1 **Distinct roles of two RDL GABA-receptors in fipronil action in the** 2 **diamondback moth (*Plutella xylostella*)**

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14 **Abstract**

15 The phenylpyrazole insecticide, fipronil, blocks insect RDL γ -aminobutyric
16 acid (GABA) receptors, thereby impairs inhibitory neurotransmission. Some insect
17 species, such as the diamondback moth (*Plutella xylostella*), possess more than one
18 *Rdl* gene. The involvement of multiple *Rdls* in fipronil toxicity and resistance remain
19 largely unknown. In this study, we investigated the roles of two *Rdl* genes, *PxRdl1*
20 and *PxRdl2*, from *P. xylostella* in the action of fipronil. Expressed in *Xenopus*
21 oocytes, *PxRDL2* receptors were 40-times less sensitive to fipronil than *PxRDL1*.
22 *PxRDL2* receptors were also less sensitive to GABA compared to *PxRDL1*.
23 Knockout of the fipronil-sensitive *PxRdl1* gene reduced the potency of fipronil by 10
24 fold, whereas knockout of the fipronil-resistant *PxRdl2* gene enhanced the potency
25 of fipronil by 4.4 fold. Furthermore, in two fipronil-resistant diamondback moth
26 field populations, the expression of *PxRdl2* was elevated by 3.7-fold and 4.1-fold,
27 respectively compared to a susceptible strain, whereas the expression of *PxRdl1* was
28 comparable among the resistant and susceptible strains. Collectively, our results
29 indicate antagonistic effects of *PxRDL1* and *PxRDL2* on the fipronil action *in vivo*

and suggest enhanced expression of fipronil-resistant *PxRdl2* potentially a new mechanism of fipronil resistance in insects.

Key words: *Plutella xylostella*, Fipronil, RDL, CRISPR-Cas9.

Introduction

Insect ionotropic GABA receptors are the primary inhibitory neurotransmitter receptor that is widely expressed throughout the insect central nervous system (Sattelle, 1990). The first insect GABA receptor gene was cloned from dieldrin-resistant *Drosophila melanogaster* and designated as *Rdl* (Resistant to dieldrin) (ffrench-Constant *et al.*, 1991). RDL GABA receptors belong to the superfamily of pentameric ligand-gated ion channels (LGICs) and thus contain a long N-terminal extracellular domain and four transmembrane regions (TM1-TM4), the second of which (TM2) provides many of the residues that line an integral chloride channel (Casida and Durkin, 2015; Nakao, 2017; Rauh, 1990; Ozoe, 2013). Due to their importance in inhibitory transmission, RDL receptors are the target of several classes of highly effective insecticides, such as dieldrin, fipronil and fluralaner (Buckingham *et al.*, 2017; Casida and Durkin, 2015; ffrench-Constant *et al.*, 2000; Nakao, 2017). Dieldrin belongs to cyclodiene insecticides which are the first generation of noncompetitive antagonists (NCAs) against the RDL receptors (Kadous *et al.*, 1983; Rocheleau *et al.*, 1993), and fipronil is a second generation NCAs blocking RDL receptors (Gupta and Anadón, 2018; Hosie *et al.*, 1995; Zhao *et al.*, 2003).

While there is only a single *Rdl* gene in many insect species, such as *D. melanogaster*, *Musca domestica*, *Apis mellifera* and *Laodelphax striatella* (Eguchi *et al.*, 2006; Jones and Sattelle, 2006; Narusuye *et al.*, 2007; Rocheleau *et al.*, 1993), lepidopteran insects, such as *Plutella xylostella*, *Bombyx mori*, *Chilo suppressalis*, and arachnids, such as *Tetranychus urticae* and *Varroa destructor*, possess at least two *Rdl* genes (Dermauw *et al.*, 2017; Ménard *et al.*, 2018; Sheng *et al.*, 2018b; Yu *et al.*, 2010; Yuan *et al.*, 2010). When expressed in *Xenopus laevis* oocytes, *V. destructor* RDL1 was less sensitive to fipronil than RDL2 and RDL3 (Ménard *et al.*,

2018). *C. suppressalis* RDL1 was more sensitive to dieldrin compare to RDL2, but their sensitivity to fipronil was similar (Sheng *et al.*, 2018b). To data, three orthologous *Rdl* genes were found from *P. xylostella* (Yuan *et al.*, 2010; Zhou *et al.*, 2008). However, the importance of individual *Rdl* gene in fipronil action is largely unclear.

