Bacterial *Rdl2* dsRNA increased the insecticidal activity of GABAR blockers and allosteric modulators against *Plutella xylostella*

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23 ABSTRACT

24 **BACKGROUND:** The application of RNAi to control pests has attracted the
 25 attention of researchers. Our results indicated that knockout of *PxRdl2* can decrease
 26 resistance to fipronil in *Plutella xylostella*, providing a suitable target gene for
 27 RNAi-based pest control.

28 **RESULTS:** The differences in the sensitivity of two established homozygous
 29 knockout strains of *P. xylostella*. (*Rdl1*KO and *Rdl2*KO) and susceptible *P. xylostella*
 30 to a series of compounds were evaluated. Quinazolines and isoxazolines both showed
 31 stronger efficacy in the *Rdl2*KO strain. Therefore, we proposed a method based on the
 32 knockdown of the *P. xylostella Rdl2* gene as a tactic to enhance the toxicity of
 33 quinazolines and isoxazolines. To reduce costs and protect dsRNA against degradation,
 34 we applied a bacterial expression system using the L4440 vector to express *PxRdl2*
 35 dsRNA in HT115 *Escherichia coli*. Transformed bacteria (dsRNA-Bac) fed through
 36 leaves combined with quinazoline and isoxazolines proved to be more effective in
 37 both the susceptible and fipronil-resistant *P. xylostella*.

38 **CONCLUSION:** Our results provide a strategy for the development of novel
 39 insecticide spray formulations containing dsRNA-Bac, which synergize with
 40 insecticide toxins by suppressing *PxRdl2*, reducing the use of pesticides in the field.

41 **Keywords:** *Plutella xylostella*; RNAi; *PxRdl*; resistance management

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45 **1 INTRODUCTION**

46 Since RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis*
 47 *elegans*¹, it has been widely used as a powerful genetic tool for research on various
 48 taxa, including plants, insects, nematodes, bacteria, fungi, ect.^{2,3}. RNAi has been
 49 considered a new generation of pest control technology because it has great
 50 advantages, such as safety for nontarget organisms and the ability to maintain
 51 pollution-free conditions. Although dsRNA injected directly into an organism is
 52 highly efficient, it is difficult to apply as pest control in the field because of the very
 53 high costs of and complexity in preparing dsRNA. Fortunately, ingestion of specific
 54 dsRNA through the oral cavity can trigger a silencing response in most body tissues in
 55 insects; this paves the way for the development of RNAi-based agricultural pest
 56 control if dsRNA can be protected from degradation in the environment and insect
 57 intestines by suitable delivery methods.

58 Currently, wrapping dsRNA in nanomaterials or expressing dsRNA in bacteria are the
 59 two main methods of dsRNA-insect delivery. It has been reported that nanomaterials
 60 can improve the efficiency of delivery allowing dsRNA to permeate cell membranes
 61 directly or by endocytosis⁴; thus, this has become a new type of delivery agent and
 62 has been used in pest RNAi control⁵. Employing bacteria as delivery vectors of
 63 dsRNA molecules is another approach that has been used in recent years and was first
 64 carried out in the bacteriophagous nematode *C. elegans*⁶. Considering the technology
 65 and economic-related issues of dsRNA synthesis *in vitro*, there have been many
 66 reports of bacteria expressing dsRNA to induce insect RNAi to control pests⁷⁻⁹. Other

67 limitations related to the application of dsRNA for pest control include the
68 identification of the appropriate target genes and increasing the insect lethality. For
69 this point, genes targeted by chemical insecticides and bio-insecticides might be ideal
70 sites for dsRNA targeting. It has been reported that silencing *Sl102*, *MvIIS* and *OfCTP*
71 through RNAi increases the toxicity of *Bacillus thuringiensis* (Bt)¹⁰⁻¹².
72 With the cultivation of cruciferous crops, the diamondback moth (DBM), *P. xylostella*,
73 has developed into a major pest worldwide¹³. Following the misuse of insecticides in
74 the field, *P. xylostella* has developed such severe resistance to most pesticides through
75 multiple mechanisms that it is difficult to control^{14,15}. Although RNAi was reported to
76 be less efficient in lepidoptera¹⁶, RNAi in *P. xylostella* through dsRNA injection or
77 oral administration demonstrated that this approach is for this species^{17,18}. Therefore,
78 the control and resistance management of *P. xylostella* may be achieved by dsRNA
79 ingestion and interference with specific target genes.

80 In insects, γ -aminobutyric acid receptors (GABARs) are the major inhibitory
81 receptors widely distributed in the central nervous system¹⁹ and are the target of
82 natural compounds (picrotoxin) and synthetic pesticides (such as dieldrin, fipronil,
83 fluxametamide and fluralaner)²⁰⁻²². According to the classification of the Insecticide
84 Resistance Action Committee (<https://irac-online.org/modes-of-action/>, IRAC),
85 Compounds that act on GABAR are divided into two categories: blockers (Category 2,
86 represented by fipronil and chlordane) and allosteric modulators (Category 30,
87 represented by broflanilide and fluxametamide) (referred to as GABAR compounds in
88 this paper). Resistance to dieldrin (*Rdl*) gene, which combines with other GABAR

subunits to constitute the target site of cyclodiene and phenylpyrazole pesticides, was cloned from dieldrin-resistant *Drosophila melanogaster* and named *Rdl*²³. There are two *Rdls* in *P. xylostella*²⁴. Our previous research showed that in *Xenopus oocytes*, the sensitivity of *PxRdl2* to fipronil was 40-fold lower than that in *P. xylostella Rdl1*. In addition, knockout of *PxRdl1* reduced the efficacy of fipronil, while knockout of *PxRdl2* increased its efficacy²⁵. This may indicate that the *PxRdl2* gene plays an important role in resistance to GABAR compounds, which is beneficial for researching toxicology and resistance to insecticides. Based on previous results, we verified whether the knockout of *PxRdl2* or *PxRdl1* has effects on other GABAR compounds. Furthermore, we considered whether it is possible to develop *PxRdl2*-based RNAi to improve the efficacy of insecticides.

