL of diethyl
163 pyrocarbonate-treated nuclease-free water (DEPC-water), dsGFP (green fluorescent protein,
164 1000 ng· μL^{-1}, negative control), and *MpGABR*-, *MpGABRAP*-, or *MpGABRB*-dsRNA (1000
165 ng/·μL^{-1}) at 25 °C for 5 h. After the leaf discs had absorbed the solution, they were placed on
166 wet filter paper on a water-soaked sponge. Subsequently, 30 wingless adult *M. persicae*,
167 starved for 24 h, were fed on the treated leaf discs for 48 h. The surviving aphids were
168 collected after exposure for qRT–PCR.

169 **2.6 Prokaryotic expression of *MpGABR* and western blotting**

170 To produce the MpGABR protein *in vitro*, an *Escherichia coli* expression system was

171 constructed.[26] Briefly, the coding sequence of *MpGABR* was inserted into the expression
172 vector PET-B2M and transformed into BL21 (*DE3*) competent cells. To induce the expression
173 of the MpGABR protein, 2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was used, and
174 then, the transformed BL21 cells were incubated at 180 rpm and 30 °C for 24 h. Finally, the
175 protein was extracted and purified using Solarbio GST-tag Purification Resin for subsequent
176 assays.

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

188 **2.7 Molecular docking**

189 In accordance with a previously reported method,[23] the protein structure and function of
190 MpGABR were predicted via the I-TASSER server (Iterative Threading ASSEmbly
191 Refinement). AutoDockTools software (v1.5.7) was used to conduct molecular docking.
192 Furthermore, PyMOL (v3.1) and Discovery Studio (v4.5) were used to evaluate the molecular

193 docking model.

194 **2.8 Site-directed mutagenesis**

195 The cDNA of *MpGABR* was inserted into the PET-B2M vector and used as the template
196 for site-directed mutagenesis. In accordance with the manufacturer's specifications, the Fast
197 MultiSite Mutagenesis Kit (TransGen, Beijing, China) was used to generate the multisite
198 mutations in MpGABR.[27] Then, the MpGABR mutants, with the mutations R224A (ARG224
199 replaced by ALA), A226T (ALA226 replaced by THR), F227Y (PHE227 replaced by TYR), and
200 T228R (THR228 replaced by ARG), were amplified, and the amplicons were used to replace
201 the *Bam*HI-*Sma*I fragment in the recombinant MpGABR plasmid.

202 **2.9 Method for *Drosophila* genome editing**

203 To generate *Drosophila* mutants bearing the $DmGABR^{R122T}$ mutation, homologous to
204 THR228 in MpGABR, the Clustered Regularly Interspaced Short Palindromic Repeats-Cas9
205 (CRISPR/Cas9) genomic editing strategy was used. First, CRISPR Optimal Target Finder
206 (http://targetfinder.flycrispr.neuro.brown.edu/) was used to generate short guide RNA
207 (sgRNA) targets without off-target hits. Then, the Precision gRNA Synthesis Kit (Thermo
208 Scientific) was used to synthesize the sgRNA. The 228 bp homologous arm in R122T, for
209 homology-directed repair, was subsequently employed to synthetize single-stranded DNA
210 (ssDNA) bearing the desired mutation ($DmGABR^{R122T}$). Subsequently, 1 nL of a mixture
211 containing 160 ng· μL^{-1} ssDNA, 300 ng· μL^{-1} both sgRNAs, or 90 ng· μL^{-1} Cas9 protein was
212 injected into *Drosophila* embryos via the Drummond Nanoject III (Broomall, PA, USA) system.
213 CRISPR/Cas9-induced mutations were verified by amplifying and sequencing gDNA flanking
214 the genome editing target site. Finally, according to the traditional topical application and

215 bioassay approach described previously,[23] the acute insect toxicity of betulin in
216 genome-modified or nonmodified (WT) 3-day-old adult flies was determined. The
217 oligonucleotide sequences used for PCR, the sgRNA synthesis template and the fragment of
218 donor DNA used for homology-directed repair (HDR) are listed in Table S3.

219 **2.10 Microscale thermophoresis (MST) assay**

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

230 **2.11 Electrophysiological recording**

231 In accordance with previously described methods,[28] oocytes isolated from mature
232 healthy *Xenopus* females were digested at 25 °C for 1 h to remove the follicular layer.
233 Individual oocytes were selected to express *MpGABR* and incubated at 18 °C with 1× Ringer's
234 solution (96 mM NaCl, 5 mM $MgCl_2$, 5 mM HEPES, 2 mM KCl, and 0.8 mM $CaCl_2$)
235 supplemented with 550 mg·mL^{-1} sodium pyruvate, 100 mg·mL^{-1} streptomycin, 50 mg·mL^{-1}
236 tetracycline, and 5% dialyzed horse serum. After 3 days of incubation, the whole-cell currents

237 were recorded via a two-electrode voltage-clamp. The data were collected and analyzed using
238 Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., Union City, CA, USA),
239 respectively.[29]

240 **2.12 Statistical analysis**

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

244 **3. RESULTS**

245 **3.1 Control efficacy of betulin against aphids**

246 Bioassays revealed that the LC_{50} values of betulin and pymetrozine against *M. persicae* at
247 48 h were 0.1641 and 1.0612 mg·mL^{-1}, respectively (Table S1). To assess the control efficacy of
248 betulin, tobacco plants infected with *M. persicae* were exposed to betulin (0.1641 mg·mL^{-1}) and
249 pymetrozine (1.0612 mg·mL^{-1}, as a positive control) in both the greenhouse and field. In the
250 greenhouse test (Fig. 1A-D), after 14 days of treatment, *M. persicae* in the CK group (negative
251 control) reproduced rapidly, and tobacco leaves turned yellow and wrinkled, whereas
252 tobacco leaves in the betulin and pymetrozine treatment groups were barely affected by the
253 aphids (Fig. 1A). The control efficacy of betulin against *M. persicae* was significantly greater
254 than that of pymetrozine after 1 day of treatment, while there was no significant difference
255 between the control efficacies of betulin and pymetrozine after 5, 9, and 14 days of treatment
256 (Fig. 1E). After 14 days of treatment, the control efficacies of betulin and pymetrozine reached
257 91.54% and 95.15%, respectively. Throughout the field test (Fig. 1F), no difference in the
258 control efficacy was observed between betulin and pymetrozine, with values of 87.79% and

259 90.14%, respectively, after 14 days of treatment (Fig. 1G). These results suggest that betulin
260 has immense potential for development as a commercial aphid insecticide like pymetrozine.

261 **3.2 Expression patterns of genes in aphids after treatment with betulin**

262 To investigate the expression patterns of genes in *M. persicae* after exposure to betulin,
263 RNA-seq was performed on *M. persicae* with 0.1641 mg·mL^{-1} betulin treatment for 48 h and
264 without (CK) (Fig. 2A). Principal coordinate analysis (PCA) revealed that the expressed genes
265 were strongly clustered in the CK group and betulin group (Fig. 2B). Compared with those in
266 the CK group, there were 130 up- and 41 downregulated significant differentially expressed
267 genes (DEGs) in *M. persicae* after betulin treatment (Fig. 2C and D, Table S4). The validation
268 by qRT–PCR revealed that the relative expression trends of the fifteen DEGs determined via
269 qRT–PCR were similar to those detected via RNA–seq, supporting the reliability of the RNA–
270 seq data (Fig. 2E). To further analyze the functions of the DEGs, GO (Gene Ontology) and
271 KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses were performed.
272 GO term enrichment demonstrated that the DEGs were enriched mainly in the $GABA_A$
273 receptor activity term (Fig. 2F). Interestingly, KEGG enrichment also found that the DEGs
274 were enriched in the GABAergic signaling related pathway and GABAergic synapse terms
275 (Fig. 2G). Additionally, the expression of DEGs related to GABR, including *MpGABR*
276 (encoding $GABA_A$ receptor), *MpGABRAP* (encoding $GABA_A$ receptor-associated protein) and
277 *MpGABRB* (encoding $GABA_A$ receptor β subunit), in the betulin group was significantly
278 lower than that in the CK group (Fig. 2H). In particular, the log_2(fold change) value of
279 *MpGABR* was the lowest, at -3.8 (Table S4). These results suggested that *MpGABR* may be a
280 candidate target for betulin against *M. persicae.*

281 **3.3 *MpGABR* expression was inhibited by betulin**

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

288 To further verify the inhibitory effect of betulin on GABR-related gene expression, the
289 relative expression of the three GABR-related genes was detected in *M. persicae* exposed to the
290 LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h. The relative expression of all three genes decreased
291 gradually as the concentration of betulin increased (Fig. 3B-D). After exposure to the LC_{30} of
292 betulin for 48 h, the relative expression of *MpGABR*, *MpGABRAP* and *MpGABRB* decreased
293 by 82.91%, 10.53%, and 11.49%, respectively. These results indicated that *MpGABR* was most
294 sensitive to betulin.