It had been reported widely that the alanine to serine or glycine mutation at 2' position (also known as A2'S or A2'G) and threonine to leucine mutation at 6' position (also known as T6'L) of RDL GABA receptor are associated with cyclodiene resistance in many insect species such as *D. melanogaster*, *M. domestica*, *Haematobia irritans*, *Blattella germanica* and *Rhipicephalus microplus* (ffrench-Constant *et al.*, 1993; Hansen *et al.*, 2005; Hope *et al.*, 2010; Navarro *et al.*, 2010; Thompson *et al.*, 1993). Cyclodiene-resistant strains carrying A2'S/G mutations exhibited a low level of cross-resistance to fipronil (Cole *et al.*, 1995; Gao *et al.*, 2007; Kristensen *et al.*, 2005; Nakao, 2017; Scott and Wen 1997). However, a different substitutions at this position, the alanine to asparagine mutation (A2'N) mutation, in RDL has been proved to profoundly decreased the antagonist activity of fipronil in *L. striatellus* and *Sogatia furcifera* (Nakao *et al.*, 2010; Nakao *et al.*, 2011, Sheng *et al.*, 2018a). The A2'S mutation in *PxRDL1* had been reported in *P. xylostella* field strains (Li *et al.*, 2006; Wang *et al.*, 2016; Yuan *et al.*, 2010). When the A2'S mutation was introduced into *PxRDL1* in an insecticide sensitive *P. xylostella* strain using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system, the A2'S mutation caused only about 3-fold increase in fipronil resistance (Guest *et al.*, 2019), indicating a limited role of this mutation in fipronil resistance and additional mechanisms underlying higher levels of fipronil resistance in field populations. Interestingly, the *PxRDL2* has a serine at the 2'position in both susceptible and resistant strains (Jouraku *et al.*, 2013; Shi *et al.*, 2015; Tang *et al.*, 2014; Yuan *et al.*, 2010). Whether *PxRDL2* is involved in fipronil action and resistance, however, remain unknown.

In this study, we evaluated the role of *PxRDLs* in fipronil action and resistance in *P. xylostella* which is one of the most destructive cosmopolitan pests, and has been

reported from over 80 countries, and feeds on brassica crops worldwide (Mohan and Gujar, 2003; Sarfraz *et al.*, 2005; Zhou *et al.*, 2011). Specifically, we compared the transcript of *PxRdl1* and *PxRdl2* between susceptible and resistant populations; and evaluated the sensitivity of *PxRDL1* and *PxRDL2* to fipronil in *Xenopus* oocytes. Furthermore, we used the CRISPR-Cas9 technology to knockout *PxRdl1* and *PxRdl2* and evaluated the susceptibility of the resultant mutants to fipronil. Our study revealed distinct roles of *PxRDL1* and *PxRDL2* in fipronil action and resistance, and provide valuable information for better understanding the mode of action of fipronil and mechanisms of fipronil resistance.

Materials and methods

Insects and chemicals

The susceptible *P. xylostella* strain (He *et al.*, 2012) was kindly provided by Dr. Minsheng You (Fujian Agriculture and Forestry University, China). Two field populations were collected from Guangzhou (GZ) city (23.24° N, 113.18° E) of Guangdong province and Fuzhou (FZ) city (26.17° N, 118.51° E) of Fujian province in 2017. The pupae were collected from fields and adults were fed on 10% (V/V) honey solution to lay eggs. The third-instar larvae of the F₁ strain were subjected to bioassay and total RNA extraction. All populations were maintained separately at 25 ± 1 °C, 60-70% relative humidity and under a 16:8 h (light: dark) photoperiod.

In addition to special instructions, all chemicals used in this research were purchased from Sigma-Aldrich (Shanghai, China), and the mature female African clawed frogs (*X. laevis*) were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China).

Bioassay

The toxicity of fipronil to *P. xylostella* was tested in leaf-dip bioassays, according to the recommended method of the Insecticide Resistance Action Committee (<https://www.irac-online.org>). The fipronil was dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled water containing 0.5% Tween-80 to generate five

120 serial dilutions. Leaves of fresh cabbage (*Brassica oleracea*, 2-4 leaf stage) were cut
121 and dipped in insecticide solution for 10 seconds with gentle agitation and placed to
122 surface-dry on paper towel. Control leaves were dipped in distilled water containing
123 0.5% tween-80 only. 10 third-instar larvae were exposed to the insecticides at each
124 concentration, which was repeated 4 times. Mortality was assessed after 48 h.

