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Realized heritability, cross-resistance patterns, and mechanisms of resistance to thiotraniliprole in *Plutella xylostella* (L.)

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Abstract: *Plutella xylostella*, a major pest of cruciferous vegetables worldwide, has developed resistance to diamide insecticides. Thiotraniliprole, a novel synthetic diamide insecticide, exhibits excellent activity against *P. xylostella*. In the present study, we aimed to confirm the resistance risk, cross-resistance, and mechanisms of resistance to thiotraniliprole in *P. xylostella*. After 40 consecutive generations of thiotraniliprole selection, we obtained a thiotraniliprole-resistance *P. xylostella* strain with a 5141.58-fold resistance ratio (RR) to thiotraniliprole. The overall realized heritability (h^2) value of resistance was estimated as 0.9 using threshold trait analysis, indicating that the risk of developing resistance to thiotraniliprole is high in *P. xylostella.* The thiotraniliprole-resistant (TR) strain showed noticeable cross-resistance to chlorantraniliprole (RR = 44 670.05), cyantraniliprole (RR = 7038.58), and tetrachlorantraniliprole (RR = 1506.01), but no cross-resistance to tolfenpyrad, indoxacarb, diafenthiuron, or abamectin compared with the susceptible (S) strain. The enzyme assay data showed that the activities of glutathione-*S* transferase (GST), carboxylesterase (CarE), and the content of cytochrome P450 monooxygenase (P450s) were significantly higher in the TR strain than in the S strain. Sequencing of the full-length *PxRyR* cDNA revealed the gene site I4790K in the TR strain with a 100% frequency. This mutation in *PxRyR* likely underlies the high-level cross-resistance between thiotraniliprole and three other diamide insecticides. These findings provide valuable information for optimizing resistance management strategies to delay thiotraniliprole resistance development and ensure sustainable control of *P. xylostella*.

Keywords: *Plutella xylostella*; thiotraniliprole; realized heritability; cross-resistance; detoxifying enzymes; ryanodine receptor; resistance mechanisms

小菜蛾对硫虫酰胺抗性的现实遗传力、交互抗性及其抗性机制

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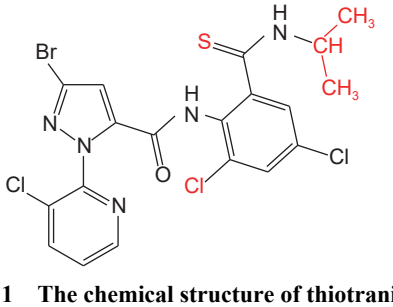
摘 要：小菜蛾是世界范围内重要的十字花科蔬菜害虫，硫虫酰胺是新型双酰胺类杀虫剂，对小菜蛾具有优异活性，本研究旨在研究小菜蛾对硫虫酰胺的抗性风险、交互抗性及其抗性机制。以硫虫酰胺对小菜蛾连续汰选 40 代，获得抗硫虫酰胺品系，其抗性倍数（RR）为 5141.58 倍，现实遗传力（h^2）为 0.9，表明小菜蛾对硫虫酰胺为高抗性风险。硫虫酰胺与氯虫苯甲酰胺（RR = 44 670.05）、溴氰虫酰胺（RR = 7038.05）、四氯虫酰胺（RR = 1506.01）存在显著交互抗性；与唑虫酰胺、茚虫威、丁醚脲和阿维菌素均无交互抗性。抗硫虫酰胺小菜蛾品系解毒酶 GST、P450s 和 CarE 的活性均显著高于敏感品系的。对抗硫虫酰胺小菜蛾 *PxRyR* cDNA 全长进行测序并比对分析发现，I4790K 氨基酸位点突变频率为 100%，这可能是与其他 3 种双酰胺类杀虫剂存在交互抗性的主要原因。该研究对延缓小菜蛾对硫虫酰胺的抗性，指导其抗性治理具有重要意义。

关键词: 小菜蛾； 硫虫酰胺； 现实遗传力； 交互抗性； 解毒酶； 鱼尼丁受体； 抗性机制
中图分类号：S433.3; S482.3 **文献标志码：**A

Plutella xylostella is a devastating pest of cruciferous vegetables that causes significant yield losses worldwide, the annual cost of controlling *P. xylostella* is estimated to be US$ 5 billion since 2012[1]. The rapid evolution of resistance in this pest, fueled by its short life cycle, high fecundity, and intensive use of insecticide, has rendered conventional chemical controls, such as organophosphates, carbamates, and pyrethroids, as well as biopesticides, including abamectin, emamectin benzoate, spinosyns, and Bt protein, ineffective[2-4]. Therefore, it is urgent to develop novel alternatives for *P. xylostella* control.

Diamide insecticides display rapid action, high target mortality, and excellent activity against lepidopteran pests that are already resistant to other insecticides. Since the commercialization of flubendiamide in 2006, followed by chlorantraniliprole (2007) and cyantraniliprole (2012), several new diamide insecticides targeting ryanodine receptor (RyR) have been developed in the past decade[5]. At present, five diamides are classified as Group 28 insecticides by the Insecticide Resistance Action Committee (IRAC). However, field-evolved resistance to diamides has been documented in multiple species, notably *P. xylostella*[6], *Tuta absoluta*[7], *Spodoptera frugiperda*[8], and *Chilo suppressalis*[9]. Among these, the resistance to chlorantraniliprole in *P. xylostella* has evolved most rapidly and extensively. Chlorantraniliprole was commercially launched in China in 2008, and control failures were reported within 2-3 years of intensive use. By 2010-2011, *P. xylostella* populations in Guangdong Province, southern China, exhibited high levels of resistance to chlorantraniliprole[6]. Subsequent studies confirmed varying resistance levels in northern and central China[10-11], as well as in Thailand, the Philippines, and Brazil. The resistance mechanisms of diamide insecticide in *P. xylostella* involve both the metabolic upregulation of detoxification enzymes and target-site mutations in the ryanodine receptor of *P. xylostella* (*PxRyR*)[12]. The increasing resistance to diamide insecticides underscores the necessity for effective resistance monitoring and the development of resistance management strategies for the pest.