295 Furthermore, after these three genes were silenced via RNAi (Fig. 3H), the expression
296 levels of *MpGABR*, *MpGABRAP* and *MpGABRB* in *M. persicae* decreased by 64.57%, 63.28%,
297 and 67.33%, respectively, compared with those in the control group (DEPC-treated water) (Fig.
298 3E-G). After exposure to the LC_{50} of betulin for 48 h, the mortality of *M. persicae* with
299 *MpGABR* silenced markedly increased, by 30.44%, compared with that in the control group,
300 whereas the mortality of *M. persicae* in the *MpGABRAP*- and *MpGABRB*-silenced groups did
301 not significantly differ from that in the control group (Fig. 3I). Additionally, western blotting
302 analysis revealed that MpGABR protein expression decreased by 69.91% after RNAi (Fig. S1A,

303 B). These results further implied that *MpGABR* might be a target for betulin against *M.*
304 *persicae*.

305 **3.5. Phylogenetic analysis of GABRs in insects**

306 Furthermore, phylogenetic analysis of GABRs in insects was performed using the amino
307 acid sequences of 71 GARB proteins from Hemiptera, Diptera, Lepidoptera, Thysanoptera,
308 Hymenoptera, and Coleoptera. MpGABR (GenBank number: XM022318019.1) was found to
309 be genetically closely related to CAI6365831.1 from *Macrosiphum euphorbiae* (Fig. 4 and Table
310 S6), indicating that MpGABR may be functionally analogous to CAI6365831.1.

311 **3.6. Responses of MpGABR to betulin**

312 To investigate the effect of betulin on the MpGABR protein, *M. persicae* was exposed to
313 the LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h. As the concentration of betulin increased, the
314 MpGABR protein content in *M. persicae* gradually decreased (Fig. 5A and B). Compared with
315 that in the CK group, the MpGABR protein content in *M. persicae* exposed to the LC_{30}, LC_{50},
316 and LC_{70} of betulin decreased by 33.68%, 44.89%, and 76.89%, respectively.

317 Moreover, the WT (wild type) MpGABR was expressed in *Escherichia coli* (Fig. 5C). The
318 binding affinity of betulin with WT MpGABR was further measured using MST. The K_d value
319 was 2.24 μM (Fig. 5D), suggesting that betulin could bind to MpGABR. Additionally,
320 voltage-clamp-based electrophysiological recording demonstrated that MpGABR strongly
321 responded to betulin (Fig. 5E). In the presence of only GABA, a fast inward current was
322 generated. As the concentration of added betulin increased, the current and channel activity
323 of MpGABR decreased correspondingly, with an EC_{50} value of 20.66 μM (Fig. 5E and F). These
324 results indicated that betulin acted as an inhibitor of MpGABR.

325 **3.5 Key sites for the binding of betulin to MpGABR**

326 To further study the binding mode of betulin in the active pocket of MpGABR, molecular
327 docking was performed to explore the structure–function relationships between betulin and
328 MpGABR. The docking results revealed that the predicted binding energy between betulin
329 and MpGABR was −6.38 kcal·mol^{-1} (Table S7), verifying that betulin could act as a specific
330 ligand for MpGABR. The three-dimensional binding pattern of betulin with MpGABR
331 indicated that the four key amino acid residues (ARG224, ALA226, PHE227 and THR228)
332 interacted with betulin in the MpGABR binding pocket (Fig. 6A). Among the four key amino
333 acid residues, ALA226 and THR228 interacted with betulin via hydrogen bonding in the
334 MpGABR binding pocket (Table S7). Betulin generated nonconventional H-bonds (C…H)
335 with ALA226 (3.31 Å) and conventional H-bonds (C-O H) with THR228 (2.16 Å). Furthermore,
336 sequence alignment of the key amino acid residues of GABR in different species from
337 Hemiptera, Diptera, Lepidoptera, Hymenoptera, Thysanoptera, and Coleoptera indicated
338 that only THR228 was conserved in Hemiptera (Fig. 6B), implying that THR228 may be an
339 essential specific site for the action of betulin on MpGABR in aphids. Mutants of aphid
340 MpGABR were subsequently constructed, including R224A (ARG224 replaced by ALA),
341 A226T (ALA226 replaced by THR), F227Y (PHE227 replaced by TYR, similar to the site in
342 *Drosophila*), and T228R (THR228 replaced by ARG, similar to the site in *Drosophila*) (Fig. 6C).
343 The binding affinity of betulin with these mutants was measured using MST. The results
344 revealed that the K_d values of R224A, A226T, F227Y, T228R, and WT were 2.31, 2.27, 2.25, 5321,
345 and 2.24 μM, respectively (Fig. 6D and E, Table S8). This result suggested that the mutation at
346 the THR228 site, with the highest K_d value, markedly reduced the binding ability of betulin to

347 MpGABR, implying that the THR228 site may be an essential specific site for the binding of
348 betulin to MpGABR.

349 **3.6 Betulin binds specifically to MpGABR via THR228**

350 To further prove that THR228 is the specific binding site for betulin in MpGABR, the WT
351 and mutant (R122T, equivalent to THR228 in MpGABR) *Drosophila* DmGABR proteins were
352 expressed in *E. coli* and extracted (Fig. 7A and D). The binding affinities of betulin and
353 pymetrozine with $DmGABR^{WT}$ and $DmGABR^{R122T}$ were evaluated using MST. As shown in
354 Fig. 7B, betulin was able to bind to $DmGABR^{R122T}$ (K_d = 342.4 μM) but not to $DmGABR^{WT}$.
355 However, pymetrozine exhibited no binding affinity for $DmGABR^{R122T}$ or $DmGABR^{WT}$ (Fig.
356 7C). Moreover, to generate *Drosophila* mutant individuals carrying $DmGABR^{R122T}$, the
357 CRISPR/Cas9 genomic editing strategy was used. After exposure to different concentrations
358 of betulin, the mortality rate of $DmGABR^{R122T}$ *Drosophila* was significantly greater than that of
359 $DmGABR^{WT}$ *Drosophila* (Fig. 7E). In addition, the LD_{50} value of betulin against $DmGABR^{R122T}$
360 *Drosophila* at 72 h was 27.19 $\mu g \cdot fly^{-1}$, whereas the LD_{50} value of betulin against $DmGABR^{WT}$
361 *Drosophila* could not be calculated because the lethal concentration exceeded 1000 $\mu g \cdot fly^{-1}$
362 (Table S9). Additionally, there was no significant difference in mortality between $DmGABR^{WT}$
363 and $DmGABR^{R122T}$ *Drosophila* after exposure to pymetrozine (Fig. 7F). The LD_{50} values of
364 pymetrozine against $DmGABR^{WT}$ and $DmGABR^{R122T}$ *Drosophila* at 72 h were 0.23 and 0.24
365 $\mu g \cdot fly^{-1}$, respectively (Table S9). Moreover, THR228 is located at the neurotransmitter-gated
366 ion-channel ligand-binding domain in MpGABR (Fig. S2), implying that betulin may play a
367 role as a competitive antagonist of MpGABR. Taken together, these findings suggested that
368 betulin bound specifically to MpGABR via THR228, resulting in the death of *M. persicae*.