125

126 *Cloning and sequence of PxRdls*

127 Total RNA was isolated from single third-instar larva of three strains (total 30
128 larvae of each strain) using the DNA/RNA/Protein Isolation Kit (TianGen, Beijing,
129 China). The first-strand cDNA was synthesized with 1 µg of total RNA using a
130 PrimeScript™ 1st Strand cDNA Synthesis Kit with gDNA Eraser (TaKaRa
131 Biotechnology, Dalian, China). For sequencing analysis of the *PxRdls*, the primers
132 used to amplify the full-length open reading frame (ORF) of *PxRdl1* (GenBank No.
133 NM_001305534.1) and *PxRdl2* (GenBank No. NM_001305535.1) are listed in Table
134 S1. The PCR products of the expected size were purified using the Easy Pure® Quick
135 Gel Extraction Kit (Transgen Biotech, Beijing, China) and cloned into TA Vector
136 (Takara Biotechnology, Dalian, China). Positive clones were selected and sequenced
137 by Beijing Genomics Institute (Beijing, China). DNA alignments were performed by
138 DNAMAN V9 (Lynnon Biosoft, CA, USA).

139

140 *Expression analysis of PxRdls mRNA*

141 To monitor the transcript level of *PxRdls*, Total RNA was extracted from 5-6
142 third-instar larvae and repeated 3 times. qRT-PCR was carried out using the SYBR®
143 Premix Ex Taq™ II (Takara Biotechnology, Dalian, China) and CFX96 Connect
144 Real-Time system (Bio-RAD, USA). The qRT-PCR program was 95 °C for 2 min,
145 followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. Melting
146 curves were prepared by increasing the temperature from 60 to 95°C to check the
147 specificity of the primers when the cycling reaction finished. qRT-PCR data was
148 normalized to an internal control, the *Actin* gene (GenBank No. JN410820.1), and
149 analyzed using the $2^{-\Delta\Delta CT}$ method.

150

151 *cRNA preparation and X. laevis oocytes injection*

152 The ORFs encoding *PxRdl*s were cloned into a pT7TS vector using the In-Fusion
153 HD Cloning Kits (Takara Biotechnology, Dalian, China) and, finally validated by
154 sequencing (Fig. 1). The linearized recombinant plasmid was used as templates to
155 synthesize capped RNAs (cRNAs) with the mMessage mMachine T7 Kit (Life
156 Technologies, Carlsbad, CA). The cRNAs were dissolved in RNase-free water at a
157 concentration of 1000 ng/μL, and finally stored at -80°C.

158 Mature female frogs were anesthetized in 1 g/L ethyl 3-aminobenzoate
159 methanesulfonate for 15-20 min. *X. laevis* oocytes at stage IV and V were harvested
160 and rinsed with Ca²⁺-free SOS solution (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5
161 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6), and then
162 treated with 1 mg/ mL type I collagenase in Ca²⁺ free SOS solution. After 1-2 h of
163 incubation at 16°C, the oocytes were gently washed with SOS solution (100 mM NaCl,
164 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.6) containing 2.5 mM
165 sodium pyruvate, 50 mg/ml gentamycin 50 mg/ ml, 100 U/ml penicillin and 100
166 mg/ml streptomycin, and then incubated in the same solution at 16°C overnight. The
167 oocytes were injected with 4.6 ng of cRNA. The injected oocytes were incubated in
168 SOS solution at 16°C for electrophysiological experiments.