In this study, two homozygous knockout strains of *P. xylostella* (*Rdl1KO* and *Rdl2KO*) and susceptible strains were established to evaluate the differences in sensitivity of 5 compounds by bioassay. The bioassay results suggested that the newly synthesized quinazolines-4a and the two new isoxazolines (fluxametamide and fluralaner) displayed greater activity in the *Rdl2KO* strain than in the susceptible strain. Furthermore, *PxRdl2* interference by dsRNA, which induced expression by transformed HT115 *Escherichia coli* (named dsRNA-Bac in this paper), could improve the efficacy of quinazoline and isoxazolines (named RNAi compound in this paper) in both susceptible and fipronil-resistant *P. xylostella*. In addition, we also verified the effect of UV radiation on dsRNA-Bac to ensure that it could remain stable when exposed to sunlight in field applications. In summary, our study provides new

insights for pest control, improving safety for nontarget organisms. Thus, the results may be beneficial for pesticide resistance management and reducing the usage of pesticides in the field, which could be part of an integrated pest management (IPM) approach.

2 MATERIALS AND METHODS

2.1 Insect strain rearing and compounds

Susceptible and fipronil-resistant *P. xylostella* were generously provided by Dr. Minsheng You (Fujian Agriculture and Forestry University, China). Two homozygous knockout strains (*Rdl1*KO and *Rdl2*KO) were previously established from susceptible strains using CRISPR/Cas9 previously²⁵, and were reared on an artificial diet for more than 30 generations in our laboratory. The four strains were kept at 25±1 °C and 65±5% relative humidity under 16:8 h (light: dark) conditions.

Compounds of fipronil (95%), fluxametamide (95%), chloraniliprole (95%), fluralaner (95%) and abamectin (95%) were purchased from TargetMol (Shanghai, China). Compounds 5a²⁶ and 4a²⁷ were synthesized in our laboratory.

2.2 Bioassays

Two bioassays were conducted in this study. To evaluate the sensitivity of the susceptible, *Rdl*KO and fipronil-resistant strains to the compounds, leaf-dip bioassays were carried out as recommended by the IRAC (<https://irac-online.org/methods/plutella-xylostella-larvae/>). The LCs of the different compounds were determined through the leaf-dip bioassays. To better simulate the outdoor spraying method applied in the field and to detect the impact of gene

133 silencing on the lethality of the compounds, RNAi compounds were subjected to
134 leaf-dip bioassays, in which dsRNA-Bac was evenly applied to the surface of the
135 leaves containing compounds.
136 For the *PxRdl2* RNAi compounds, a sublethal dose (LC₂₀) allowed the assessment of
137 any increase in the mortality rate caused by RNAi-induced gene silencing. Third
138 instar larvae were fed with leaves containing *PxRdl2* dsRNA-Bac (or *GFP*
139 dsRNA-Bac) and compounds. Each treatment used at least 10 larvae with 4
140 replications. Mortality was recorded every 12 h for 2 days. The theoretically reduced
141 dosage was calculated by the following formula: (LCs – LC₂₀ of the *PxRdl2* RNAi
142 compounds)/LCs × 100%. The impact of *PxRdl1* dsRNA on the chemical toxicity of *P.*
143 *xylostella* was also assessed as described above, with the expectation that the LC₅₀ of
144 each compound dosage was used.

145 **2.3 In vitro synthesis of *PxRdl*s dsRNA**

146 Total RNA was extracted from 3rd instar larvae or bacteria using an RNAsimple Total
147 RNA Kit (TianGen, Beijing, China). The extracted RNA (1 µg) was used to reverse
148 transcribe cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect
149 Real Time) (TaKaRa Biotechnology, Dalian, China).
150 A 428-bp fragment of *PxRdl1* and a 441-bp fragment of *PxRdl2* were amplified from
151 cDNA extracted from larvae. The specific fragments were purified using an
152 E.Z.N.A.® Gel Extraction Kit (OMEGA Bio-tek, Guangzhou, China). The PCR
153 products were used as templates for the next PCR and primers containing the T7
154 polymerase promoter sequence at their 5' ends were carried out. The PCR products

were used as templates to synthesize specific dsRNAs according to the manufacturer's instructions using the *in vitro* Transcription T7 Kit (TaKaRa Biotechnology, Dalian, China).

The 289-bp *GFP* fragment was amplified from the cloning vector pEGFP-N1-ro1*GFP* (Plasmid #82369, Addgene), and then, a similar method was applied to synthesize specific *GFP* dsRNA. All the primers in this paper were designed using Primer Premier 5 (Premier, Canada) and are shown in Table 1.