369 **4. DISCUSSION**

370 Although chemical pesticides are effective in pest control, the long-term unreasonable
371 application of these substances has led the emergence of resistant pests, environmental
372 deterioration, and deleterious effects on nontarget organisms, including humans, raising
373 widespread and intense concerns.[30] These challenges have made it urgent for the
374 development of alternative strategies for pest control. Recently, the utilization of plant
375 secondary metabolites as insecticides has become increasingly popular as an eco-friendly and
376 biocontrol approach.[31-33] Our previous study revealed that *P. davidiana*, a wild relative of
377 cultivated peach, strongly resists *M. persicae* by accumulating high contents of betulin.[3, 5] In
378 addition, betulin, a lupane-type triterpene, possesses potent insecticidal activity and is a
379 promising substance for the development of novel insecticides for aphid control. In this study,
380 the insecticidal effect of betulin was further evaluated by comparing the control efficacy of
381 betulin with that of pymetrozine against aphids in greenhouses and fields (Fig. 1A-G). These
382 results indicate that betulin has a similar control effect to pyrhidone and has immense
383 potential for development as a plant-derived insecticide.

384 Terpenes are a diverse group of plant secondary metabolites that can increase the
385 resistance of plants to insect herbivores through direct [34] and indirect [35] defense mechanisms.
386 In direct defense against herbivores, triterpenes play important roles in diverse biological
387 activities, including antiparasitic, insecticidal, and antifeedant activities.[36, 37] Azadirachtin, a
388 tetracyclic triterpenoid compound isolated from the Indian neem tree (*Azadirachta indica*), is
389 one of the most prominent commercial biopesticides, exhibiting strong insect antifeedant
390 properties as well as growth- and reproduction-regulating effects.[38, 39] Besides, triterpene

391 glycoside compounds play crucial roles in the defense of tobacco (*N. attenuata*) against
392 tobacco hornworm (*Manduca sexta*) larvae.[40] Although betulin exhibits various
393 pharmacological activities,[14-17] reports on its insecticidal activity are limited. Encouragingly,
394 the effects of betulin and its derivatives on pests have attracted increasing attention. Betulinic
395 acid and its derivatives showed larvicidal activity against *Aedes aegypti* larvae.[41]
396 Betulin-cinnamic acid-related hybrid compound 5b exhibited strong aphicidal activity, and
397 compound 2l could destroy the ultrastructure of midgut cells and significantly inhibit the
398 activity of α-amylase in diamondback moth (*Plutella xylostella* L.) larvae.[42] Our previous
399 studies also indicated that betulin possesses potent insecticidal activity and is a key
400 endogenous secondary metabolite related to the defense of peach against *M. persicae*.[5]
401 Elucidating the insecticidal mechanism of betulin against aphids will provide a basis for the
402 development of novel aphicides and sustainable strategies for aphid control.

403 GABA receptors have been confirmed to be targets of terpenoids that impair insect
404 neuronal function in herbivores.[19] GABRs are heteropentameric ligand-gated ion channels in
405 the central nervous system that conduct chloride and bicarbonate ions. These receptors are
406 targets of numerous drugs for the treatment of neuropsychiatric disorders.[20] A variety of
407 terpenoids act as positive allosteric modulators or noncompetitive antagonists of GABRs,
408 such as diterpenoids (isopimaric acid and miltirone), sesquiterpenoids (picrotoxin, bilobalide
409 and ginkgolides) and monoterpenoids (α-thujone and thymol).[19] In this study, both GO and
410 KEGG enrichment analyses revealed that the DEGs identified by RNA-seq were enriched in
411 GABAergic signaling-related pathways (Fig. 2F and G). Additionally, the expression of the
412 DEGs related to GABRs, particularly *MpGABR* (Table S4), in the betulin group was

413 significantly lower than that in the CK group (Fig. 2H). Besides, the relative expression of
414 *MpGABR*, *MpGABRAP* and *MpGABRB* decreased gradually after *M. persicae* was exposed to
415 the LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h (Fig. 3B-D). Among them, *MpGABR* was the most
416 sensitive to betulin, and its expression was reduced by 82.91% after exposure to the LC_{30} of
417 betulin for 48 h. Furthermore, compared with the control group, the *M. persicae* group with
418 *MpGABR* silenced by RNAi presented a significant increase in mortality, by 30.44%, after 48 h
419 of exposure to the LC_{50} of betulin (Fig. 3I). Collectively, these results suggest that betulin may
420 have insecticidal effects on aphids by inhibiting *MpGABR* expression.

421 Additionally, betulin has been reported to be able to bind to GABRs.[43] We further
422 investigated the interaction of betulin with the MpGABR protein. The MST assay revealed
423 that betulin was able to bind to MpGABR (K_d = 2.24 μM) (Fig. 5D), which is consistent with
424 previous findings showing that betulin binds to GABA receptors in mouse brains *in vitro*.[44]
425 Voltage-clamp-based electrophysiological recordings indicated that betulin acted as an
426 inhibitor (EC_{50} = 20.66 μM) for MpGABR (Fig. 5E and F). Subsequent molecular docking
427 analysis suggested that four key amino acid residues (ARG224, ALA226, PHE227 and
428 THR228) interact with betulin in the MpGABR binding pocket (Fig. 6A), among which only
429 ALA226 and THR228 interact with betulin via hydrogen bonding (Table S7). The results of
430 the sequence alignment revealed that only THR228 was conserved in Hemiptera (Fig. 6B).
431 Furthermore, evaluation of the ability of betulin to bind to MpGABR mutants with mutations
432 at those four sites revealed that the ability of betulin to bind to T228R was significantly
433 weaker than its ability to bind to the WT (Fig. 6D and E, Table S8), indicating that THR228 is
434 an essential specific site for the binding of betulin to MpGABR. Moreover, to further prove

435 that THR228 is the specific binding site for betulin in MpGABR, the binding affinities of
436 betulin with the WT and mutant (R122T, equivalent to THR228 in MpGABR) *Drosophila*
437 DmGABR proteins were assessed using MST. The results showed that betulin was able to
438 bind to $DmGABR^{R122T}$ (K_d = 342.4 μM) but not $DmGABR^{WT}$ (Fig. 7B). Additionally, after
439 exposure to different concentrations of betulin, the mortality rate of $DmGABR^{R122T}$ *Drosophila*
440 was significantly greater than that of $DmGABR^{WT}$ *Drosophila* (Fig. 7E and Table S9). Similarly,
441 a previous study indicated that the R122G amino acid site substitution, generated via RNA
442 editing, affects the sensitivity of *Drosophila* to fipronil.[45] Together, these findings suggest that
443 betulin binds specifically to MpGABR via THR228, acting as an inhibitor of MpGABR and
444 causing aphid death.

445 GABRs belong to the cysteine loop (Cys)-loop superfamily of neurotransmitter receptors
446 and are essential heteropentameric ligand-gated ion channels in the central nervous system.
447 After GABA binds to the extracellular Cys-loop of a GABR,[46] the GABR is activated and opens
448 up its central pore to allow chloride ions to pass through, thereby hyperpolarizing neurons
449 and attenuating excitatory neurotransmission.[47] In insects, GABRs play an important role in
450 circadian rhythms,[48] sleep,[49] movement,[50] olfactory memory,[51] and so on. Additionally, GABR
451 is a critical target for a variety of insecticides and ectoparasiticides. Insecticides targeting
452 GABRs are categorized into noncompetitive antagonists (NCAs) and competitive antagonists
453 (CAs) according to their different binding sites. NCAs block chloride channels in nerve cells
454 by interacting with amino acid residues of the GABA-gated chloride channel, causing a
455 conformational change in the receptor, which interferes with the normal function of the
456 central nervous system and ultimately leads to insect death.[19, 52] A series of first- and

457 second-generation NCA insecticides have been successfully developed. Picrotoxinin, isolated
458 from *Anamirta cocculus* fruit, is the oldest natural NCA and is toxic to houseflies.[53] Among the
459 first-generation NCAs, polychlorocycloalkanes, including dieldrin and lindane, were
460 commonly used as pesticides in the middle and late 20th centuries.[54, 55] As second-generation
461 NCAs, fipronil and its derivatives are commercially available phenylpyrazole insecticides
462 that have been widely used for agricultural pest control.[56, 57] Additionally, CAs bind to
463 orthogonal binding sites, such as the GABA recognition site in the extracellular region,
464 competitively inhibiting the binding of GABA to its receptor, leading to toxic effects in insects.
465 Given the lack of interference from existing insect resistance mechanisms, CAs hold promise
466 for the development of efficient new insecticides. Novel 1,
467 6-dihydro-6-iminopyridazine-derived insecticides, as CAs, have insecticidal properties
468 against common cutworms and houseflies.[58] Besides, nootkatone, a sesquiterpenoid, also acts
469 as a CA to induce insect mortality.[59] Fluralaner, an isooxazoline ectoparasiticide, inhibits both
470 parasites and *Aedes aegypti* by acting as a CA.[60,61] Our results revealed that the binding site
471 (THR228) for betulin in MpGABR was located in the extracellular neurotransmitter-gated
472 ion-channel ligand-binding domain (Fig. S2), implying that betulin acts as a CA of MpGABR.
473 Our work may have provided a novel target for developing insecticides. In this study, betulin,
474 on the one hand, inhibited the expression of *MpGABR* and, on the other hand, specifically
475 bound to MpGABR through THR228, acting as an inhibitor of MpGABR and causing aphid
476 death (Fig. 8).