169

170 *Two-electrode voltage-clamp (TEVC) recordings*

171 Oocytes 2-4 days post-injection were kept in SOS solution and clamped at -80
172 mV. The currents were recorded by the two electrode voltage-clamp (TEVC) method
173 using an Oocyte Clamp OC-725C amplifier (Warner Instruments, Hamden, CT, USA)
174 with continuous perfusion at a rate of 3 mL/min. The recording pipettes were filled
175 with 3 M KCl, delivering an electrical resistance of 0.5-2 MΩ. GABA was dissolved
176 in SOS solution, and fipronil was dissolved in DMSO and then diluted with SOS
177 solution with a final DMSO concentration of less than 0.1% (v/v). GABA
178 concentration response curves were obtained by perfusing oocytes with GABA
179 concentrations from 1 μM to 1 mM. Responses to GABA at each concentration were

180 normalized to the maximum current induced by 1 mM GABA. Fipronil inhibition
181 curves were generated by inhibiting the response to GABA (EC_{50}). The oocytes were
182 first stimulated with GABA (EC_{50}) twice, followed by perfusing the fipronil alone
183 for 5 min, and finally GABA (EC_{50}) was co-applied with fipronil. Each experiment
184 was performed on 5-6 different oocytes obtained from at least two frogs.

185

186 *sgRNA preparation and eggs microinjection*

187 The single guide RNA (sgRNA) for editing *PxRdl1* and *PxRdl2* gene were
188 designed using CRISPR RGEN Tools (<http://www.rgenome.net/>). After the off-target
189 analysis, the sgRNA-*PxRdl1* target sequence (5' TTAGCGTATAAAAAAAGGCCAGG
190 3') was selected at exon 4 of *PxRdl1* (Fig. 5A), and the sgRNA-*PxRdl2* target
191 sequence (5'CCTGGCGCTGCCGTCATCCTGGC 3') was selected at exon 1 of
192 *PxRdl2* (Fig. 6A). The sgRNAs were prepared following the instruction (Guide-it™
193 sgRNA *In Vitro* Transcription and Screening Systems, TaKaRa, Japan). Briefly,
194 SgRNA synthesis templates were amplified firstly, and the PCR product was run and
195 analyzed on 2% agarose gel to confirm the size of the PCR product is about 130bp.
196 Then combine 20 μ L *in vitro* transcription reaction solution contain 5 μ L of PCR
197 product, 7 μ L of Guide-it *In Vitro* Transcription Buffer, 3 μ L of Guide-it T7
198 Polymerase Mix and 5 μ L of RNase Free Water. The transcription reaction solution
199 was incubated at 37°C for 4 h to maximize sgRNA yield. Following incubation, 2 μ L
200 of DNase I was added and incubated at 37°C for 15 min. Finally the sgRNA was
201 purified with Guide-it IVT RNA Clean-Up Kit (TaKaRa, Japan) and stored at -80°C
202 for injection step.

203 Microinjection of Cas9 protein and sgRNA was conducted as the method
204 described by Huang *et al.*, (2016). In brief, susceptible strains of female *P. xylostella*
205 were allowed to lay eggs on parafilm sheets treated previously with cabbage juice.
206 The fresh eggs were collected within 30 min and microinjected immediately with the
207 mixture of 300 ng/ μ L Cas9-N-NLS Nuclease (GenCrispr, China) and 150 ng/ μ L
208 sgRNA. The Cas9 protein and sgRNA were mixed well and incubated at 37°C for 15
209 min before injection. Injection was carried out using an Eppendorf TransferMan® 4r

micromanipulator, Eppendorf FemtoJet[®] 4x programmable microinjector and Eppendorf Femtotip II injection capillaries (Eppendorf, Hamburg, Germany). The injection conditions were injection pressure (p_i) 600 hPa, compensation pressure (p_c) 80 hPa and injection time (t_i) 0.8s. Eggs after injection were incubated at $25 \pm 1^\circ\text{C}$, 60-70% relative humidity for hatching.

Identification of *PxRdl* mutants

About 500 fresh eggs were injected with the mixture of Cas9 and sgRNA. Finally, 43 and 37 of the eggs injected with sgRNA-*PxRdl1* or sgRNA-*PxRdl2* successfully developed to moths (named as G_0 progeny). Single-pair mating between G_0 and wild type adults were set up to generate G_1 progeny.

The G_0 progeny were collected after oviposition, and the genomic DNA was extracted individually using TIANamp Genomic DNA Kit (TianGen, Beijing, China). The detection primers (Table S1) were designed to amplify the target region flanking the desired cleavage site. The PCR product was directly sequenced to identify the detailed indels of genomic DNA. qRT-PCR was performed to measure the transcript level of *PxRdl*s in homozygous mutant strains.

