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Short title: RyR mutations and diamide resistance in diamondback moth

Varying contributions of three ryanodine receptor point mutations to diamide insecticide resistance in *Plutella xylostella*

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Abstract

BACKGROUND: Although decoding the molecular mechanisms underlying insecticide resistance has often proven difficult, recent progress has revealed that specific mutations in the ryanodine receptor (RyR) of the diamondback moth, *Plutella xylostella*, can confer resistance to diamide insecticides. The extent to which specific RyR mutations contribute to the diamide resistance phenotype, the associated genetic traits and fitness costs remain limited.

RESULTS: Three field-evolved PxRyR mutations (G4946E, I4790M, and I4790K) were respectively introgressed into a common susceptible background strain (IPP-S) of *P. xylostella* with marker-assisted backcrossing. The mutations alone can result in moderate to high levels of resistance to five commercial diamides (flubendiamide, chlorantraniliprole, cyantraniliprole, tetraniliprole, and cyclaniliprole), and the resistance intensity mediated by the three mutations was hierachial in order of I4790K (1199- to >2778-fold) > G4946E (39- to 739-fold) > I4790M (16- to 57-fold). Flubendiamide resistance was autosomal and incompletely recessive, and was significantly linked with the introgressed mutations in the three constructed strains. In addition, the resistance levels to flubendiamide of hybrid progeny from any two resistant strains fell in between the status of their parents. Furthermore, by comparing the net replacement rate, the fitness of 4946E, 4790M and 4790K strains were 0.77, 0.93 and 0.92 relative to the IPP-S strain respectively.

CONCLUTION: Three independent PxRyR mutations confer varying degrees of resistance to diamides in *P. xylostella*. Among the three mutations, I4790K confers highest levels of resistance (> 1000-fold) to all five commercial diamides. The findings can guide resistance management practices for diamides in *P. xylostella* and other arthropods.

Key words: Ryanodine receptor; diamide resistance; diamondback moth; genetics; fitness cost

1 INTRODUCTION

Diamides are a novel class of insecticide with fast action, high target-mortality, and an excellent toxicological profile.[1] They can selectively activate the insect ryanodine receptor (RyR), disrupting calcium homeostasis by uncontrolled loss of intracellular calcium stores, ultimately leading to the death of the insect.[2] Diamides were first introduced onto the market with the launch of the phthalic acid diamide, flubendiamide, in 2006.[3] Subsequently, the anthranilic acid diamides chlorantraniliprole and cyantraniliprole were commercialized.[4,5] Cyclaniliprole and tetraniliprole are newly registered diamide insecticides.[6,7] These five diamides are classified as Group 28 insecticides, RyR modulators, by the Insecticide Resistance Action Committee.[8] Because of their suitable biological, ecological, and toxicological properties, diamides can be effectively used to control insect pests that are resistant to other classes of insecticides.[3,9,10]

The diamondback moth, *Plutella xylostella*, is one of the most destructive pests of cruciferous crops, accounting for US $4-5 billion worth of annual economic losses globally, and is the most widely distributed Lepidopteran pest.[11,12] *Plutella xylostella* has developed resistance to as many as 97 chemicals,[13] and was the first species to evolve high levels of resistance to diamides under field conditions in China,[14] Thailand, and the Philippines.[15] As a result of this rapid evolution of resistance to diamides, mechanisms involved in the resistance phenotype of *P. xylostella* have been extensively studied. Documented mechanisms for resistance to diamides (*e.g.* chlorantraniliprole) in *P. xylostella* are mainly associated with mutations in the target site, ryanodine receptor.[15-17]

High levels of diamide resistance were first reported in field populations in 2012,[14] prompting full-length mRNA sequencing of a wild type RyR from a susceptible *P. xylostella* laboratory population (*PxRyR*, GenBank accession no. JN801028).[18] The >16 kb *PxRyR* coding sequence produced a predicted 5164 amino acid protein and this gene model was adopted as a reference for comparing substitutions identified among different populations or species. A point mutation (G4946E) encoding a glycine to glutamic acid substitution in PxRyR codon 4946 was first identified in field populations of *P. xylostella*.[15] The homologous mutation (G4946E/V) was later independently detected in diamide resistant populations of *Tuta absoluta* and *Chilo suppressalis*.[19,20] A second key RyR mutation, I4790M, was also identified in diamide resistant

populations of *P. xylostella*.[16] The homologous mutation has also been found in *T. absoluta*, *C. suppressalis* and two *Spodoptera* species, *S. exigua* and *S. frugiperda*.[19,21-23] Recently, a novel mutation at this same codon position (I4790K) was reported from a Japanese field population of *P. xylostella*.[17] Collectively, a limited number of specific substitution mutations in the diamide target, the RyR, have repeatedly caused high level resistance among numerous insect species and control failure in agriculture.