477 **5. CONCLUSION**

478 Betulin, a key metabolite in the aphid-resistant wild peach *P. davidiana*, possesses potent

479 aphicidal effects on *M. persicae.* This study confirmed that betulin exhibited excellent control
480 efficacy against *M. persicae* in both greenhouse and field experiments. RNA-seq, qRT–PCR
481 and western blotting assays revealed that betulin significantly inhibited the expression of
482 *MpGABR* in aphids. In addition, RNAi-mediated silencing of *MpGABR* markedly increased
483 the sensitivity of aphids to betulin. Moreover, MST and voltage-clamp-based
484 electrophysiological recording assays indicated that betulin was able to bind to MpGABR (K_d
485 = 2.24 μM) and acted as an inhibitor (EC_{50} = 20.66 μM) of MpGABR. Molecular docking
486 analyses suggested that the amino acid residue THR228, which is highly conserved in
487 Hemiptera, might be a critical specific binding site for betulin in MpGABR. Mutagenesis and
488 genome editing assays revealed that betulin bound specifically to this amino acid residue in
489 aphids but not in *Drosophila*, resulting in aphid death. Collectively, the results suggest that the
490 aphicidal effects of betulin on aphids occur in a two-pronged manner: on the one hand,
491 betulin inhibits *MpGABR* expression; on the other hand, it specifically binds to MpGABR via
492 THR228 and acts as an inhibitor of MpGABR. Elucidating the insecticidal mechanism of
493 betulin against aphids will provide a basis for the development of novel insecticides and
494 sustainable strategies for aphid control.

495 **ASSOCIATED CONTENT**

496 **Supporting Information**

497 The Supporting Information are available free of charge on the ACS Publications website.
498 Protein expression of MpGABR after RNAi (Figure S1); Betulin binding site on the domain of
499 MpGABR (Figure S2); LC_{50} values of betulin and pymetrozine against *M. persicae* at 48 h
500 (Table S1); Primers used for cloning, RNAi, heterologous expression, and qRT-PCR (Table S2);

501 Oligonucleotide sequences for PCR, the sgRNA synthesis template and the fragment of donor
502 DNA for homology-directed repair (HDR) (Table S3); Screening of genes significantly
503 differentially expressed in *M. persicae* in response to betulin at 48 h post treatment (Table S4);
504 Complete sequence information for the $GABA_A$ receptor gene of *M. persicae* (Table S5);
505 Sequences and relevant information for phylogenetic analysis of $GABA_A$ receptor (Table S6);
506 Binding energy and nonbonding interactions between betulin and $GABA_A$ receptor (Table S7);
507 Parameters of dose-response curves from microscale thermophoresis experiments (Table S8);
508 LD_{50} values of betulin and pymetrozine against *D. melanogaster* at 72 h (Table S9).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GABA, Gamma-aminobutyric acid; GABR, $GABA_A$ receptor; GABRB, $GABA_A$ receptor β

545 subunit; GABRAP, $GABA_A$ receptor associated protein; MST, microscale thermophoresis;
546 PCA, principal coordinates analysis; DEGs, differentially expressed genes; GO, Gene
547 Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; K_d, dissociation constant; RDL,
548 resistance to dieldrin; NCA, noncompetitive antagonists; CA, competitive antagonists.

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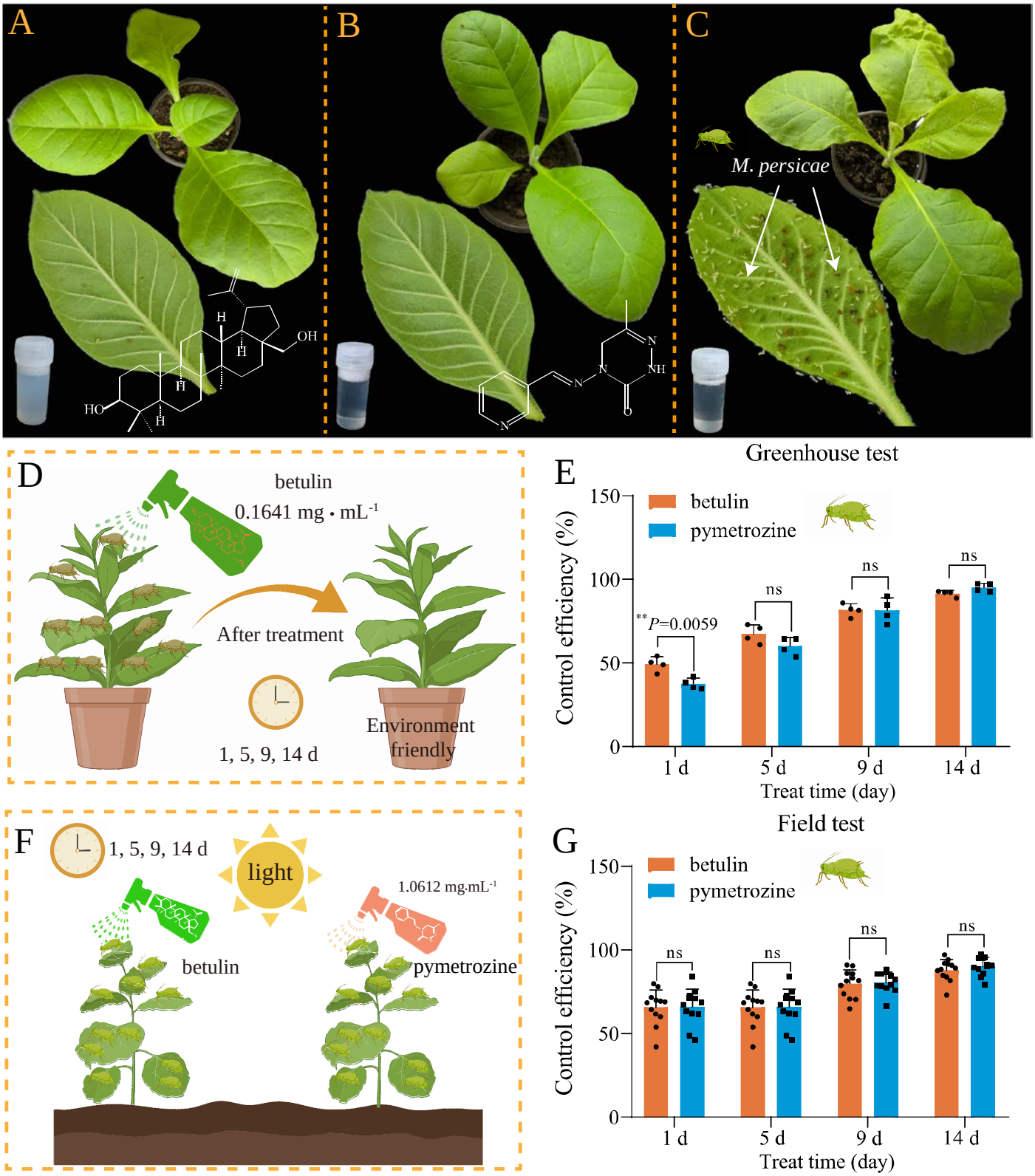


Figure 1. The aphicidal activity of plant-derived derivative betulin against *M. persicae*. (A-G) Representative images of the control effects of betulin (A, 0.1641 mg·mL^{-1}, 48 h LC_{50} value of betulin), pymetrozine (B, 1.0612 mg·mL^{-1}, a positive control), and CK (C, a negative control) against *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.