2.4 Construction of transformed HT115 *Escherichia coli* expressing dsRNA and overexpression of dsRNA

The 289-bp *GFP*, 428-bp *PxRdl1* and 441-bp *PxRdl2* fragments were cloned into L4440 vectors (Plasmid #1654, Addgene) via homologous recombination^{28,29}. The recombinant L4440 vectors were transformed into HT115 *E. coli* competent cells. To produce dsRNA, the transformed bacteria were induced by isopropyl β -D-1-thiogalactopyranoside (IPTG). dsRNA was extracted from the bacterial cells for subsequent qRT-PCR absolute quantification.

2.5 qRT-PCR absolute quantification of dsRNA produced by the transformed bacteria

qRT-PCR was carried out using SYBR[®] Premix Ex Taq[™] II (Takara Biotechnology) and a CFX96 Connect Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Based on the absolute quantification method³⁰, the quantity of the dsRNA was confirmed by threshold (CT) values, which relate to an established standard curve. The standard curve for dsRNA was established by plotting the logarithm of a

10-10⁵-fold dilution of the starting solution of template cDNA with inserts against the corresponding CT values.

2.6 Pretreatment of bacteria

Bacteria were collected by centrifugation at 5000×g for 5 min at 4°C and suspended in DEPC-H₂O. To inactivate the bacteria and promote the release of dsRNA, sonication treatment was carried out by a low-temperature ultrahigh-pressure cell crusher (Guangzhou Juneng Nano & Biotechnology Co., Ltd, Guangzhou, China) at 1800 bar. LB agar plates containing 100 µg mL⁻¹ ampicillin were used to evaluate whether the bacteria were totally inactivated.

2.7 qRT-PCR relative quantification of *PxRdl*s transcription

dsRNA-Bac (100, 200 or 400 ng µL⁻¹) was evenly applied to the surface of the leaves, and then naturally air-dried. Gene analysis was performed by qRT-PCR, which was carried out by using gene-specific primers, after 2 days of feeding the dsRNA-Bac-covered leaves to 3rd instar *P. xylostella* larvae. Gene-specific primers were designed to test a segment of the mRNA external to the segment targeted by the dsRNA. The qRT-PCR data were normalized to an internal control (*Actin* gene, GenBank No. JN410820.1) and analysed by the 2^{-ΔΔCT} method³¹. Each treatment used 5 larvae with 3 replications. The controls received *GFP* dsRNA-Bac.

2.8 UV irradiation on dsRNA-Bac

To better simulate the effects of field applications, a UV irradiation experiment was carried out to determine the stability of dsRNA-Bac. *PxRdl2* dsRNA-Bac and *GFP* dsRNA-Bac were placed on the surface of glassware. The glassware was placed on an

199 HD-650 desktop clean bench (Su Jing Purification Equipment, Zhejiang, China). A
200 UV lamp was used to irradiate the glassware for 0 h, 0.5 h, 1 h, 2 h or 4 h (254 nm, 8
201 W). Before UV irradiation, absolute quantification was carried out on the dsRNA-Bac.
202 The treated dsRNA-Bac was fed to *P. xylostella* through leaves for gene expression
203 analysis after two days.

204 **2.9 Statistical analysis**

205 For the leaf-dip bioassay data, SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used to
206 calculate the LCs. The LCs were analysed using regression-probit analysis, and “Log
207 base 10” was selected for transformation. The LC₅₀ values were confirmed to be
208 significantly different if the 95% fiducial limits did not overlap. The other data were
209 analysed using GraphPad Prism, version 5.0 (GraphPad Software Inc., La Jolla, CA,
210 USA). Gene expression was analysed by one-way ANOVA and Tukey’s test (alpha =
211 0.05). Survival curves of the *P. xylostella* 3rd instar larvae were compared using
212 log-rank analyses.

213 **3 RESULTS**

214 **3.1 Toxicological responses of the two *Rdl* knockout strains to five compounds**

215 To test roles of the two *Rdl* homologous genes in the interaction with the five tested
216 compounds, the responses of the two knockout strains and susceptible strain were
217 measured (Table 2 and Fig. 1: data for fipronil were adapted from our previous
218 publication²⁵). The *Rdl1*KO strain showed greater resistance (2.85-fold) than the
219 susceptible strain to the newly synthesized compound quinazoline (4a). In contrast,
220 the toxicity of 4a to the *Rdl2*KO strain increased significantly as the resistance ratio

(RR) decreased to 0.45. These results revealed that while *PxRdl1* and *PxRdl2* are both vital determinants of the sensitivity of *P. xylostella* to 4a, they have adverse effects. Such a tendency was also observed in response to fipronil²⁵ and 5a treatment (unpublished data). The toxicity of fluxametamide to the *Rdl1*KO and *Rdl2*KO strains was significantly increased relative to that to the susceptible strain. Unlike fluxametamide, knockout of *PxRdl1* had no significant effect on fluralaner susceptibility, but knockout of *PxRdl2* increased fluralaner susceptibility (2.50-fold). No difference in toxicity was observed in the three strains for abamectin. The negative control, chloraniliprole, exhibited toxicity similar to that of the two *Rdl* knockout strains compared with that of the susceptible strain. The increased susceptibility to knockout of *Rdl2* suggested that it could be an ideal target for RNAi in the development of synergists with GABAR compounds.