Functional association between these three RyR substitution mutations and diamide resistance has been demonstrated by several approaches. The significant role of the G4946E mutation in diamide resistance has been confirmed with decreased calcium release responses to diamides in Sf9 cells expressing the mutant *PxRyR* allele,[24] reduced binding of native membrane preparations from a resistant *P. xylostella* to radioligand,[25] and recreation of this RyR mutation using CRISPR/Cas9-mediated homology directed repair in *Drosophila melanogaster* and *S. exigua*.[26,27]

The role of I4790M in conferring moderate level resistance to diamides was demonstrated by introgression of the mutant allele into the wild type background of *S. exigua* (obtaining 21- to 25-fold resistance to diamides)[22] and by CRISPR/Cas9-mediated knockin of this RyR mutation into the wild type *P. xylostella* (conferring 6.0- to 40.5-fold resistance to diamides).[28] More recently, the functional significance of I4790K in diamide resistance was revealed by nearly abolished calcium release responses to diamides in the HEK 293T cell line expressing the mutant *Bombyx mori* RyR.[17]

The Insecticide Resistance Action Committee have provided guidelines which aim to prolong insecticide efficacy using a three-pronged approach: tracking of resistance episodes, identification of resistance mechanisms, and prediction of future resistance events.[29] Modeling the spread of diamide resistance will contribute to the goal of effective resistance management practices, however, this approach would benefit from a better understanding of the intensity, inheritance and fitness costs that different RyR point mutations have on the diamide resistance phenotype. Based on evidence from *S. exigua* and *P. xylostella*, G4946E or I4790M mutation knockin experiments can confer varying levels of resistance to diamides, and resistance is almost completely recessive.[27,28] Interestingly, the fitness cost of RyR mutations are less

predictable. A *P. xylostella* strain exhibiting > 27,000-fold chlorantraniliprole resistance generated fewer, smaller larvae, which took longer to develop and were less likely to successfully pupate than controls in the absence of insecticide.[30] In contrast, a Sudlon strain only showed mild fitness costs, with a 7-14% delay in development across various larval and pupal stages.[31] However, a systemic evaluation of the contribution, genetics, and fitness of these mutations (G4946E and I4790M/K) to diamide resistance in a species has not yet been conducted.

In the current study, the G4946E, I4790M, and I4790K alleles of *PxRyR* were identified among field populations of *P. xylostella* and introgressed into the genetic background of a susceptible reference strain, IPP-S. The contribution these three introgressed alleles had on the diamide resistance phenotype was determined, then inheritance and genetic linkage between flubendiamide and resistance was tested in each strain. Finally, we compared a series of fitness traits between the three introgression strains and susceptible reference. This study provides systemic information about the extent of diamide resistance mediated by RyR mutation in *P. xylostella* and its associated genetic traits. Our findings can be used to guide diamide resistance management practice for this species and other arthropods pests.

2 METHODS AND MATERIALS

2.1 Insects and rearing

Two field populations were collected from China in 2019, FX-19 from the Fengxian district of Shanghai and BY-19 from the Baiyun district of Guangzhou. More than 200 larvae or pupae were collected from each sampling site. The larvae were maintained in the laboratory on radish seedlings (*Raphanus sativus* L.) until pupation. Adults were fed on 10% honey solution and radish seedlings were provided for oviposition.

The susceptible *P. xylostella* strain IPP-S was obtained from Institute of Plant Protection, Chinese Academy of Agricultural Sciences. The IPP-S strain has been maintained in the laboratory without exposure to any insecticides, and reared on an artificial diet for 11 years according to a protocol developed by Anthony Shelton (Cornell Univeristy, https://shelton.entomology.cornell.edu/). For construction of the near-isogenic lines, matings were established then eggs collected on aluminum foil sheets treated with cabbage juice and

transferred onto artificial diet.

All populations were maintained separately at 25 ± 1 ºC, 60 ± 5% relative humidity (RH) and a photoperiod of 16:8 (light: dark) h.

2.2 Chemicals and reagents

Formulated insecticides used for bioassays were chlorantraniliprole (Coragen, 20% suspension concentrate, DuPont Crop Protection, USA), flubendiamide (Phoenix, 20% wettable powder, Nihon Nohyaku Co., Ltd., Japan) and cyantraniliprole (Benevia, 10% oil dispersion, DuPont Crop Protection, USA). Tetraniliprole (90% technical material) was a gift from Bayer Crop Science (China) Co., Ltd. Cyclaniliprole (100% certified reference material) was bought from AccuStandard, Inc. Connecticut, USA. Acetone (> 99.5% analytical reagent) and Triton X-100 (98%) were purchased from Sinopharm Chemical Reagent Co., Ltd. China and Solarbio Science and Technology Co., Ltd. Beijing, China, respectively. Technical tetraniliprole and cyclaniliprole were dissoved respectively in acetone to make 10 mg/mL stock solutions.