Thiotraniliprole is a novel synthetic insecticide commercialized by Zhejiang Udragon Bioscience Co. Ltd.. The chemical structure is shown in **Scheme 1**. The molecular formula of thiotraniliprole is $C_{19}H_{15}BrCl_3N_5OS$, with a relative molecular mass of 547.68, and a CAS number of 1442448-92-1. Thiotraniliprole exhibits low toxicity in mammals and demonstrates high activities against the *Mythmna separata*, *P. xylostella*, *C. suppressalis*, and *Hyphantria cunea*[13-14]. The symptoms of its toxicity-rapid cessation of feeding, lethargy, and muscle paralysis-mirror those of chlorantraniliprole, indicating that it likely acts on the ryanodine receptor.



Scheme 1 The chemical structure of thiotraniliprole

In the present study, we aimed to determine the potential risk of resistance development in *P. xylostella* to thiotraniliprole and to explore the underlying resistance mechanisms. A laboratory-suscepti-

ble strain of *P. xylostella* (termed S) was exposed to thiotraniliprole for 40 generations, resulting in the development of a highly resistant strain (termed TR). The cross-resistance patterns of thiotraniliprole with other insecticides were assessed, and the biochemical mechanisms of resistance were explored by enzyme assays. Furthermore, target-site amino acid substitutions in *PxRyR* were identified in the TR strain. Our findings provide guidelines for developing effective resistance management strategies against *P. xylostella* for thiotraniliprole.

1 Materials and Methods

1.1 Instruments, chemicals, and commercial kits

The primary instruments employed in this study included an Ultraviolet-Visible Spectrophotometer (Shanghai Jinghua Technology Instrument Co., Ltd., Shanghai, China), a Spectrophotometer Multiskan Sky (Thermo Fisher Scientific, Inc., USA), and a 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc., USA).

All insecticides used for the bioassay were as follows: thiotraniliprole (95% TC) from Zhejiang Udragon Biological Technology Co., Ltd. (Hangzhou, China); chlorantraniliprole (95% TC) and cyantraniliprole (90% TC) from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China); tetrachlorantraniliprole (95% TC) from Shenyang Sciencreat Chemicals Co., Ltd. (Shenyang, China); diafenthiuron (95% TC) from Jiangsu Changqing Agrochemical Co., Ltd. (Jiangdu, China); abamectin (92% TC) from Zhejiang Qianjiang Biochemical Co., Ltd. (Haining, China); indoxacarb (95% TC) from Nantong Shizhuang Chemical Co., Ltd (Nantong, China); and tolfenpyrad (97% TC) from Jiangxi Zhengbang Technology Co., Ltd. (Nanchang, China).

To perform the enzyme assays, Protein Quantification Kit (Catalog No.: A045-4), Carboxyesterase Test Kit (Catalog No.: A133-1-1), Glutathione *S*-transferase (GSH-ST) Assay Kit (Catalog No. A004-1-1) and Insect Cytochrome P450 Elisa Kit (Catalog No.: H303) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

For quantitative real-time PCR (RT-qPCR) and *PxRyR* full-length amplification, the following commercial kits were employed: UNlQ-10 Column Trizol Total RNA Isolation Kit (Catalog No.: B511321), SanPrep Column DNA Gel Extraction Kit (Catalog No.: B518131) and ONE-4-ALL Genomic DNA Mini-Preps Kit (Catalog No.: B618503-0100) from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China); FastKing RT Kit (Catalog No.: R116-02) and SuperReal PreMix Plus (SYBR Green) Kit (Catalog No.: FP205-02) from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China).

1.2 Insects and resistance selection

The susceptible parental *P. xylostella* (S) strain was reared and maintained in the laboratory for over 10 years without exposure to insecticides. A thiotraniliprole-resistant (TR) strain was selected for 40 consecutive generations from the S strain under thiotraniliprole pressure. For each resistance selection, 2000 larvae were transferred to cabbage leaves treated with thiotraniliprole at 50% lethal concentration (LC_{50}) for generations F_1 to F_{17}, and 50 mg/L for generations F_{18} to F_{40}. After 48 h, surviving larvae were shifted to fresh, untreated cabbage leaves. All populations were maintained under the controlled condition of (25 ± 1) °C, 60%-70% relative humidity, and a photoperiod of 16 : 8 (L : D) h.

1.3 Bioassays

Leaf-dipping method, as described by Ismail et al [15], was used to determine the susceptibility of second-instar larvae of *P. xylostella* to insecticides. The cross-resistance patterns were evaluated for chlorantraniliprole, cyantraniliprole, tetrachlorantraniliprole, diafenthiuron, abamectin, indoxacarb, and tolfenpyrad. The insecticides were first dissolved in *N, N*-dimethylformamide, and then diluted with distilled water containing 0.1% Tween 80 to produce five to seven serial concentrations. Cabbage leaf discs (5.0 cm diameter) were dipped in the insecticide solution for 10 s, and the control discs were treated with 0.1% Tween 80 solution only. The leaf discs were naturally dried at (25 ± 2)℃, after which 10 second-instar larvae were placed in separate plastic Petri dishes. Each treatment was replicated three times.