Statistical analysis

The bioassay, qRT-PCR and electrophysiology data are presented as the means $\pm$ standard error (SE). For the bioassay, the LC_{50} of fipronil was obtained by a linear regression program using SPSS 22.0 (SPSS Inc., IL). For electrophysiology analyses, the EC_{50} and IC_{50} were obtained by a nonlinear regression program using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The mRNA expression significance was analyzed with one-way ANOVA followed by Tukey's test ($p < 0.01$) using SPSS 22.0 (SPSS Inc., IL). The significance of LC_{50} , EC_{50} and IC_{50} were analyzed with Student's *t*-test ($\alpha=0.05$) using Stata v. 14 (StataCorp LLC, College Station, TX, USA).

Results

Resistance to Fipronil

Fipronil toxicity analysis was performed on the two field (GZ and FZ) F₁ strains and the susceptible strain by leaf-dip bioassays. The two field strains exhibited high levels of fipronil resistance, with resistance ratios that were 1686-fold for the GZ populations and 953-folds for FZ populations relative to the susceptible strain (Table 1).

Mutation screening of PxRDLs in field resistant populations

Results from sequence analysis showed that, for PxRDL1, compared with the amino acid sequence from the susceptible strain, A2'S mutation was present in the GZ population with a frequency of 73.33%, while the frequency was 33.33% in the FZ population. Furthermore, another mutation, T6'M, was detected in the GZ population with a lower frequency of 23.33% and higher frequency of 56.67% in the FZ population (Fig.1 and Table 2). Individuals carrying A2'S and T6'M double mutations in PxRDL1 were not found in any of the populations examined. There were only few mutations with low frequency (3.33%-18.33%) been detected in the N-terminal extracellular domain and TM3-TM4 loop of PxRDL2 (Table S2).

Functional and pharmacological characterization of PxRDLs in Xenopus oocytes

To compare the sensitivity of the PxRDLs to fipronil, PxRDLs were expressed in *Xenopus* oocytes, and the potencies of fipronil as an antagonist and GABA as an agonist were evaluated under the TEVC condition. The EC₅₀ value of GABA in activating currents in oocytes expressing PxRDL2 was 153.40 μM, which was ≈ 7-fold higher than that in oocytes expressing PxRDL1 (Fig. 3; Table 3). The IC₅₀ for fipronil in oocytes expressing PxRDL2 was much higher than that in oocytes expressing PxRDL1, with an IC₅₀ value of 5.23 μM (Fig. 4; Table 3).

Over-expression of PxRDL2 transcript in the resistant strains

qRT-PCR was performed to compare the expression of the *PxRdls* genes among

the susceptible and two resistant strains. The results showed that the transcript level of *PxRdl1* was 6.92-fold higher than that of *PxRdl2* in the susceptible strain (Fig. 2A). There was no significant difference in the transcript level of the *PxRdl1* among the three strains. However, the transcript level of *PxRdl2* was 4.11-fold higher in the GZ strain and 3.70-fold higher in the FZ strain compared to the susceptible strain (Fig. 2A).

Construction of homozygous *PxRdl* mutant *P. xylostella* strains

To further evaluate the role of *PxRDL2* in the action of fipronil, we used CRISPR-Cas9 to construct KO-*PxRdl1* and KO-*PxRdl2* strains, respectively. G_1 progeny of *PxRdl1* mutants were obtained and multiple deletion types were found in target sequence, such as 4-nt deletion (Fig. 5B) and 2, 3, 12, 13-nt deletion (data not shown). There were also multiple deletion types, such as 1, 6, 18-nt deletion (data not shown) and a 52-nt deletion near the target sequence been detected in the G_1 progeny of *PxRdl2* mutants (Fig. 6B).

The 4-nt deletion of *PxRdl1* (7 pairs) and 52-nt of *PxRdl2* (9 pairs) heterozygous mutant G_1 adults were set up single-pair mating to obtain G_2 progeny. 62 pairs of 4-nt deletion of *PxRdl1* and 70 pairs of 52-nt deletion of *PxRdl2* adults were set up single-pair mating to obtain G_3 progeny, respectively. Then the single-pair mating was performed progeny by progeny, until the male and female adults were both homozygous mutant (Fig. S1). Genomic DNA sequencing of G_2 progeny adults confirmed that 5 pairs of 4-nt deletion of *PxRdl1* and 4 pairs of 52-nt deletion of *PxRdl2* adults were homozygous mutant. All adults of the G_3 progeny generated from homozygous mutant G_2 progeny were still set up single-pair mating and genomic DNA sequencing to confirm they were homozygous mutant. In conclusion, we successfully obtained a homozygous mutant strain (KO-*PxRdl1*) that has 4-nt deletion at exon 4 of *PxRdl1*, and a homozygous mutant strain (KO-*PxRdl2*) that has 52-nt deletion at exon 1 of *PxRdl2*. The 4-nt and 52-nt deletion both resulted in frame shift and premature stop codons (Fig. 5D and Fig. 6D).