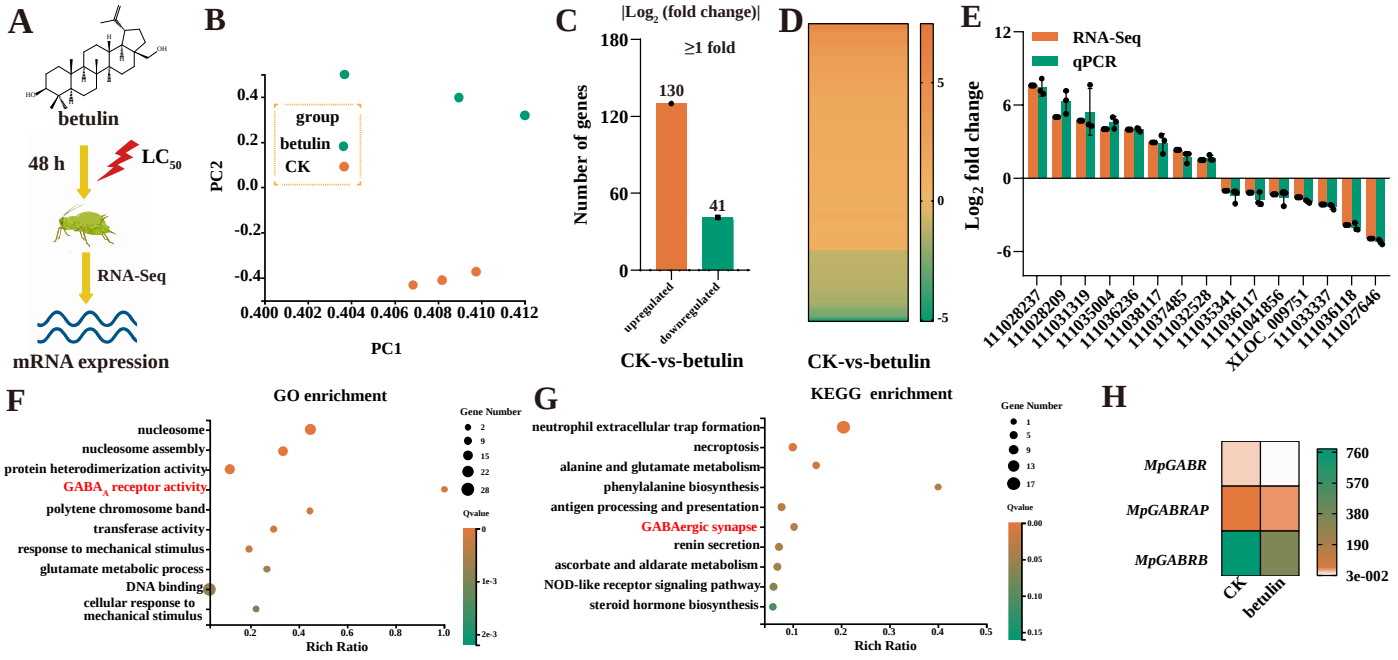


Figure 2. RNA-Seq analysis revealed candidate targets for betulin against *M. persicae*. (A) Diagram of the candidate targets for betulin against aphids as determined by RNA-Seq. Water containing 0.1% (v/v) Tween-80 and 3% (v/v) acetone was used as the control treatment (CK). (B) Principal coordinates 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.

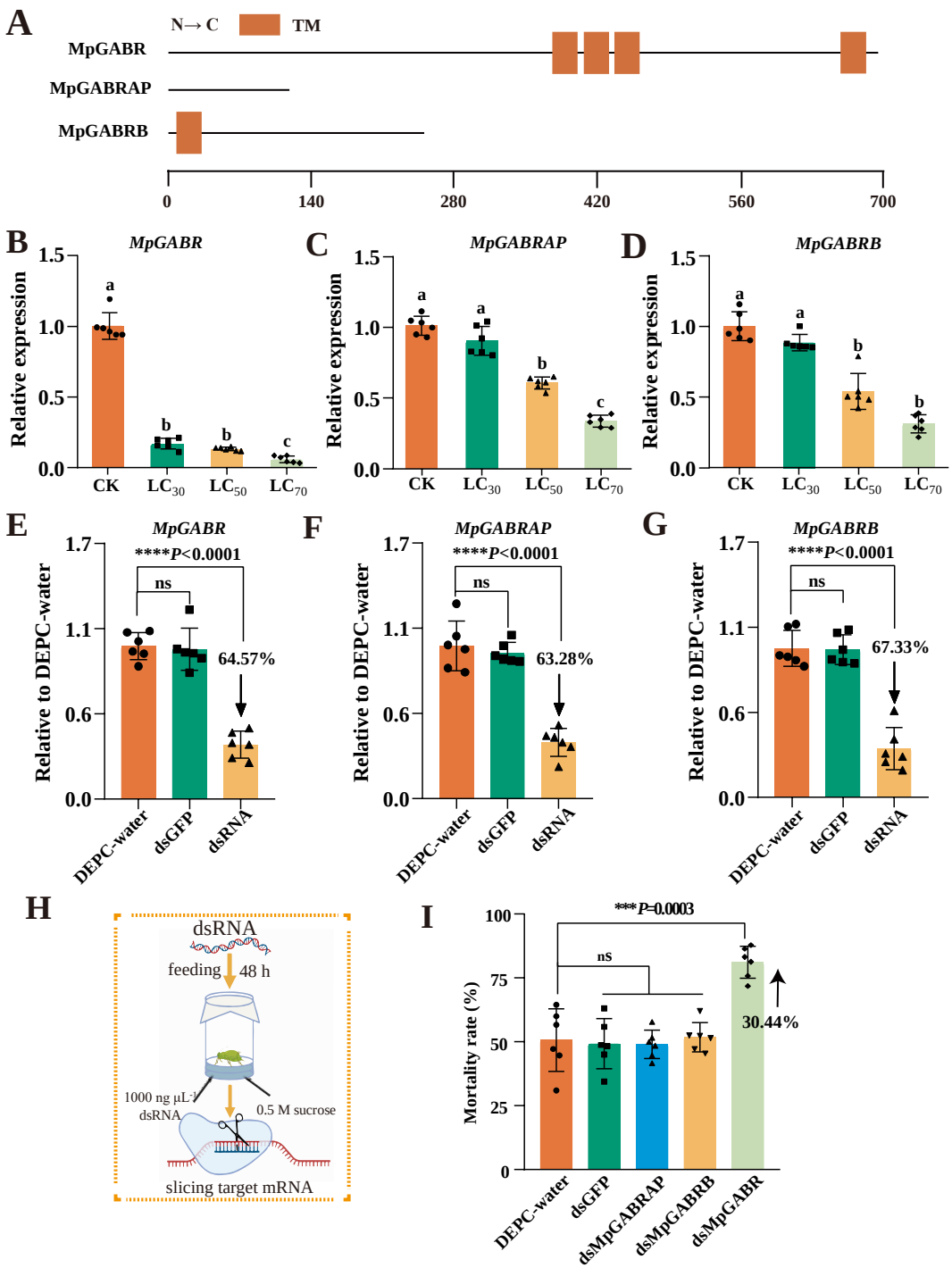
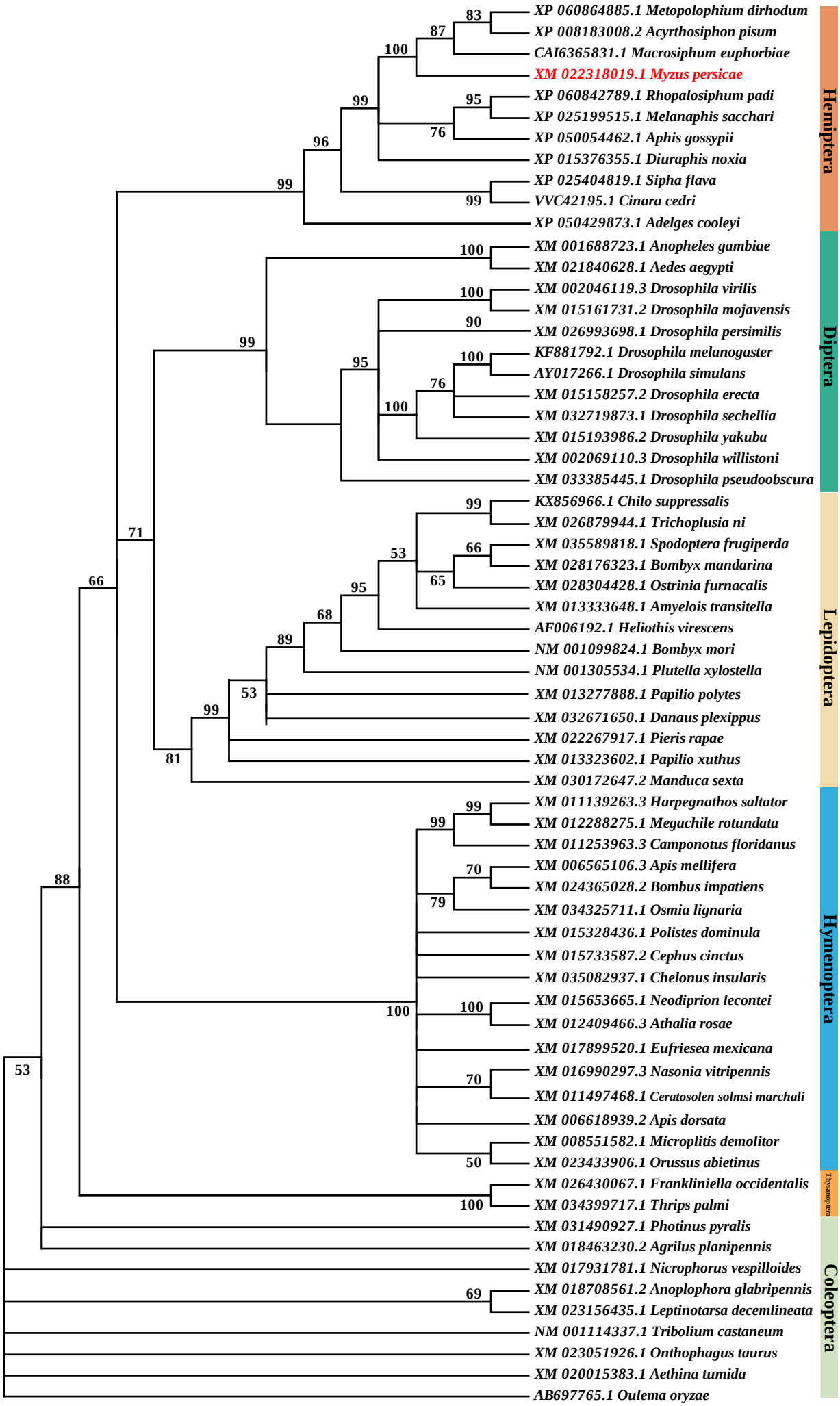