3.2 Production of bacteria expressing *PxRdls* dsRNA

To construct a vector expressing dsRNA, the *GFP* and *PxRdls* PCR products were cloned into the L4440 vector, which was induced by the addition of IPTG to overexpress dsRNA under the double T7 promoters and used to transform HT115 *E. coli* cells. The quantity of dsRNA produced by the HT115 *E. coli* (Fig. 2a) was quantified by qRT-PCR (Fig. 2b, Fig. 2c, Fig. 2d). For *GFP*, the PCR efficiency ($E = 95.516\%$) was calculated according to the slope and the coefficient of correlation (R^2) of the standard curve (slope = -3.358, y intercept = 18.835, $R^2 = 0.996$). For *PxRdl1*, the PCR efficiency ($E = 108.930\%$) was calculated according to the slope and the coefficient of correlation (R^2) of the standard curve (slope = -3.125, y intercept =

243 12.116, $R^2 = 0.998$). For *PxRdl2*, the PCR efficiency ($E = 92.780\%$) was calculated
244 according to the slope and the coefficient of correlation (R^2) of the standard curve
245 (slope = -3.508, y intercept = 5.898, $R^2 = 0.998$).

246 **3.3 Gene silencing in *P. xylostella* by feeding individuals leaves containing** 247 **dsRNA-Bac**

248 Quantitative analysis of the expression of the two *PxRdl* genes in 3rd instar larvae was
249 performed by qRT-PCR two days after the larvae were fed leaves containing dsRNA
250 produced by HT115 *E. coli*. The results demonstrated that the transcription levels of
251 the targeted *PxRdl1* and *PxRdl2* genes were significantly affected by the dsRNA
252 treatment and were positively correlated with the experimental dose used ($P < 0.05$)
253 (Fig. 3a, Fig. 3b).

254 **3.4 The effect of UV irradiation on dsRNA-Bac**

255 After dsRNA-Bac was irradiated with UV for different times, the treated dsRNA-Bac
256 was fed to 3rd instar larvae through leaves. The gene expression analysis data showed
257 that dsRNA-Bac had strong stability and could maintain activity under strong UV
258 irradiation for 1 h. The activity of dsRNA-Bac decreased when exposed to UV for
259 more than 2 h ($P < 0.05$) (Fig. 3c).

260 **3.5 *PxRdl2* dsRNA-Bac enhances the toxicity of quinazolines and isoxazolines in** 261 **susceptible *Plutella xylostella***

262 RNAi compounds were used to test the efficacy of the simultaneous administration of
263 *PxRdl2* dsRNA-Bac and compounds (fipronil, quinazolines and isoxazolines). This
264 experiment aimed to more closely reproduce the possible effects of the two active

265 ingredients (dsRNA and compounds) applied in the field. The results showed that
 266 simultaneous administration of *PxRdl2* dsRNA-Bac and compounds led to obviously
 267 higher mortality in *PxRdl2*-silenced larvae than in controls (Fig. 4) (log-rank test for
 268 3rd instar larvae exposed to different compounds: fipronil: chi-square = 4.501, $df = 3$,
 269 $P = 0.0339$; fluxametamide: chi-square = 14.80, $df = 3$, $P = 0.0001$; fluralaner:
 270 chi-square = 12.33, $df = 3$, $P = 0.0004$; 5a: chi-square = 14.95, $df = 3$, $P = 0.0001$; 4a:
 271 chi-square = 34.26, $df = 3$, $P < 0.0001$).

272 In addition, to further evaluate the effect of RNAi compound-mediated toxicity
 273 enhancement, the theoretical reduction in compound usage was calculated. When the
 274 compounds were used alone, the LCs^b in Table 3 shows the dose corresponding to
 275 mortality, indicating the final mortality of *PxRdl2* RNAi compounds after 48 h.
 276 According to the calculation results, the enhanced toxicity of the compounds mediated
 277 by RNAi could reduce the doses needed of different compounds (fipronil: 45.00%;
 278 fluxametamide: 56.82%; fluralaner: 45.45%; 5a: 59.31%; and 4a: 72.36%) (Table 3).

279 **3.6 *PxRdl1* dsRNA-Bac-mediated gene silencing led to reduced toxicity of** 280 **quinazolines and isoxazolines in susceptible *Plutella xylostella***

281 The LC₅₀ values of fipronil and the quinazolines and isoxazolines were used for a
 282 *PxRdl1* RNAi compound bioassay similar to that for *PxRdl2*. The data showed that
 283 *PxRdl1* silencing reduced the toxicity of fipronil and the quinazolines and
 284 fluxametamide, except for fluralaner (Fig. 5) (log-rank test of the 3rd instar larvae for
 285 different compounds, fipronil: chi-square = 11.11, $df = 3$, $P = 0.0009$; fluxametamide:
 286 chi-square = 4.672, $df = 3$, $P = 0.0307$; fluralaner: chi-square = 0.5698, $df = 3$, $P =$

0.4503; 5a: chi-square = 4.159, $df = 3$, $P = 0.0414$; 4a: chi-square = 8.844, $df = 3$, $P = 0.0029$).

3.7 *PxRdl2* dsRNA-Bac enhances the toxicity of quinazoline and isoxazolines in fipronil-resistant *Plutella xylostella*

Fipronil-resistant *P. xylostella* are extremely resistant to fipronil, and its resistance has increased 1881-fold. For 4a, the resistance also increased 14-fold. Compound 5a and two isoxazolines had good effects against fipronil-resistant *P. xylostella*. These 3 compounds were used to the *PxRdl2* RNAi compounds. The data showed that these three compounds could increase the toxicity to fipronil-resistant *P. xylostella* of *PxRdl2* RNAi compounds (Fig. 6) (log-rank test of 3rd instar larvae for different compounds, fluxametamide: chi-square = 11.47, $df = 3$, $P = 0.0007$; fluralaner: chi-square = 4.964, $df = 3$, $P = 0.0259$; 5a: chi-square = 14.65, $df = 3$, $P = 0.0001$). In addition, the enhanced toxicity mediated by RNAi could reduce the dose required of different compounds, such as fluxametamide (58.06%), fluralaner (43.14%), and 5a (45.61%) (Table 4).