1.4 Estimation of realized heritability

Realized heritability (h^2) for thiotraniliprole resistance was estimated as described by Tabashnik et al [16].

$$h^2 = R/S$$

where R is the response to selection and S is the selection differential.

$$R = (\log \text{final } LC_{50} - \log \text{initial } LC_{50})/n$$

where the final LC_{50} was the LC_{50} of TR F_{18}; the initial LC_{50} was the LC_{50} of the parental generation before selection; and n was the number of selections.

$$S = i\delta p$$

where i is the intensity of selection; δp is the phenotypic standard deviation.

$$\delta p = [1/2(\text{initial slope} + \text{final slope})]^{-1}$$

$$i \approx 1.583 - 0.019\,333\,6p + 0.000\,042\,8p^2 + 3.651\,94/p$$

where p is the average survival rate of each generation selection.

The required generation(G) of a 10-fold increase under the pressure of 50%-90% in the field can be predicted by realized heritability.

$$G = 1/(h^2 S)$$

1.5 Enzyme assays

To obtain the enzyme sources of glutathione *S*-transferase (GST), carboxylesterase (CarE), and cytochrome P450 monooxygenase (P450s), 0.1 g third-instar larvae from both the S strain and TR strain (F_{40}) were homogenized in ice-cold PBS buffer solution (0.1 mol/L, pH 7.4). For GST and CarE, the homogenates were centrifuged at 4 °C, 3,000 r/min for 20 min. For P450s, the homogenates were centrifuged at 4 °C, 11 000 r/min for 30 min. The supernatants were collected, and the protein content was determined using the bicinchoninic acid (BCA) method with a Protein Quantification Kit according to the manufacturer's instructions. Three replicates were performed for each treatment group. Absorbance measurements were performed using Ultraviolet-Visible Spectrophotometer and Spectrophotometer Multiskan Sky.

GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate with a Glutathione *S*-transferase (GSH-ST) Assay Kit, and absorbance was measured at 412 nm. The CarE activity assay was performed according to the instructions of the Carboxyesterase Test Kit using α-naphthyl acetate (α-NA) as the substrate, and the change in absorbance at 450 nm was measured from the 10^{th} s to the 190^{th} s.

A commercially available Insect Cytochrome P450 ELISA Kit was used to measure the content of P450s. The samples were added to the enzyme wells pre-coated with antibodies. After an incubation of 30 min at 37 °C, horseradish peroxidase (HRP)-labeled recognition antigen was added. This step allowed both the solid-phase antigen and the formed immune complex to bind. The bound HRP catalyzed the conversion of tetramethylbenzidine (TMB) from a blue color to yellow in the presence of an acid-stop solution. The absorbance was measured at a wavelength of 450 nm, and a standard curve was established to calculate the P450s enzyme content in different samples.

1.6 RNA extractions, cDNA synthesis, and *PxRyR* sequencing

Total RNA was extracted from three 4-instar flash-frozen *P. xylostella* larvae using the UNlQ-10 Column Trizol Total RNA Isolation Kit following the manufacturer's instructions. One μg of total RNA was used for cDNA synthesis with FastKing RT Kit. To amplify the full-length of *PxRyR*, 15 pairs of primers (**Table 1**) were designed based on *PxRyR* sequence (JN801028.1) registered in GenBank, using Primer Premier (version 6.0, Premier Biosoft International, Palo Alto, CA). All the PCR products were purified and sequenced by Sangon Biotech (Shanghai, China). Sequence alignment was performed using the DNAman software (version 8.0, Lynnon Biosoft, Quebec, Canada).

1.7 Quantitative real-time PCR

CYP6BG1, *CYP321E1*, *GSTu1*, *GST2L*, and *CE* have been reported to be associated with *P. xylostella* resistance to chlorantraniliprole. Thiotraniliprole has a similar mode of action to chlorantraniliprole. The relative mRNA expression levels of these five genes were measured using quantitative real-time polymerase chain reaction (RT-qPCR) in TR and S strains of *P. xylostella*. Total RNA was extracted and first-strand cDNA was synthesized as described in Section 1.6. Reactions were performed in a 20 μL mixture containing 10 μL SuperReal PreMix (2 ×), 0.4 μL ROX Reference Dye (50 ×), 3 pmol/mL forward primer, 3 pmol/mL reverse primer, and 1 μL synthesized cDNA. After incubation at 95 °C for 5 min, a total of 40 cycles of PCR were conducted under the following conditions: 10 s at 95 °C, 20 s at 55 °C, and 32 s at 72 °C. Primers used are listed in **Table 2**. Each experiment included three biological replicates. The RT-qPCR data were analyzed and normalized to ribosomal protein L32 using the *Ct* values with the $2^{-\Delta\Delta Ct}$ method.