Figure 3. *MpGABR* expression in aphids was significantly inhibited by treatment with betulin. (A) Schematic drawing of MpGABR, MpGABRAP and MpGABRB. TM, transmembrane helices. (B-D) qPCR expression analysis of *MpGABR* (B), *MpGABRAP* (C) and *MpGABRB* (D) transcripts in *M. persicae* exposed to LC_{30}, LC_{50}, and LC_{70} of betulin for 48 h. The bars represent the average (± SD). Different letters above the error bars indicate significant difference (ANOVA, Tukey's test, $P < 0.05$). (E-G) qPCR expression analysis of *MpGABR* (E), *MpGABRAP* (F) and *MpGABRB* (G) after RNAi at 48 h post-dsRNA feeding relative to the expression levels after DEPC-water treatment. (H) Schematic drawing of the RNAi assay in *M. persicae*. (I) 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.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XP 050054462.1 Aphis gossypii
XP 015376355.1 Diuraphis noxia
XP 025404819.1 Sipha flava
VVC42195.1 Cinara cedri
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XM 001688723.1 Anopheles gambiae
XM 021840628.1 Aedes aegypti
XM 002046119.3 Drosophila virilis
XM 015161731.2 Drosophila mojavensis
XM 026993698.1 Drosophila persimilis
KF881792.1 Drosophila melanogaster
AY017266.1 Drosophila simulans
XM 015158257.2 Drosophila erecta
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XM 015193986.2 Drosophila yakuba
XM 002069110.3 Drosophila willistoni
XM 033385445.1 Drosophila pseudoobscura
KX856966.1 Chilo suppressalis
XM 026879944.1 Trichoplusia ni
XM 035589818.1 Spodoptera frugiperda
XM 028176323.1 Bombyx mandarina
XM 028304428.1 Ostrinia furnacalis
XM 013333648.1 Amyelois transitella
AF006192.1 Heliothis virescens
NM 001099824.1 Bombyx mori
NM 001305534.1 Plutella xylostella
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XM 012288275.1 Megachile rotundata
XM 011253963.3 Camponotus floridanus
XM 006565106.3 Apis mellifera
XM 024365028.2 Bombus impatiens
XM 034325711.1 Osmia lignaria
XM 015328436.1 Polistes dominula
XM 015733587.2 Cephus cinctus
XM 035082937.1 Chelonus insularis
XM 015653665.1 Neodiprion lecontei
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XM 017899520.1 Eufriesea mexicana
XM 016990297.3 Nasonia vitripennis
XM 011497468.1 Ceratosolen solmsi marchali
XM 006618939.2 Apis dorsata
XM 008551582.1 Microplitis demolitor
XM 023433906.1 Orussus abietinus
XM 026430067.1 Frankliniella occidentalis
XM 034399717.1 Thrips palmi
XM 031490927.1 Photinus pyralis
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XM 023156435.1 Leptinotarsa decemlineata
NM 001114337.1 Tribolium castaneum
XM 023051926.1 Onthophagus taurus
XM 020015383.1 Aethina tumida
AB697765.1 Oulema oryzae
Hemiptera
Diptera
Lepidoptera
Hymenoptera
Thysanoptera
Coleoptera

Figure 4. Phylogenetic analysis of GABR in insects. Maximum likelihood based phylogenetic tree of GABR from six orders: Hemiptera, Diptera, Lepidoptera, Thysanoptera, Hymenoptera, and Coleoptera. Protein sequence alignment was performed using MUSCLE in MEGA X. The sequences used for constructing the tree were listed in Table S6.