4 DISCUSSION

Recent studies have shown that the existence of multiple copies of homologous *Rdl* further enriches the functional library of GABARs in insects^{24,32,33}. These different *Rdl* subunits may combine to produce GABARs with a series of physiological and pharmacological properties^{32,33}. It has been reported that knockout of *Rdl1* or *Rdl2* alone does not affect the survival of *H. armigera* or *P. xylostella*^{25,34}. These results indicated the functional redundancy of multiple homologous *Rdl* genes in *H. armigera*

309 and *P. xylostella*. We also tried to knockout both *Rdl* genes in *P. xylostella*, but
 310 unfortunately, no viable homozygous knockouts were obtained (the associated data
 311 and process were not shown in this paper). This may further illustrate the functional
 312 redundancy of the two *Rdls*, which cannot be deleted at the same time.

313 Three strains of the *Rdl* genotype in *P. xylostella* were used in bioassays of different
 314 compounds to clarify the role of the two *Rdls* as anti-compounds. Data from our
 315 previous bioassays showed that the *Rdl1*KO strain obtained 10.4-fold resistance to
 316 fipronil and that the *Rdl2*KO strains obtained 4.4-fold sensitivity to fipronil compared
 317 to the sensitivity of susceptible strains²⁵. For 4a, knockout of *PxRdl1* significantly
 318 increased resistance, by 2.85-fold, whereas knockout of *PxRdl2* significantly
 319 increased susceptibility, by 2.21-fold. 5a is a possible antagonist of GABAR²⁶, our
 320 unpublished results also proved that 5a has a similar trend to 4a and fipronil. This
 321 suggested that both *PxRdl1* and *PxRdl2* were involved in mediating fipronil and
 322 newly synthesized quinazolines toxicity. Furthermore, *PxRdl2* played an important
 323 role in anti-quinazolines activities, and *PxRdl2* was more tolerant than *PxRdl1* to
 324 quinazolines. In addition, different pharmacological properties between the two
 325 *PxRdls* may resolve their counter contribution to the sensitivities of *P. xylostella* to
 326 these compounds.

327 The novel isoxazoline insecticides fluxametamide and fluralaner were also used in the
 328 sensitivity tests of the three strains. The toxicity of the two isoxazolines to the three
 329 strains showed different trends, which may verify the novel mode of action compared
 330 to that of fipronil and two quinazolines. The key mode of action of avermectin in

331 invertebrates is to activate glutamate-gated chloride channels (GluCl_s), although it has
332 also been proposed that avermectin binds to and activates GABAR³⁵. However, in our
333 study, knockout of *PxRdl1* or *PxRdl2* did not affect sensitivity to abamectin,
334 confirming that abamectin may not be active on *PxRdl1* or *PxRdl2* individually.
335 Susceptibility to the negative control, chloraniliprole, was not significantly affected
336 by knockout of either *PxRdl1* or *PxRdl2*. In summary, in pharmacological reactions,
337 our results indicated that *PxRdl2* plays an important role in resisting quinazolines and
338 isoxazolines.

339 Pest control based on RNAi, due to its specificity and minimal or nonexistent impact
340 on nontarget species, provides new opportunities for the development of sustainable
341 IPM plans. Baum et al³⁶ demonstrated that genetically engineered dsRNA expression
342 in maize (*Zea mays*) could cause larval mortality in the western corn rootworm
343 (WCR), *Diabrotica virgifera*. This aroused great interest from researchers related to
344 the use of dsRNA produced by transgenic plants as a novel pest control agent. This
345 new type of pest control tool has been widely applied in coleoptera because of its high
346 RNAi efficiency³⁷. Therefore, it should be further studied and expanded to control
347 other important agricultural pests, such as lepidopteran. The reaction efficiency of
348 lepidopteran insects to RNAi is uneven, and the dsRNA delivery mode will affect its
349 interference efficiency. It has been reported that *H. armigera* and *S. littoralis* were
350 sensitive to orally delivered dsRNA^{10,38}. Moreover, there were also reports of RNAi in
351 *P. xylostella* using orally delivered dsRNA³⁹, which could provide us with references
352 to control *P. xylostella* using RNAi.