Table 1 Primers used for the full-length amplification of *PxRyR*

Primer No.	Primer name	Sequence (5′–3′)
1	RT1305-P1-F	ATGGCGGAAGCGGAAGG
	RT1305-P1-R	TCCATCTTGCCCTCTTCGTG
2	RT1305-P2-F	CTCCCATCATCAAGTATGGAGACTC
	RT1305-P2-R	AGATCGTCACCCACTCCGTT
3	RT1305-P3-F	CATAGACCACATCGAGAAGACCAC
	RT1305-P3-R	GGTCCAGCATATAACCATAGACTAGC
4	RT1305-P4-F	GATGGACGTATGGGCTTAATGAG
	RT1305-P4-R	GCAACGAGTAATTTTGAATCGTAAC
5	RT1305-P5-F	TCACTATGAACGATATCAAAGGTCTAC
	RT1305-P5'-F	AAGGTCTACACCGCGACTATGAT
	RT1305-P5'-R	GCCCACTGGTGTGGCTTC
	RT1305-P5-R	CGTGGACTTGCAGAGATTGGT
6	RT1305-P6-F	GGAAGGTCCCTGCACAGTCT
	RT1305-P6'-F	GCTCAGTGCCGCTGTGTTG
	RT1305-P6'-R	TGATCTCTACGTAACGTCTCAACTG
	RT1305-P6-R	GGTAGGTCCGACTGTTTGATCTCT
7	RT1305-P7-F	TCTCAACGACATACAACTACGACAC
	RT1305-P7-R	AGAAGCGCAGGAAGTCCAGAT
8	RT1305-P8-F	AGTTTGATGGAGAATACTGAGCTGG
	RT1305-P8-R	CGTGCTGCCGTAGTATTTTGC
9	RT1305-P9-F	GTCCATGTTGCTGAAACTGCTG
	RT1305-P9-R	CGTGGAGGGTTTCAGCATCT
10	RT1305-P10-F	CTACACAAAGCGAAACAGAACAGAC
	RT1305-P10-R	TTCTTGAGCAGTTGGTTCATGTG
11	RT1305-P11-F	CTTACTCCGACTCCCCTCACG
	RT1305-P11-R	CTCGCTTGCGTTGAATGGAC
12	RT1305-P12-F	CACTGTCGAGAGAATTGTGGCTAT
	RT1305-P12'-F	AGAATTGTGGCTATGAGCAAAGTAC
	RT1305-P12'-R	CCGATGGCTTTGAAGAAGTTAG
	RT1305-P12-R	GGATGACCTCGGTGAGTGTGT
13	RT1305-P13-F	ATCTGCACCGTGGACTACCTACT
	RT1305-P13-R	GTCTGCGATTTTCTGCTTTATGTT
14	RT1305-P14-F	TATGGGTGATGATGATGATGACAGT
	RT1305-P14'-F	TATGGGTGATGATGATGATGACAGT
	RT1305-P14'-R	CCAGTTTACGGGCTATCTCCTT
	RT1305-P14-R	TACTTGACGCGGACCTTCTTCT
15	RT1305-P15-F	TGAAGAAGACGAAGACCCGATAG
	RT1305-P15-R	CTACTCTCCCATGGCGTCCTC

Note: ' Inner primers for nested PCR.

1.8 Genomic DNA extraction and PCR amplification

To extract genomic DNA from a single *P. xylostella*, fourth-instar larvae were selected individually and frozen in liquid nitrogen. Twenty-four isolated samples were prepared for the TR and S strains of *P. xylostella*. Genomic DNA was extracted using a ONE-4-ALL Genomic DNA Mini-Preps Kit. Two pairs of primers (4790-F: 5′- GGAGGCAGCGGTG AAGAAGACGAAG -3, ′4790-R: 5′- CCTTCAAA TGGTAGTACCCGATCAG - 3′; 2447-F: 5′ - GCTAT CTGGACTTCCTGCG -3, ′2447-R: 5′ - GATCAGCA GCCGGATCACC - 3′) flanking the two substitutions (I4790K and C2557W) were used to amplify 148 bp and 592 bp DNA fragments, respectively. The PCR reaction system consisted of 10 μL 2 × Phanta Flash Master Mix, 2 μL genomic DNA template, 0.8 μL of each primer (10 μmol/L), and 6.4 μL ddH_2O, bringing the total volume to 20 μL. The PCR cycling was performed with an initial denaturation at 98 °C for 30 s, followed by 35 cycles (98 °C for 10 s, 58 °C for 5 s, and 72 °C for 5 s) with a final extension at 72 °C for 1 min. The PCR products were purified using a SanPrep Column DNA Gel Extraction Kit and directly sequenced using forward primers (4790-F or 2447-F) by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Table 2 Primers used for RT-qPCR

Primer No.	Primer name	Sequence (5′-3′)
1	CYP6BG1-F	GCAGTGAAGTTGGCTACGAC
	CYP6BG1-R	GTAGTCCTCCACCACCTCAC
2	CYP321E1-F	TCGCCGAAAGTCTTCCAAAT
	CYP321E1-R	CGCTGGATTCGTCTTTCATC
3	GSTu1-F	AGCACAGATGAATCCGCAGAAAGAG
	GSTu1-R	TTTAGCTTCCGTCGGGTAGAGAGG
4	GST2L-F	CGAGTTCAAGCCCAAGACCA
	GST2L-R	CCTTCTCATCTTCTTCGTAGTGGA
5	CE -F	CCGTAGAGATGCGGCTAAAG
	CE -R	AGGTGAACGACGGATAGACG

1.9 Data analysis

Bioassay data were analyzed using probit analysis to determine the LC_{50} value, 95% confidence interval (CI), and linear regression equation using the Statistical Product and Service Solutions Program (SPSS, version 19.0, IBM). A non-overlap of 95% CI was used as the criterion for the significance of differences. Resistance ratios (RR) were calculated as the LC_{50} value of the test strain divided by the LC_{50} value of the S strain. Cross-resistance ratios (CR) were determined by dividing the LC_{50} of each insecticide for the TR strain by the corresponding LC_{50} for the S strain. Enzyme assay data were statistically analyzed using Duncan's new multiple-range method. Student's

t-tests were employed to compare the relative expression levels between different *P. xylostella* strains.