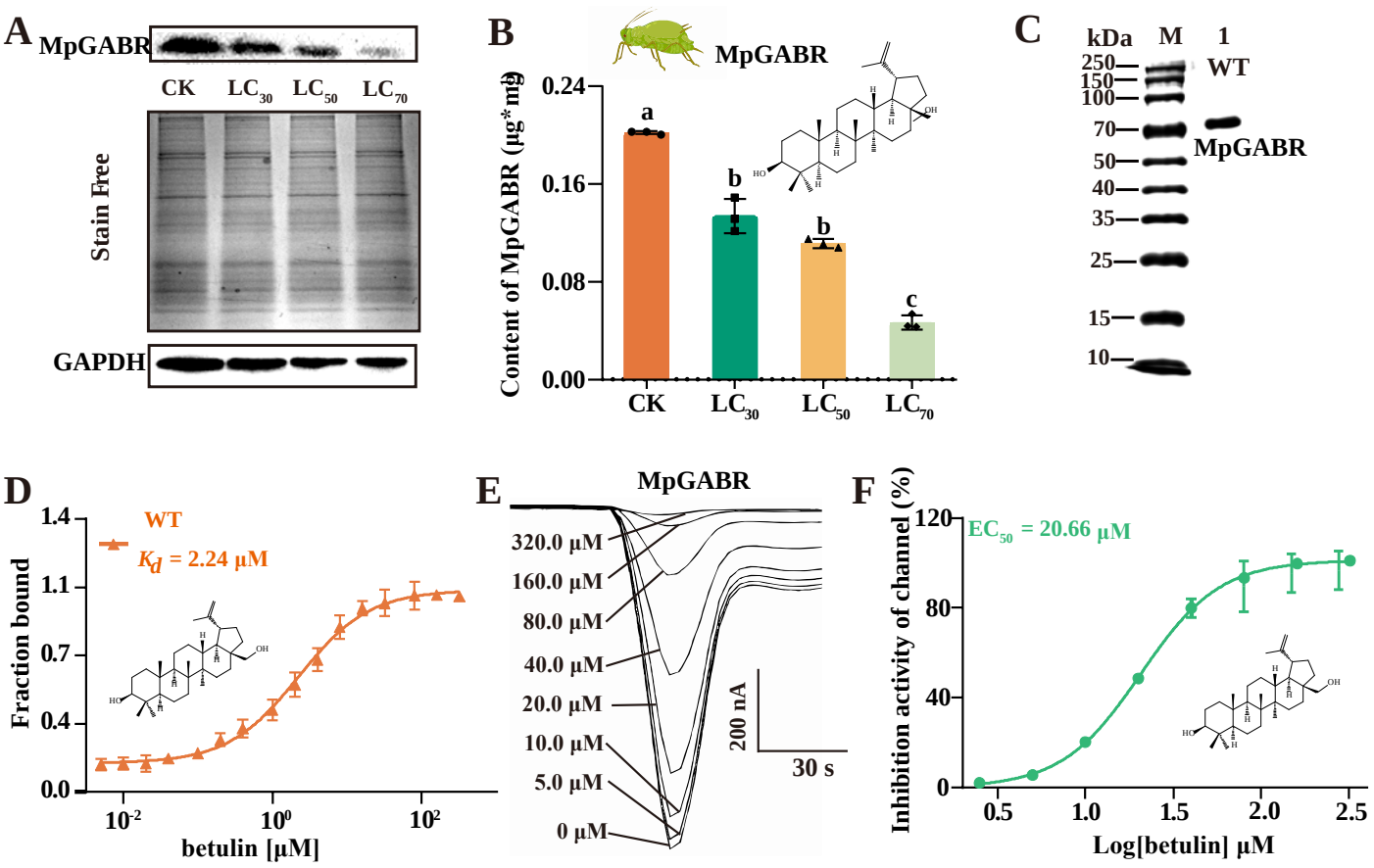

Figure 5. The interaction of betulin with MpGABR protein. (A, B) Western blotting analysis of MpGABR protein after betulin treatment for 48 h at three different concentrations (LC_{30}, LC_{50} and LC_{70}). The bars represent the average (± SD). Different letters above the error bars indicate significant difference (ANOVA, Tukey's test, $P < 0.05$). (C) Expression of recombinant wild type-aphid MpGABR (1, WT) protein by *Escherichia coli*. (D) Quantification of the binding affinity of betulin with wild type-aphid MpGABR using MST. Current responses (E) and inhibition activity (F) of MpGABR induced by different concentration of betulin in the presence of GABA. 0 μM MpGABR indicates the presence of only GABA.

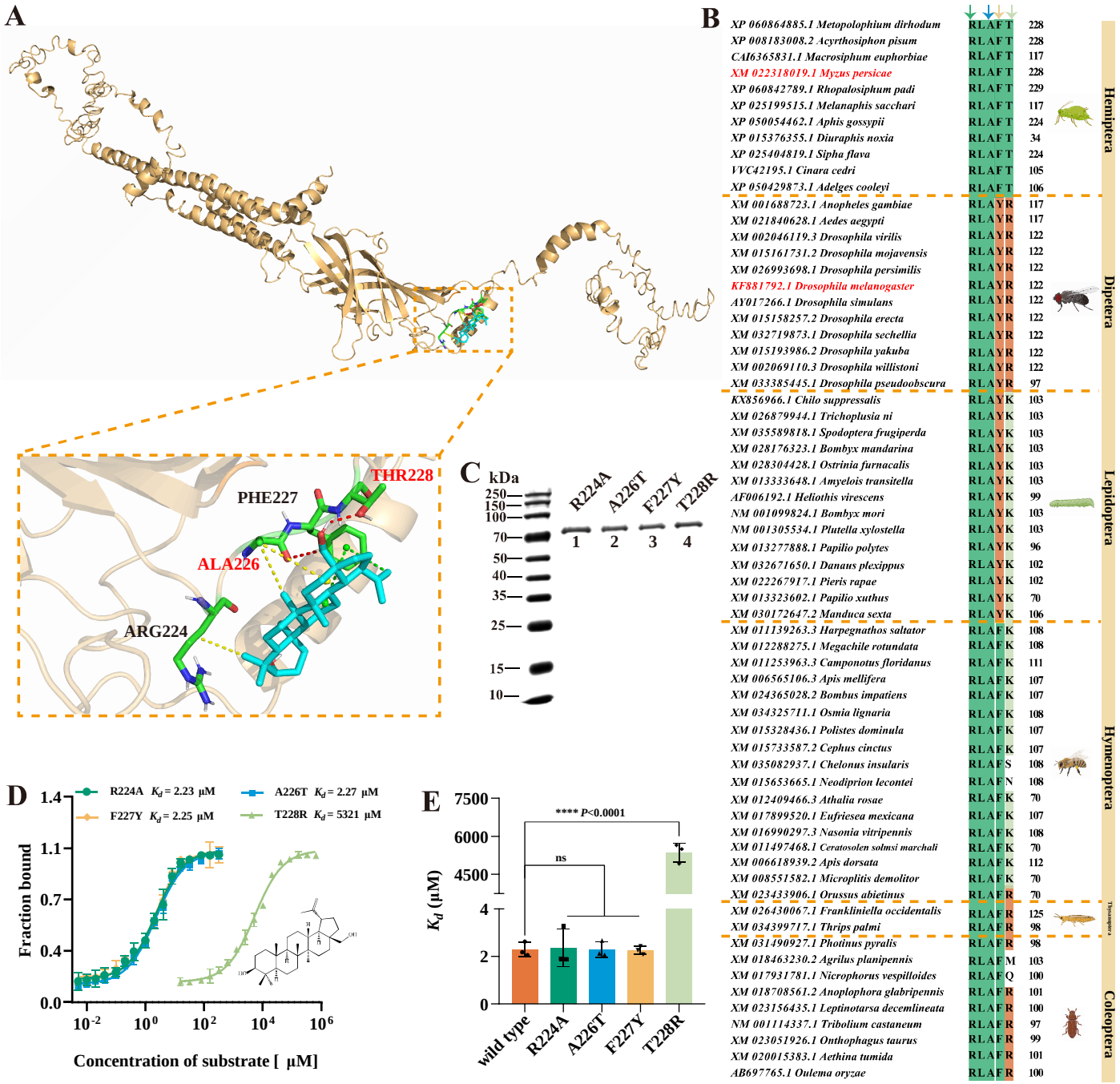

Figure 6. Molecular docking, binding site and inhibitory effect of betulin on MpGABR. (A) Best conformations of betulin docked to the binding pocket of MpGABR in aphids. An enlarged view of the betulin binding sites in MpGABR is indicated by a dashed frame. Potential Pi–alkyl (green), Alkyl (yellow), and hydrogen bond (red) interactions are indicated by dashed lines. (B) Sequence alignment of the key amino acids bound to betulin. The conserved residues among the different species in GABR are shown in orange, cyan, and light green. (C) Expression of the recombinant mutation type-aphid MpGABR by *Escherichia coli*. Lane 1: R244A, Lane 2: A226T, Lane 3: F227Y, Lane 4: T228R. (D, E) Quantification of the binding affinity of betulin with wild-type and mutant type-aphid MpGABR using MST. The bars represent the average (± SD) values. * indicates a significant difference (Student's *t* test, ****$P < 0.0001$).