353 In our previous research, *PxRdl2* was confirmed to play an important role in the fight
354 against fipronil and the function of *PxRdl2* in fighting quinazolines and isoxazolines
355 was verified in this article. This discovery allows the extension of a new pest control
356 method aimed at enhancing the toxicity of insecticides through RNAi-mediated
357 *PxRdl2* gene silencing in *P. xylostella*. However, the development of efficient, safe
358 and economically sustainable RNAi delivery strategies is vital to achieve this goal. In
359 this paper, we probed the utilization of bacteria as potential carriers of dsRNA
360 targeting the *PxRdl2* gene and evaluated their effects on quinazolines and isoxazolines
361 for the control of *P. xylostella*. The recombinant vector L4440 was transformed into
362 HT115 *E. coli* to induce the expression of a specific dsRNA fragment, which may be
363 the preferred method for the mass production of dsRNA. Expression of dsRNA by
364 bacteria was effective in silencing the target gene *PxRdl2* and showed a quantitative
365 dependence when administering *P. xylostella* dsRNA-Bac via leaves (Fig. 3a, Fig. 3b).
366 It was previously reported that the dsRNA molecule was protected by the bacterial
367 envelope and was not easily degraded (in both the environment and the insect gut),
368 which could allow dsRNA to exist/release for a longer time^{38,40}. This provides support
369 for the application of RNAi technology in the field, where light (UV) is an important
370 factor affecting the effect of dsRNA. In the laboratory, UV light is an important means
371 for sterilization and digestion of nucleic acids. To better simulate field light factors,
372 UV irradiation was carried out to test the stability of dsRNA-Bac. Compared with *in*
373 *vitro* synthesized dsRNA⁴¹, our data showed that dsRNA-Bac was resistant to UV
374 exposure and needed to be continuously irradiated with strong UV for more than 2 h

375 obviously to lose part of its activity (Fig. 3c). It was speculated that dsRNA-Bac may
376 be more stable under the protection of the bacterial envelope than dsRNA directly
377 exposed to the external environment. This experiment proved the stability of
378 dsRNA-Bac under light exposure, because UV radiation in the laboratory is several
379 times stronger than the intensity of solar UV radiation in the field, which laid the
380 foundation for the use of dsRNA-Bac for RNAi in the field.

381 Feeding *P. xylostella* dsRNA-Bac confirmed that the target gene (*PxRdl1*) was
382 successfully silenced, which prompted us to evaluate the effect of *PxRdl1* gene
383 silencing on insecticidal activity. The data suggested that *PxRdl2* gene silencing
384 induced by dsRNA-Bac has a strong synergistic effect with quinazolines and
385 isoxazolines. Significantly, the mortality of larvae increased in the case of
386 simultaneous feeding of compounds and dsRNA-Bac (Fig. 4). In addition, the
387 theoretically reduced doses of the compounds were calculated and ranged from
388 45.00% to 72.36%. To further verify these results, *PxRdl1* dsRNA-Bac was used for
389 RNAi compound treatment. For fipronil and the quinazolines and fluxametamide, the
390 results showed that the influence of *PxRdl1* was the opposite of the influence of
391 *PxRdl2* (Fig. 5), which further verifies that *PxRdl2* is a key gene in the response to the
392 compounds. However, fluralaner did not show the exact opposite results (Fig. 5c), and
393 more research may be required to explore the mechanism driving this response. There
394 was high resistance to fipronil and 4a in the fipronil-resistant *P. xylostella* (Table 2).
395 Fortunately, 5a and two isoxazolines were very effective against the fipronil-resistant
396 strain. In the subsequent *PxRdl2* RNAi compounds, silencing the *PxRdl2* gene also

397 increased the toxicity of the three compounds to the fipronil-resistant strain (Fig. 6).
 398 The calculated theoretical reduction in the doses of the three compounds was from
 399 43.14% to 58.06% (Table 4).
 400 In summary, oral *PxRdl2* dsRNA-Bac combined with GABAR compounds could
 401 increase the activity of the compounds against *P. xylostella*, and these combinations
 402 are suitable for both susceptible and resistant strains. Stable dsRNA-Bac can be
 403 prepared by bacteria in large quantities, which greatly reduces the associated costs,
 404 supporting the development of pesticide sprays. Therefore, a spray may be developed
 405 that includes dsRNA-Bac as a synergist of insecticides, which is beneficial for
 406 reducing pesticide doses, delaying pest resistance and protecting nontarget organisms.

407

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417 **Competing financial interests**

418 The authors declare no competing financial interests.

419

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Figure Legends

Fig. 1 Resistance levels of the *Rdl1*KO (*PxRdl1* knockout strain) and the *Rdl2*KO (*PxRdl2* knockout strain) to chemical insecticides compared with the susceptible strain of *Plutella xylostella*. Resistance ratio = LC_{50} of *Rdl1*KO or *Rdl2*KO divided by LC_{50} of susceptible. * mean their LC_{50} s are significantly different from susceptible.

Fig. 2 Production of HT115 *Escherichia coli* cells expressing dsRNA. **a** Expression of dsRNA by transformed HT115 *E. coli*; the total RNA were carried out to RT-PCR, and the production of amplification were resolved on 1% agarose gel. The specific primers used for *GFP*, *PxRdl1* or *PxRdl2* genes produced amplicons of the expected fragments in transformed HT115 *E. coli* (lines 1, 2 and 3), whereas the same primers did not produced any fragment from non-transformed HT115 *E. coli* (WT) (lines 4, 5 and 6). **b c** and **d** Standard curves used on qRT-PCR absolute quantification of *GFP*, *PxRdl1* and *PxRdl2* dsRNA produced by *E. coli* suspensions.

Fig. 3 mRNA expression of *PxRdls* gene in *Plutella xylostella* 3rd instar larvae orally treated for 2 days with dsRNA-Bac. **a** and **b** The *PxRdl1* and *PxRdl2* gene are down-regulated upon ingestion of dsRNA-Bac administered by leaves, which was dose-dependent; **c** The *PxRdl2* dsRNA-Bac treated different UV irradiation times (0h, 1h, 2h, 4h) was fed to *P. xylostella* 3rd instar larvae for 2 days. UV irradiation for less than 1h had no obvious effect on the activity of dsRNA-Bac. *GFP* dsRNA-Bac were used as control. The values are the mean $\pm$ standard errors (* $P < 0.05$, Tukey' s test).