2 Results

2.1 Selection of TR strain

The TR strain of *P. xylostella* (F_{40}) was derived from the S strain after 40 generations of thiotraniliprole selection. The resistance development trends of the TR strain are listed in **Table 3**. During the selection process, the RR development was slow from F_0 to F_4, with the TR strain developing only a 3.63-fold resistance. Subsequently, the RR increase accelerated from F_4 to F_{18}, reaching a 1669.06-fold at F_{18}. From F_{26} to F_{40}, the RR increased more gradually. Ultimately, we obtained a *P. xylostella* strain with high resistance (RR = 5141.58) to thiotraniliprole.

Table 3 Selection of thiotraniliprole resistance in *P. xylostella*

Generation	N[a]	LC_{50}/(mg/L) (95% CI)	Slope ± SE	χ^2 (df)	RR
F_0(S[b])	180	0.072 (0.064-0.082)	4.468 ± 0.222	4.610 (3)	1.00
F_2	180	0.066 (0.043-0.093)	3.766 ± 0.346	6.376 (3)	0.92
F_4	180	0.261 (0.171-0.523)	2.49 ± 0.276	5.628 (3)	3.63
F_6	210	1.303 (1.097-1.569)	2.727 ± 0.274	1.178 (3)	18.10
F_8	210	3.025 (2.447-3.640)	2.527 ± 0.269	0.585 (3)	42.01
F_{10}	210	6.076 (5.173-7.129)	3.020 ± 0.284	3.804 (3)	84.39
F_{12}	210	12.177 (7.972-17.287)	1.199 ± 0.222	1.368 (3)	169.13
F_{14}	210	31.695 (25.876-39.466)	2.202 ± 0.251	4.836 (3)	440.21
F_{16}	210	58.349 (45.950-75.287)	1.829 ± 0.238	0.066 (3)	810.40
F_{18}	210	120.172 (96.233-156.346)	2.013 ± 0.247	2.040 (3)	1669.06
F_{22}	180	219.301(185.760-265.168)	5.411 ± 0.923	1.707 (3)	3045.85
F_{24}	210	265.14(244.477-285.059)	12.587 ± 2.120	0.436 (3)	3682.50
F_{26}	210	294.37(264.124-329.789)	8.006 ± 1.002	2.001(3)	4088.47
F_{28}	210	310.763(284.962-343.192)	9.928 ± 1.852	0.209(3)	4316.15
F_{30}	210	315.939(290.764-339.918)	12.382 ± 2.088	4.409(3)	4388.04
F_{32}	210	337.283(312.301-362.244)	12.474 ± 2.062	1.442(3)	4584.49
F_{34}	210	357.39(318.217-404.565)	7.463 ± 1.668	0.167(3)	4963.75
F_{36}	210	366.092(335.063-396.457)	11.005 ± 1.935	0.276(3)	5084.61
F_{38}	210	366.946(336.809-403.451)	10.147 ± 1.865	0.868(3)	5096.47
F_{40}	210	370.194(339.359-408.399)	9.914 ± 1.849	0.308(3)	5141.58

Note: [a] Total number of *P. xylostella* larvae for bioassay; [b] Susceptible strain of *P. xylostella*.

2.2 Cross-resistance of *P. xylostella* to thiotraniliprole and other insecticides

The TR strain (F_{40}) was used to test cross-resistance to different insecticides (**Table 4**). The TR strain exhibited no significant cross-resistance (RR < 2.5) to tolfenpyrad, indoxacarb, diafenthiuron, or abamectin, but high cross-resistance(RR > 1500) to chlorantraniliprole, cyantraniliprole, and tetrachlorantraniliprole.

2.3 Estimation of realized heritability

After 18 generations of selection with thiotraniliprole, the LC_{50} value increased from 0.072 to 120.172 mg/L and the slopes ranged from 4.468 to 2.013. Threshold trait analysis was performed to estimate the realized heritability (h^2) of *P. xylostella* resistance to thiotraniliprole. The mean overall h^2 was 0.90, with a selection response R of 0.179 and a selection differential S of 0.198 (**Table 5**). However, the h^2 value in the initial eight generations (F_0-F_8, h^2 = 1.08) was higher than that in the last 11 generations (F_8-F_{18}, h^2 = 0.55). The higher h^2 in F_0-F_8 demonstrated that the evolution of the resistance of *P. xylostella* to thiotraniliprole was faster in the initial selection stage. When h^2 was 0.9, it was estimated that two to five generations would be required to achieve a 10-fold resistance under selective pressure resulting in 50%-90% mortality with thiotraniliprole (**Fig. 1**).

2.4 Activities/content of detoxification enzymes

To determine the role of detoxification enzymes in TR, enzyme assays to measure the activities of GST, CarE, and the content of P450s were performed in both

Table 4 Cross-resistance of S and TR strains of *P. xylostella* to other insecticides