299 *Impact of PxRDL disruption on fipronil toxicity*

300 qRT-PCR results showed that there was no significant difference in individual
301 *PxRdl* transcript level between wild-type and *PxRdl* knockout strains (Fig. 2B).
302 Bioassay results showed that compared to the wild-type strain, KO-*PxRdl1* larvae
303 exhibited 10.41-fold fipronil resistance; however, KO-*PxRdl2* larvae were 4.4-fold
304 more sensitive to fipronil (Table 4).

305

306 **Discussion**

307 In this study, we evaluated the roles of *PxRDL1* and *PxRDL2* in fipronil action
308 and resistance. Our functional expression of the *PxRDL* receptors in *Xenopus* oocytes
309 showed that the *PxRDL1* monomeric receptor is significantly more sensitive to
310 fipronil than the *PxRDL2* monomeric receptor. Our CRISPR-Cas9 experiments
311 showed that *PxRDL1* knockout insects are more resistant to fipronil, whereas
312 *PxRDL2* knockout insects are more sensitive to fipronil. Furthermore, besides
313 detection of two previously identified A2'S and T6'M mutations in *PxRDL1* from two
314 fipronil-resistant field strains, we discovered an elevated level of *PxRDL2* transcripts
315 (but not *PxRDL1* transcripts) in both strains. Our results suggest an antagonistic effect
316 of *PxRDL2* on the action of fipronil and elevated expression of *PxRDL2* as a potential
317 new mechanism of fipronil resistance.

318 Earlier studies reported that *P. xylostella* possess three orthologous *Rdl* genes.
319 *PxRDL1* and *PxRDL3* (GenBank No. EF156251) both have an amino acid alanine at
320 2' position, and *PxRDL2* has a 2' serine (Yuan *et al.*, 2010; Zhou *et al.*, 2008). We
321 failed to amplify *PxRDL3* by RT-PCR although we attempted using various PCR
322 primer pairs. Therefore, in this study we evaluated the sensitivity of *PxRDL1* and
323 *PxRDL2* to fipronil in *X. laevis* oocytes. *PxRDL1* and *PxRDL2* exhibited different
324 sensitivities to both fipronil and GABA (Figs. 3 and 4). *PxRDL2* was 40.23-fold less
325 sensitive to fipronil than *PxRDL1* (Fig. 4). *PxRDL2* is also less sensitive to GABA
326 compared to *PxRDL1* (Fig. 3). These results are consistent with those from a
327 functional analysis of RDLs from *V. destructor* (Ménard *et al.*, 2018). Like *PxRDL1*,
328 *V. destructor* RDL2 and RDL3 monomeric receptors have an amino acid alanine at 2'

position and are more sensitive to fipronil and GABA. Like *PxRDL2*, the *V. destructor* RDL1 monomeric receptor has 2' serine and exhibits a lower fipronil sensitivity and GABA affinity (Ménard *et al.*, 2018). However, *C. suppressalis* RDL1 and RDL2 which have 2' alanine and 2' serine, respectively, display a similar sensitivity to fipronil (Sheng *et al.*, 2018b). Nevertheless, *C. suppressalis* RDL1 was more sensitive to dieldrin than RDL2 (Sheng *et al.*, 2018b). These results suggest that additional unidentified sequence features contribute to the low sensitivity to fipronil of *V. destructor* RDL1 and *PxRDL2* receptors.