574 **Fig. 4** *PxRdl2* RNAi-compounds in susceptible *Plutella xylostella* 3rd instar larvae
 575 Susceptible 3rd instar larvae were treated for 2 days with leaves containing with
 576 *PxRdl2* dsRNA-Bac (corresponding to 400 ng μL^{-1} of dsRNA) and with compounds
 577 (LC_{20}). **a** for fipronil, * $P = 0.0339$; **b** for fluxametamide, *** $P = 0.0001$; **c** for
 578 fluralaner *** $P = 0.0004$; **d** for 5a *** $P = 0.0001$; **e** for 4a *** $P < 0.0001$ (based on
 579 log-rank test).

580 **Fig. 5** *PxRdl1* RNAi-compounds in susceptible *Plutella xylostella* 3rd instar larvae
 581 Susceptible 3rd instar larvae were treated for 2 days with leaves containing with
 582 *PxRdl1* dsRNA-Bac (corresponding to 400 ng μL^{-1} of dsRNA) and with compounds
 583 (LC_{50}). **a** for fipronil, *** $P = 0.0009$; **b** for fluxametamide, * $P = 0.0307$; **c** for
 584 fluralaner $P = 0.4503$; **d** for 5a * $P = 0.0414$; **e** for 4a ** $P = 0.0029$ (based on
 585 log-rank test).

586 **Fig. 6** *PxRdl2* RNAi-compounds in fipronil-resistant *Plutella xylostella* 3rd instar
 587 larvae
 588 Fipronil-resistant 3rd instar larvae were treated for 2 days with leaves containing with
 589 *PxRdl2* dsRNA-Bac (corresponding to 400 ng μL^{-1} of dsRNA) and with compounds
 590 (LC_{20}). **a** for fluxametamide, *** $P = 0.0007$; **b** for fluralaner, * $P = 0.0259$; **c** for 5a,
 591 *** $P = 0.0001$ (based on log-rank test).

592 Table 1 Detailed information of primers used in this study.

Primer name	Nucleotide sequence (5'-3')	Function
<i>GFP</i> -F	CAGTGCTTCAGCCGCTACCC	Amplification of <i>GFP</i>
<i>GFP</i> -R	AGTTCACCTTGATGCCGTTCTT	
<i>Rdl1</i> -F	AGCAATACTGGATTCATT	Amplification of <i>PxRdl1</i>
<i>Rdl1</i> -R	TCTCTATATGGCACAGTTGT	
<i>Rdl2</i> -F	TGGTCTTCGCTAGTCTTT	Amplification of <i>PxRdl2</i>
<i>Rdl2</i> -R	AATTTGTTGATTCCCTTC	
T7- <i>GFP</i> -F ^a	TAATACGACTCACTATAGGGC AGTGCTTCAGCCGCTACCC	<i>In vitro</i> synthesis of <i>GFP</i> dsRNA
T7- <i>GFP</i> -R ^a	TAATACGACTCACTATAGGG AGTTCACCTTGATGCCGTTCTT	
T7- <i>Rdl1</i> -F ^a	TAATACGACTCACTATAGGG AGCAATACTGGATTC	<i>In vitro</i> synthesis of <i>PxRdl1</i> dsRNA
T7- <i>Rdl1</i> -R ^a	TAATACGACTCACTATAGGGT CTCTATATGGCACA	
T7- <i>Rdl2</i> -F ^a	TAATACGACTCACTATAGGGT GGTCTTCGCTAGTCT	<i>In vitro</i> synthesis of <i>PxRdl2</i> dsRNA
T7- <i>Rdl2</i> -R ^a	TAATACGACTCACTATAGGGA TTTGTTGATTCCCTTCG	
qPCR-AQ- <i>GFP</i> -F	GCCGCTACCCCGACCACAT	Absolute quantification of dsRNA produced by the transformed bacteria
qPCR-AQ- <i>GFP</i> -R	CGCCCTCGAACTTCACCTC	
qPCR-AQ- <i>Rdl1</i> -F	GCGTAGAGACATTATCAGTT	
qPCR-AQ- <i>Rdl1</i> -R	AATTCGTTGCTCGTAGTAG	
qPCR-AQ- <i>Rdl2</i> -F	GGCTGCTGAGAAGAAA	
qPCR-AQ- <i>Rdl2</i> -R	GGGAGTGTGGGACCTA	
qPCR- <i>Actin</i> -F	TGGCACCACACCTTCTAC	Relative quantification of <i>PxRdls</i> transcription
qPCR- <i>Actin</i> -R	CATGATCTGGGTCATCTTCT	
qPCR- <i>Rdl1</i> -F	GAAGTTGCCTCCAGACTGC	
qPCR- <i>Rdl1</i> -R	CCACCCTTTGAATGTGCC	
qPCR- <i>Rdl2</i> -F	GTCAGTCAGCTACGACAAACG	
qPCR- <i>Rdl2</i> -R	AAATCCAGGGTAAAATCCAT	

^aThe bold nucleotide sequences are the T7 promoter.

Table 2 Toxicity of different compounds to susceptible, fipronil-resistant and *Rdl*KO *Plutella xylostella*.