Insecticide	Strain	LC_{50}/(mg/L)(95% CI)	Slope ± SE	χ^2(df)	CR[a]
thiotraniliprole	TR[b]	370.194(339.359-408.399)	9.914 ± 1.849	0.308(3)	5141.58
	S[c]	0.072(0.064-0.082)	4.468 ± 0.222	4.610 (3)	
chlorantraniliprole	TR	982.741(922.271-1049.751)	8.210 ± 1.013	2.783(4)	44 670.05
	S	0.022(0.019-0.026)	4.034 ± 0.379	2.070 (3)	
tolfenpyrad	TR	5.584(4.909-6.341)	4.018 ± 0.363	0.623 (3)	1.94
	S	2.872(2.317-3.433)	2.852 ± 0.298	4.179 (3)	
indoxacarb	TR	1.719 (1.475-1.985)	3.485 ± 0.315	3.689 (3)	1.03
	S	1.664(0.783-2.806)	2.360 ± 0.259	7.663 (3)	
diafenthiuron	TR	25.000(16.789-37.226)	3.101 ± 0.288	6.193 (3)	1.10
	S	22.777(20.677-25.081)	6.826 ± 0.580	4.876 (3)	
abamectin	TR	0.384 (0.321-0.486)	2.632 ± 0.272	3.633 (3)	2.37
	S	0.162 (0.066-0.684)	2.001 ± 0.189	6.866 (3)	
cyantraniliprole	TR	872.784(773.263-981.451)	4.118 ± 0.383	3.293(3)	7038.58
	S	0.124 (0.084-0.188)	3.528 ± 0.313	7.272 (3)	
tetrachlorantraniliprole	TR	102.409 (76.594-137.097)	5.276 ± 0.422	6.784 (3)	1506.01
	S	0.068 (0.061-0.075)	4.693 ± 0.465	1.735 (3)	

Note: [a] Cross-resistance ratio; [b] thiotraniliprole resistance strain; [c] Susceptible strain.

Table 5 Estimated realized heritability of resistance to thiotraniliprole in *P. xylostella*

Generation	*N*	Initial LC_{50}/(mg/L)	Final LC_{50}/(mg/L)	*R* [a]	Initial slope	Final slope	*I* [b]	Δp [c]	*S* [d]	h^2 [e]
F_0-F_8	8	0.072	3.025	0.203	4.468	2.527	0.655	0.286	0.187	1.08
F_8-F_{18}	11	3.025	120.172	0.145	2.527	2.013	0.601	0.441	0.265	0.55
F_0-F_{18}	18	0.072	120.172	0.179	4.468	2.013	0.642	0.309	0.198	0.90

Note: [a] Response to the selection; [b] Selection intensity; [c] Phenotypic variance; [d] Selection differential; [e] Estimated realized heritability of resistance.

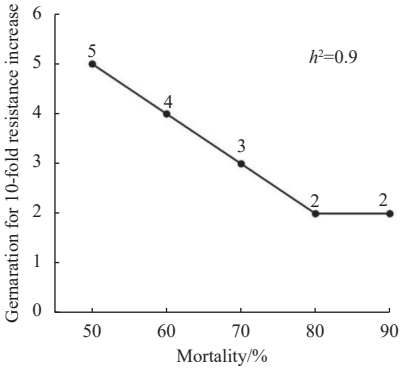


Fig. 1 The predicted generations of *P. xylostella* required 10-fold resistance to thiotraniliprole in the field at the overall h^2 (slope = 3.241)

the TR (F_{40}) and S strains. As shown in **Table 6**, the activities of GST and CarE, the content of P450s in the TR strain were higher than those in the S strain, respectively.

2.5 Expression levels of five detoxification enzyme-related genes in different *P. xylostella* strains

The RT-qPCR results revealed upregulation of *CYP6BG1* (3.41-fold), *CYP321E1* (1.78-fold), *GSTu1* (1.49-fold), and *CE* (1.69-fold) in TR (**Fig. 2**). *GST2L*

Table 6 Activities of GST, CarE, and content of P450s in various *P. xylostella* strains

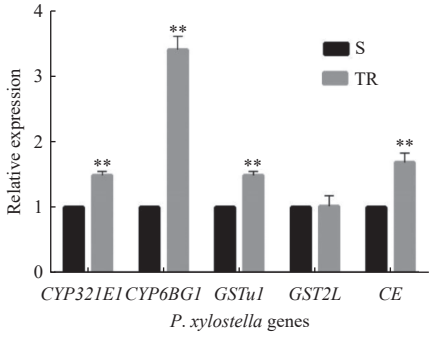
Enzyme	Strain	Enzyme activity/(U/mg protein or ng/mL)*	Rate
GST	S	8.80 ± 0.21	2.67
	TR	23.50 ± 1.22a	
CarE	S	4.69 ± 0.16	1.35
	TR	6.35 ± 0.92a	
P450s	S	17.06 ± 0.25	1.33
	TR	22.71 ± 0.50a	

Note: *Enzyme activity and content are presented as mean ± SD; "a" represents the significant difference in enzyme activity or content between the TR and S strains of *P. xylostella* ($P < 0.05$).

did not show significant upregulation at the mRNA level in the TR strain compared with that in the S strain. *CYP6BG1* was the most highly upregulated detoxification enzyme gene.

2.6 Identification of the mutations in *P. xylostella* TR strain

To identify the mutations, the *PxRyR* fragments from six groups of larvae samples collected from the TR strain were amplified and sequenced, covering the



Relative mRNA expression level was measured using RT-qPCR; values are presented as mean ± SE, $^{**}P < 0.01$.

Fig. 2 Relative expression levels of five *P. xylostella* genes from the S and TR strains

entire coding region. Sequence alignment of TR strain with other *P. xylostella* sequences registered in GenBank (JX467684.1, JQ769303.1, JN801028.1, JF927788.1, JF926694.1, and JF926693.1) revealed that there are two amino acid substitutions in the conserved regions of TR strain *PxRyR* sequence (numbering based JN801028) (**Fig. 3**): (1) at position 2447, a cysteine residue (TGT) is replaced by a tryptophan (TGG; C2447W); (2) at position 4 790, isoleucine (ATA) is replaced by a lysine (AAA; I4790K).