Wei *et al.*, (2015) knocked down the transcript level of the *L. striatellus Rdl* gene using RNA interference (RNAi), and found the effect of fipronil was significantly reduced. Similarly, Jia *et al.*, (2019) also found that knockdown the transcript level of *Rdl1* or *Rdl2* gene both significantly reduce the mortality of *C. suppressalis* treated with fluralaner (mainly acting on RDL GABAR). CRISPR-Cas9 technology has been successfully applied in insects to clarify the relationship between targets and insecticide action *in vivo* (Homem and Davies, 2018; Wang *et al.*, 2017), as it can block the gene function completely. For instance, Wang *et al.*, (2020) proved the *P. xylostella* nicotinic acetylcholine receptors (nAChRs) $\alpha 6$ gene is the main target of spinosyns, and knockout of this gene caused over 200 fold spinosyns resistance. In this study, our results revealed distinct roles of *PxRDLs* in the action and resistance of fipronil in *P. xylostella*. Knockout of *PxRdl1* gene conferred resistance to fipronil, and *PxRDL1* receptors are highly sensitive to fipronil, confirming that *PxRDL1* is a main target of fipronil. However, knockout of fipronil-resistant *PxRdl2* gene enhanced insect sensitivity to fipronil, revealing an antagonistic effect of *PxRDL2* on the action of fipronil in *P. xylostella*. Our qRT-PCR results showed that the transcript level of *PxRdl1* did not change in the *PxRdl2* knockout strain, and *vice versa* (Fig. 2B), suggesting no obvious genetic compensation at the transcriptional level. Furthermore, knockout of *PxRdl1* and *PxRdl2* didn't induce lethality. These results provide evidence for functional redundancy regarding the role of *PxRDL1* and *PxRDL2* in neuronal signaling, despite their distinct contributions to the action of fipronil.

The presence of multiple *Rdl* genes was thought to be the result of gene duplication or conversion events that occurred recently to enhance the tolerance to naturally occurring toxins or insecticides (Ménard *et al.*, 2018; Meng *et al.*, 2019; Sheng *et al.*, 2018b). In this study, we showed that *PxRDL2* was more resistant to fipronil than *PxRDL1*. We speculate that the increased transcript level of *PxRdl2* gene in the two fipronil resistant field strains may be associated with fipronil resistance. Furthermore, although how *PxRDL2* antagonizes the action of fipronil remains to be determined, enhanced level of *PxRdl2* transcripts could impose a greater antagonism on the action of fipronil, thereby, confer resistance to fipronil.

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Disclosure

The authors declare no competing financial interest.

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Table 1 Resistance to fipronil in field-collected strains of *P. xylostella*.

Strain	N ^a	LC ₅₀ (95% CI) (mg L ⁻¹) ^c	Slope ± SE	RR ^b
Susceptible	237	0.29 (0.24-0.34) a	1.94±0.19	
GZ	241	488.97 (402.81-605.28) c	1.65±0.18	1686
FZ	236	276.32 (222.30-332.66) b	1.80±0.19	953

^aNumbers of larvae used in bioassay.

^bRR(resistance ratio)=LC₅₀ (GZ or FZ) /LC₅₀(Susceptible) .

^c Lower case letters(a, b and c) indicate significance between different strains. Statistical analysis was determined by Student's *t*-test (alpha=0.05)

Table 2 The amino acid mutation frequencies of the *PxRDL1* subunit in the GZ and FZ strains.

Strain	Mutation	Location	Frequency (%)
GZ	A2'S	TM2	73.33

	T6'M	TM2	23.33
	D472N	C-terminal extracellular domain	16.67
FZ	A2'S	TM2	33.33
	T6'M	TM2	56.67

Table 3 Potencies of GABA and fipronil in *Xenopus* oocytes injected with *PxRdl* cRNAs

Variants	GABA	Fipronil		
	EC ₅₀ ± SEM (μM) ^a	Hill Slope (95% CI)	IC ₅₀ ± SEM (μM) ^b	Hill Slope (95% CI)
RDL1-WT	20.79 ± 5.99 b	1.32 (0.93-1.70)	0.13 ± 0.01 e	1.63 (1.07-2.19)
RDL1-A2'S	74.87 ± 11.43 c	2.00 (1.52-2.48)	0.55 ± 0.33 f	0.67 (0.41-0.96)
RDL1-T6'M	12.98 ± 3.77 a	1.30 (0.88-1.71)	0.48 ± 0.25 f	0.68 (0.44-0.92)
RDL2-WT	153.40 ± 18.55 d	1.67 (1.32-2.02)	5.23 ± 3.10 g	0.93 (0.50-1.35)

^a Lower case letters(a to d) indicate significance between different treatments. Statistical analysis was determined by Student's *t*-test (alpha=0.05)

^b Lower case letters(e to g) indicate significance between different treatments. Statistical analysis was determined by Student's *t*-test (alpha=0.05)

Table 4 Toxicity of fipronil to susceptible and *PxRdl* knockout *P. xylostella*.