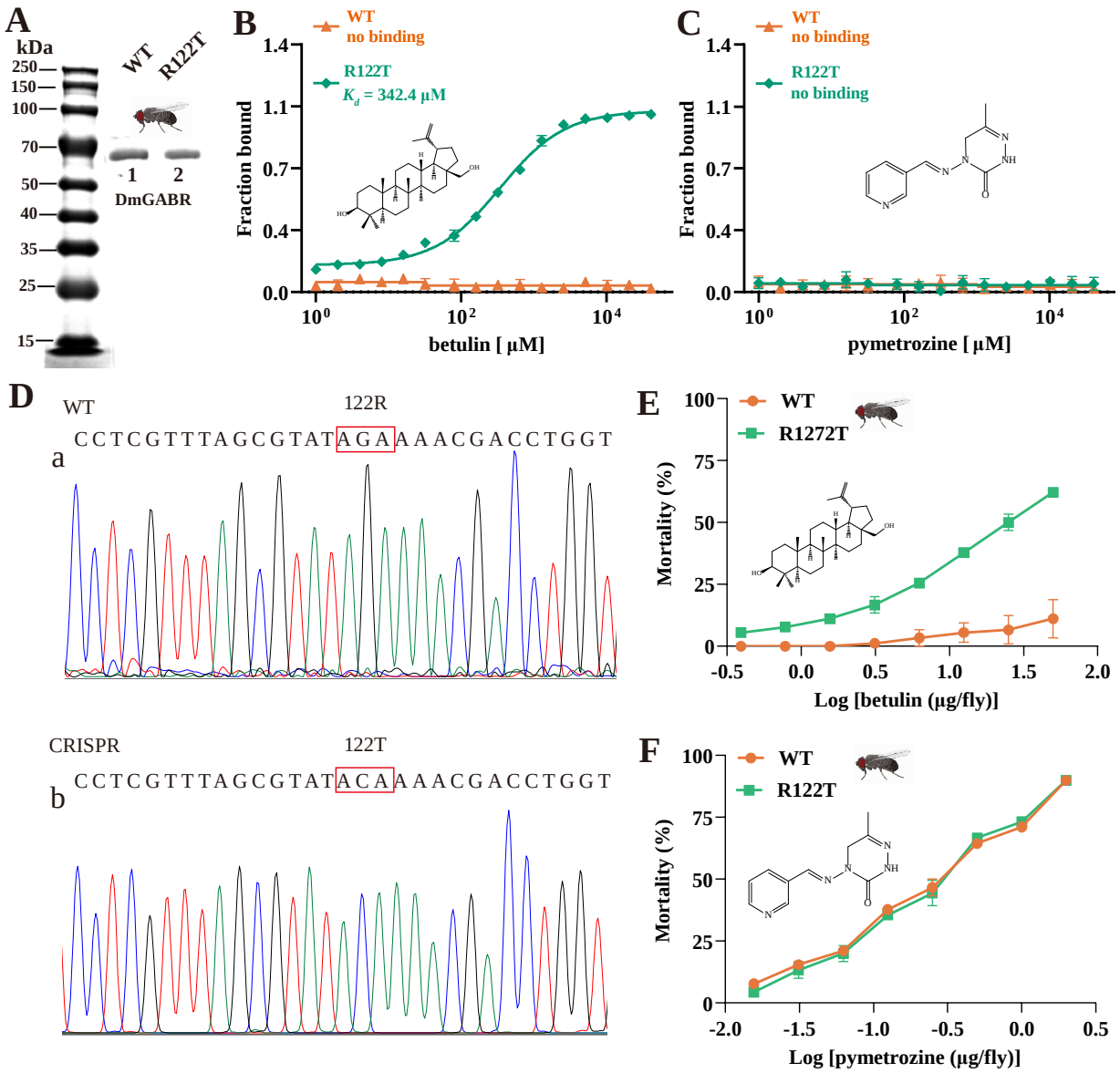

Figure 7. Gene editing in *Drosophila* validated the species-specific binding site of betulin. (A) Expression of the recombinant wild type (WT) and mutation type (R122T) of DmGABR (*Drosophila melanogaster*) by *E. coli*. Lane 1: wild type, Lane 2: R122T. (B, C) Quantification of the binding affinity 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*.

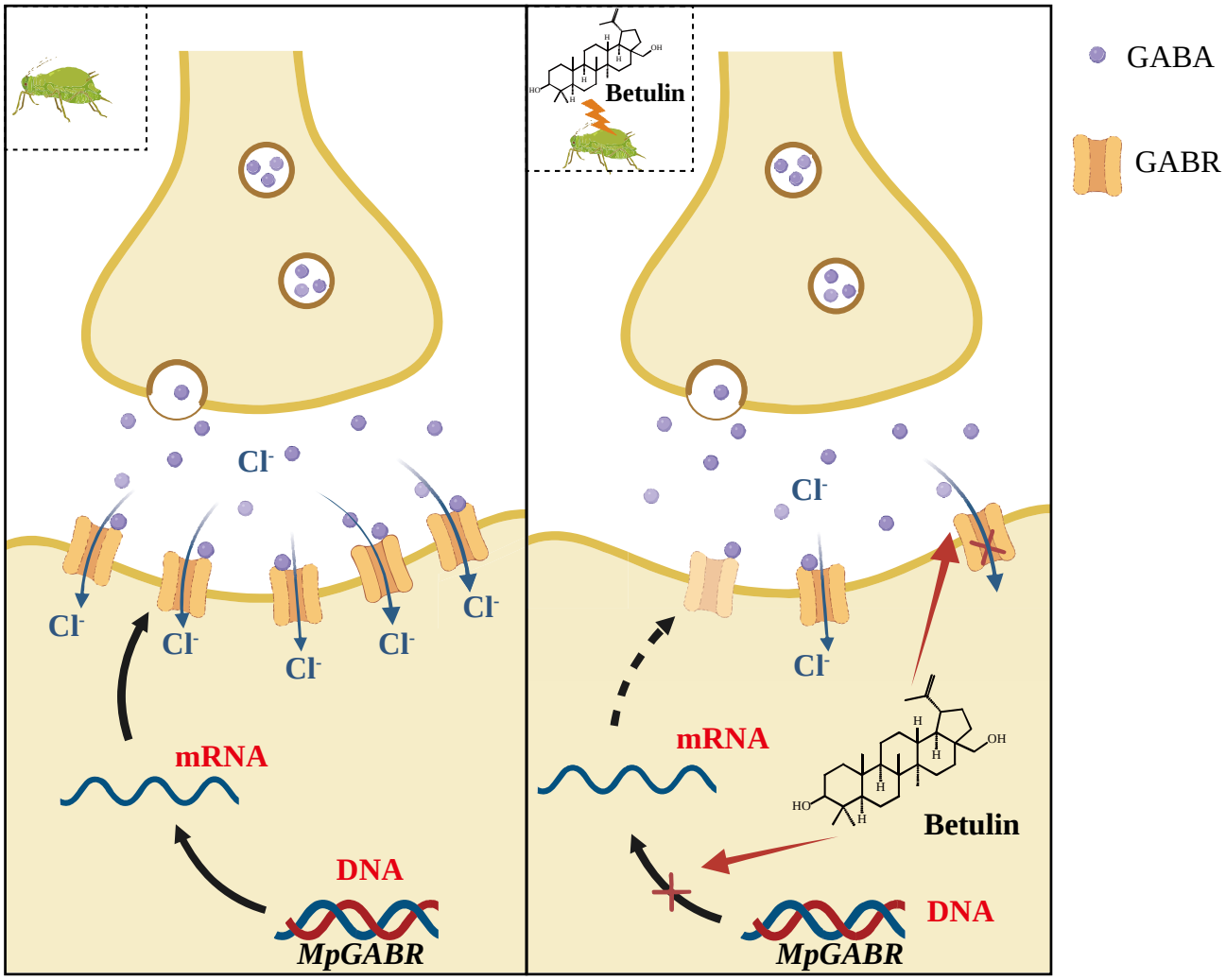

Figure 8. Proposed model for the mechanism of action against *Myzus persicae* by targeting $GABA_A$ receptors (GABR). After exposure to betulin, the expression of *MpGABR* was inhibited the level of MpGABR protein is decreased, resulting in a decrease in the channel of chloride ion influx. Besides, betulin directly and specifically binds to the amino acid residue THR228 of MpGABR, thereby disabling it.