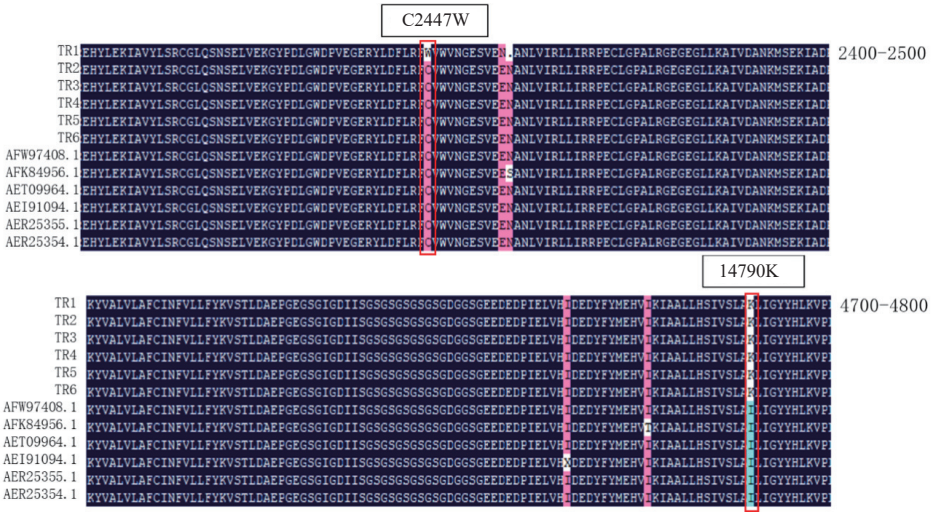


Fig. 3 Alignment of multiple amino acid sequences of *PxRyR* in the TR strain and *P. xylostella* sequences from GenBank

We further investigated the frequency of two substitutions (C2447W and I4790K) in both the TR and S strains. A total of 24 4-instar larvae were selected from each strain and the genomic DNA was extracted. Two pairs of primers were designed and synthesized to amplify the fragment containing the substitutions. The results showed that AAA was detected in all TR samples, while all S samples showed ATA (**Fig. 4**). However, TGT was not detected in any of the TR samples. These results indicate that I4790K may be the primary mutation in *PxRyR* of *P. xylostella* TR strain.

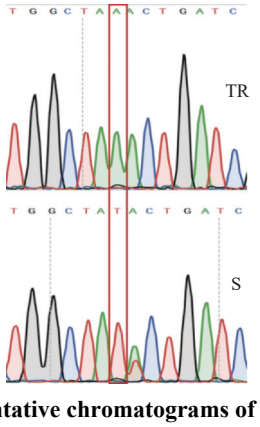


Fig. 4 Representative chromatograms of sequencing PCR amplicons of the *PxRyR* fragment in the TR and S strains

3 Discussion

Thiotraniliprole, a novel insecticide commercialized by Zhejiang Udragon Bioscience Co., Ltd., has been approved for use in China as a 10% SC for the control of *P. xylostella*. Known for its destructive impact, *P. xylostella* has the potential to develop resistance to new pesticides within 2-3 years of field application [17]. Insecticide resistance poses a major risk to the product life cycle and sustainability of integrated pest management programs for *P. xylostella*. The evolution of resistance can be postponed if effective resistance management strategies are employed during insecticide application. To determine the resistance risk of *P. xylostella* to thiotraniliprole, an

insecticide-susceptible strain of *P. xylostella* was continuously selected using thiotraniliprole in the laboratory for 40 generations, and h^2 was estimated using threshold trait analysis. Furthermore, the cross-resistance patterns and resistance mechanisms were investigated.

Cross-resistance is an important component of resistance management programs and a valuable tool for assessing the modes of action of insecticides. In the present study, The TR strain of *P. xylostella* (F_{40}) showed no notable cross-resistance to tolfenpyrad, indoxacarb, diafenthiuron, or abmectin compared with the S strain. However, there was noticeable cross-resistance to chlorantraniliprole (RR = 44 670.05), cyantraniliprole (RR = 7038.58), and tetrachlorantraniliprole (RR = 1506.01). Previous studies have demonstrated cross-resistance among diamide insecticides. Liu et al observed high cross-resistance to chlorantraniliprole (RR = 24.3) and flubendiamide (RR = 14.1) in a cyantraniliprole-selected population[18]. Similarly, cross-resistance has been reported between chlorantraniliprole and flubendiamide in *P. xylostella* populations in Brazil [19]. To mitigate the resistance development of *P. xylostella*, it is advisable to avoid the exclusive use of thiotraniliprole and other Group 28 insecticides throughout a crop cycle. Instead, apply thiotraniliprole and other Group 28 insecticides within a limited time window to prevent consecutive insect pest generations from being exposed to the same mode of action.

h^2 can effectively be employed in evaluating the rate of resistance development and is the proportion of phenotypic variation accounted for by additive genetic variation, which varies with genetic variation and environmental variance[20]. In the present study, the *P. xylostella* strain developed high-level resistance (RR = 1669.06) to thiotraniliprole after successive 18 generations of selection in the laboratory. The value of overall h^2 was estimated as 0.90, only two generations were needed to develop the resistance level to a 10-fold under 90% selection pressure, suggesting a higher risk of resistance to thiotraniliprole. The higher h^2 value indicates the possibility of the rapid development of resistance under the continuous use of thiotraniliprole in the field. Under laboratory conditions, selection pressure and gene mutations may induce higher phenotypic variation; however, field conditions are homogeneous, and multiple factors such as the migration of populations, climatic conditions, and insecticide rotation are related to the development of resistance in pest populations, and the actual rate of field-evolved resistance development could be lower than the predicted rate in the laboratory. However, resistance to chlorantraniliprole and cyantraniliprole has evolved in *P. xylostella* field population[21], therefore, it is essential to implement strict resistance management measures and monitor the development of resistance in field pest populations.