Strain	N ^a	LC ₅₀ (95% CI) (mg L ⁻¹) ^c	Slope ± SE	RR ^b
Susceptible	238	0.22 (0.19-0.26) b	2.66±0.02	—

KO- <i>PxRdl1</i>	242	2.29 (1.90-2.76) c	2.27±0.22	10.41
KO- <i>PxRdl2</i>	239	0.05 (0.04-0.06) a	2.58±0.01	0.23

^aNumbers of larvae used in bioassay.

^bRR(resistance ratio)= LC_{50} (KO-*PxRdl1* or KO-*PxRdl2*) / LC_{50} (Susceptible) .

^cLower case letters(a, b and c) indicate significance between different strains. Statistical analysis was determined by Student's *t*-test (alpha=0.05)

Captions of figures

Fig.1 The alignment of amino acid sequence differences between *PxRDL1*-WT, *PxRDL1*-A2'S, *PxRDL1*-T6'M and *PxRDL2*-WT. WT, the wild type *PxRDL1* and *PxRDL2*; A2'S, the alanine to serine mutation in *PxRDL1*; T6'M, the threonine to methionine mutation in *PxRDL1*. The shown sequences also were the sequences that expressed in *Xenopus laevis* oocytes.

Fig.2 The expression profile of *PxRdls* mRNA in third-instar larvae of different strains. (A) The mRNA expression level of susceptible and two field strains. (B) The mRNA expression level of susceptible and two *PxRdls* knockout strains. Error bars indicate the SE of the mean of three independent replicates. Uppercase letters (A and B) indicate significance between *PxRdl1* and *PxRdl2* in the same strain. Lower case letters (a and b) indicate significance among different strains. The significance was analyzed with one-way ANOVA followed by a Tukey test ($P < 0.01$).

Fig.3 Dose-response curves of GABA-induced current in oocytes injected with *PxRdl* cRNAs. Each point represents the mean $\pm$ SE of responses in 5-6 oocytes from at least two *Xenopus laevis*.

Fig.4 Fipronil inhibition of GABA-induced currents in oocytes injected with *PxRdl* cRNAs. (A) Examples of fipronil inhibition of currents in channels containing *PxRdl* cRNAs (B) Fipronil concentration-response curves in channels containing *PxRDL1*. (C) Fipronil concentration-response curves in channels containing *PxRDL2*. Each point represents the mean $\pm$ SE of responses in 5-6 oocytes from at least two *Xenopus laevis*.

Fig.5 The CRISPR-Cas9 mediated mutation type of *PxRdl1* gene. (A) Schematic representation of sgRNA and *PxRdl1* DNA sequences. The protospacer DNA is shown in yellow and protospacer adjacent motif (PAM) sequence is shown in purple. Cleavage sites are represented by red arrows. (B) PCR was performed using primers designed to detect the 4-nt deletion in *PxRdl1*. M, marker. WT, wild type genomic DNA. (C) Direct sequencing chromatograms of PCR products amplified from genomic DNA of wild type, heterozygous mutant and homozygous mutant (-4nt deletion). The locations of -4nt deletion and PAM were marked by green and red box, respectively. (D) The deduced peptide sequence from partial of exon 4 of *PxRdl1*, the stop code was marked by red box.

Fig.6 The CRISPR-Cas9 mediated mutation type of *PxRdl2* gene. (A) Schematic representation of sgRNA and *PxRdl2* DNA sequences. The protospacer DNA is shown in yellow and protospacer adjacent motif (PAM) sequence is shown in purple. Cleavage sites are represented by red arrows. (B) PCR was performed using primers designed to detect the 52-nt deletion in *PxRdl2*. M, marker.

WT, wild type genomic DNA. (C) Direct sequencing chromatograms of PCR products amplified from genomic DNA of wild type, heterozygous mutant and homozygous mutant (-52nt deletion). The locations of -52nt deletion and PAM were marked by green and red box, respectively. (D) The deduced peptide sequence of *PxRDL2*, the 52nt deletion caused premature translation termination of *PxRDL2*.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6