Compound ^a	Strain ^b	N ^c	Slope $\pm$ SE	LC ₅₀ (mg L ⁻¹) (95% FL ^d)	RR ^e
fipronil ²⁵	susceptible	238	2.66 $\pm$ 0.02	0.22 (0.19-0.26)	-
	<i>Rdl</i> 1KO	242	2.27 $\pm$ 0.22	2.29 (1.90-2.76)	10.41
	<i>Rdl</i> 2KO	239	2.58 $\pm$ 0.01	0.05 (0.04-0.06)	0.23
	resistant	243	4.43 $\pm$ 0.48	413.83 (366.49-459.29)	1881.05
fluxametamide	susceptible	246	2.65 $\pm$ 0.43	0.079 (0.051-0.59)	-
	<i>Rdl</i> 1KO	252	4.06 $\pm$ 0.44	0.042 (0.037-0.047)	0.53
	<i>Rdl</i> 2KO	253	3.21 $\pm$ 0.37	0.043 (0.038-0.050)	0.54
	resistant	247	2.19 $\pm$ 0.21	0.31 (0.18-0.51)	3.92
fluralaner	susceptible	243	4.40 $\pm$ 0.85	0.010 (0.0090-0.012)	-
	<i>Rdl</i> 1KO	242	5.53 $\pm$ 0.73	0.0090 (0.0060-0.015)	0.90
	<i>Rdl</i> 2KO	252	4.67 $\pm$ 0.48	0.0040 (0.0040-0.0050)	0.40
	resistant	246	2.44 $\pm$ 0.31	0.065 (0.033-0.333)	6.50
5a	susceptible	244	3.16 $\pm$ 0.35	1.74 (0.86-3.18)	-
	resistant	244	3.19 $\pm$ 0.41	5.24 (4.58-5.94)	3.01
4a	susceptible	241	3.01 $\pm$ 0.34	3.93 (3.41-4.60)	-
	<i>Rdl</i> 1KO	242	5.32 $\pm$ 0.58	11.19 (10.32-12.17)	2.85
	<i>Rdl</i> 2KO	251	2.50 $\pm$ 0.29	1.78 (1.51-2.11)	0.45
	resistant	250	4.41 $\pm$ 0.50	57.95 (40.35-79.44)	14.75
abamectin	susceptible	252	1.69 $\pm$ 0.24	0.012 (0.0080-0.015)	-
	<i>Rdl</i> 1KO	236	2.30 $\pm$ 0.27	0.015 (0.012-0.018)	1.25
	<i>Rdl</i> 2KO	254	2.33 $\pm$ 0.25	0.016 (0.014-0.020)	1.33
chloraniliprole	susceptible	236	1.30 $\pm$ 0.19	1.32 (0.88-1.81)	-
	<i>Rdl</i> 1KO	233	1.41 $\pm$ 0.19	1.70 (1.23-2.27)	1.29
	<i>Rdl</i> 2KO	238	1.11 $\pm$ 0.18	1.58 (1.03-2.25)	1.20

^a4a is a compound synthesized by Shuai Yang et al., 2020; 5a is a compound synthesized by Xunyu Jiang et al., 2020 (same below) (Only susceptible and resistant data is showed).

^bSusceptible: susceptible strain; *Rdl*1KO: *PxRdl1* knockout strain; *Rdl*2KO: *PxRdl2* knockout strain; resistant: fipronil-resistant strain.

^cNumber of larvae tested.

^d95% Fiducial limits.

^eResistance ratio was calculated by LC₅₀ of *Rdl*1KO, *Rdl*2KO or resistant divided by LC₅₀ of susceptible.

Table 3 The LCs of 5 compounds in susceptible *Plutella xylostella*

597

compound	LC ₂₀ ^a	LC ₄₅ ^b (45%) ^c	LC ₅₅ ^b (55%) ^c	LC ₆₀ ^b (60%) ^c	LC ₆₅ ^b (65%) ^c	LC ₈₀ ^b (80%) ^c	Reduce dosage(%) ^d	LC ₅₀ ^e
fipronil	0.11	0.20					45.00%	0.22
fluxametamide	0.038		0.088				56.82%	0.079
fluralaner	0.0060			0.011			45.45%	0.010
5a	0.94				2.31		59.31%	1.74
4a	2.07					7.49	72.36%	3.93

^aLC₂₀ for *PxRdl2* RNAi-compounds.

^bLCs: The dose corresponding to the mortality of the compounds used alone.

^cThe final mortality of *PxRdl2* RNAi-compounds after 48 h.

^dTheoretically reduce dosage = (LCs – LC₂₀ of the *PxRdl2* RNAi compounds)/LCs × 100%.

^eLC₅₀ for *PxRdl1* RNAi-compounds.

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Table 4 The LCs of 5 compounds in fipronil-resistant *Plutella xylostella*

compound ^a	LC ₂₀ ^b	LC ₄₀ ^c (40%) ^d	LC ₅₀ ^c (50%) ^d	Reduce dosage(%) ^e
fipronil	267.24	-		
fluxametamide	0.13		0.31	58.06%
fluralaner	0.029	0.051		43.14%
5a	2.85		5.24	45.61%
4a	37.34	-		

^aHigh resistance to fipronil and 4a, no RNAi-compounds.

^bLC₂₀ for *PxRdl2* RNAi-compounds.

^cLCs: The dose corresponding to the mortality of the compound used alone.

^dThe final mortality of *PxRdl2* RNAi-compounds after 48 h.

^eTheoretically reduce dosage = (LCs – LC₂₀ of the *PxRdl2* RNAi compounds)/LCs × 100%.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6