The major detoxifying enzymes P450s, CarE, and GST have been reported to participate in insecticide resistance in insects. In *P. xylostella*, inhibitors, enzyme assays, and transcriptome profiling have been used to identify the roles of these enzyme families in the resistance to diamides. Hu et al speculatedthatGSTmaybethemaindetoxificationmechanism responsible for chlorantraniliprole resistance, noting a significant increase in GST activity[22]. Liu et al found the P450s activity in a chlorantraniliprole-resistant strain was notably higher than that in a chlorantraniliprole-susceptible strain[23]. Furthermore, the overexpression of *CYP6BG1* and *CYP321E1* in *P. xylostella* has been linked to resistance to chlorantraniliprole[24-25]. Knockdown of *PxGST2L* was demonstrated to reduce GST activity and increase the toxicity of chlorantraniliprole in *P. xylostella*[26]. *GSTu1* was confirmed to be involved in chlorantraniliprole resistance via direct degradation of this insecticide[27]. Beyond GST-, P450s-, and CarE-related genes, transcriptome profiling in previous studies revealed that the UDP-glycosyltransferase gene *UGT2B17* and flavin-dependent monooxygenase gene *PxFMO2* are overexpressed in diamide-resistant *P. xylostella* strains[28-29], indicating that the biochemical mechanism of diamide resistance is complex, and may vary among *P. xylostella* populations under different conditions. In this study, biochemical analyses revealed that the GST activity in the TR strain was 2.67-fold higher than that in the S strain, whereas the content of P450s and activity of

CarE increased slightly. However, the mRNA expression level of *GSTu1* was modestly upregulated in the TR strain, and *GST2L* was not significantly upregulated at the mRNA level, suggesting that GST is involved in the detoxification of thiotraniliprole in *P. xylostella* and that other GST genes may also contribute to resistance. Although the content of P450s varied minimally between the TR and S strains, *CYP6BG1* was upregulated at the mRNA level. Therefore, the mechanisms of the metabolic resistance of *P. xylostella* to thiotraniliprole need to be investigated in the future.

Target-site mutations in RyRs are closely associated with diamides resistance[12]. Several such mutations contribute to diamide insecticide resistance in *P. xylostella*. The first identified mutation, G4946E, was detected in a single *P. xylostella* population in Mississippi[30]. The function of the G4946E mutation in diamide resistance has been demonstrated using the Ca^{2+} fluorescence ratio in *Spodoptera frugiperda* (Sf9) cell lines[31]. At present, the G4946E mutation has been reported in at least 10 different countries, including the USA, Japan, India, Thailand, and China, making it the most prevalent mutation[30]. In addition to *P. xylostella*, the G4946E mutation is associated with diamide resistance in several other agricultural insects such as *Tuta absoluta*, *C. suppressalis*, and *Spodoptera exigua*[6,8,32]. I4790M was identified in a diamide-resistant population of *P. xylostella* in China[33]. Wang et al constructed a knock-in homozygous strain (I4790M-KI) of *P. xylostella,* with moderate levels of resistance to flubendiamide[34]. The mutation I4790K was first observed in *P. xylostella* strain collected from the field in Japan in 2017, which showed high resistance to cyantraniliprole. Recently, *P. xylostella* harboring the I4790K mutation has been found in Australia and China[35-36], respectively. The frequency of the I4790K mutation in the field was significantly lower than that of the G4946E mutation in Japan[37-38]. In addition, two amino acid substitutions, E1338D and Q4594L, in the *PxRyR* have also been reported to be involved in resistance to chlorantraniliprole in China[3], although their exact role in resistance requires further investigation. Different independent mutations have been reported to confer varying degrees of resistance to diamides in *P. xylostella*, including G4946E, I4790K, and I4790M[36]. I4790K has shown the highest levels of resistance (RR > 1000) to five commercial diamides, including cyantraniliprole and chlorantraniliprole[37] Neither I4790K nor G4946E confers significant negative effects on diamide resistance[38]. In this study, the I4790K mutation was detected at a high frequency in the *PxRyR* of the TR strain, likely contributing to the notable cross-resistance between thiotraniliprole and other diamide insecticides. This high mutation frequency results in substantial resistance to thiotraniliprole in *P. xylostella* TR strain.

4 Conclusion

Our study predicted a high risk of *P. xylostella* developing resistance to thiotraniliprole and revealed significant cross-resistance to chlorantraniliprole, cyantraniliprole, and tetrachlorantraniliprole. To expand the efficacy of thiotraniliprole in the field, its use should be avoided in areas where *P. xylostella* has demonstrated high resistance to chlorantraniliprole. Resistance management measures, such as the application of synergists, rotation, and mixing with insecticides from different chemical groups, should be implemented. Furthermore, we found that GST, P450s, and CarE were involved in resistance to thiotraniliprole, and the mutation I4790K in *PxRyR* was identified in the TR strain. This study elucidated the resistance mechanism of *P. xylostella* to thiotraniliprole in the laboratory, whether thiotraniliprole will induce other mutations in insect RyRs remains to be verified in future.

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