2.3 Bioassays

Toxicity of five diamides against three introgression strains were measured using the diet-overlay bioassay method.[28] Briefly, 5 ~ 7 serial dilutions of each diamide were prepared in distilled water containing 0.1% (w/v) Triton X-100. Liquid artificial diet (900 μL) was poured into each well of 24-well plate (diameter = 15.6 mm, Corning Inc. USA). After about 1 h when the diet cooled and solidified, 100 μL of insecticide solution was applied onto the diet surface of every well, then kept at room temperature to air dry. One second-instar larva was placed into each well, and 48 larvae were tested at each concentration. Control diet was treated with distilled water containing 0.1% Triton X-100 solution. All plates were kept at 25 ± 1 ℃, 60 ± 5% RH and 16: 8 (L: D) h in a photoperiod incubator then mortality was recorded after 48 h treatment. Larvae were considered dead if they did not move when gently prodded with a probe. Data were calculated by probit analysis using PoloPlus program.[32]

2.4 Genotyping of the G4946E and I4790M/K mutations of PxRyR

Genomic DNA from *P. xylostella* individuals were extracted with the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Scientific Inc., Union City, CA, USA). Two pairs of

primers (4946-F: 5'-ACTGGCGCTACCAAGTGTGGAAGG-3', 4946-R: 5'-GTTTCCCGTTATGCGTGACAGACTG-3'; 4790-F: 5'-GGAGGCAGCGGTGAAGAAGACGAAG-3', 4790-R: 5'-CCTTCAAATGGTAGTACCCGATCAG-3') flanking the PxRyR G4946E and I4790M/K mutation sites were used to amplify 193-bp and 148-bp DNA fragments, respectively. The PCR solution consisted of 12.5 μL 2× Taq Master Mix (Novoprotein Scientific Inc. China), 1 μL gDNA template, 1 μL of each primer (10 μM) and 9.5 μL PCR-grade water to a final volume of 25 μL. DNA amplification was performed for 35 cycles (94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) with a final extension at 72 °C for 10 min. The PCR products were purified by an AxyPrep^{TM} DNA Gel Extraction Kit (Axygen Scientific Inc., Union City, CA, USA), and directly sequenced with the forward primer (4946-F or 4790-F) by TSINGKE Biological Technology (Nanjing, China). Genotypes were identified from the chromatogram and denoted as susceptible homozygote ($4946^{G/G}$ and $4790^{I/I}$), heterozygote ($4946^{G/E}$, $4790^{I/M}$ and $4790^{I/K}$) and resistant homozygote ($4946^{E/E}$, $4790^{M/M}$ and $4790^{K/K}$) (Fig. 1).

2.5 Introgression of *PxRyR* mutant alleles into the susceptible IPP-S strain

A marker-assisted introgression strategy was employed to construct three strains carrying PxRyR mutations I4790K, I4790M or G4946E, in the IPP-S genetic background (Fig. 2). The G4946E mutation strain was developed from a field population FX-19. Briefly, the mutant G4946E allele identified in population FX-19 was introgressed into the susceptible IPP-S strain by single-paired crossing between a mutant homozygote from FX-19 ($4946^{E/E}$) and a wild type homozygote from IPP-S ($4946^{G/G}$), followed by three rounds of backcrossing with IPP-S and molecular marker assisted selection. The final G4946E introgression strain is referred to as "4946E". The mutant I4790M and I4790K alleles from the BY-19 population were also successfully introgressed into the susceptible IPP-S strain by similar approach, and the two introgression strains were named as "4790M" and "4790K" respectively. Through genotyping-assisted backcrossing (Fig. 2), each introgressed strain (4946E, 4790M and 4790K) was homozygous for the corresponding mutant allele and shared about 94% (15/16) of its genetic background with that of the recipient IPP-S strain.

2.6 Inheritance and genetic linkage analysis of resistance to flubendiamide

The 4th instar male and female larvae of *P. xylostella* were visually determinated based on the

color of the fifth abdominal segment.[33] Thirty male moths from the susceptible IPP-S strain were mass-crossed with 30 virgin female moths from introgression strains 4946E, 4790M or 4790K. The reciprocal crosses were also performed. Toxicity responses of the hybrid F_1 progeny from the reciprocal crosses to flubendiamide were measured using the diet-overlay method. The dominance value (*D*) was calculated using Stone's formula[34]: $D = (2X_2 - X_1 - X_3)/(X_1 - X_3)$, where X_1, X_2 and X_3 are logarithms of LC_{50} values for mutation introgressed strain, F_1 hybrids and IPP-S strain, respectively. *D* values ranged from -1 (completely recessive) to +1 (completely dominant).

For genetic association analysis between introgressed mutations (G4946E, I4790M or I4790K) and the flubendiamide resistance phenotype, thirty male F_1 progeny (from the introgressed resistant males × IPP-S females) were mass-backcrossed with 30 females of the introgressed resistant strain (Fig. 3). Newly hatched larvae of the backcross progeny (BC) were treated with 0.15 μg/cm^2 of flubendiamide, and the survivors (BC-treated) were collected after 48 h treatment. The DNA from random selected parents (IPP-S and mutation introgressed strains), BC-treated survivors and BC-untreated individuals were extracted for genotyping.

2.7 Life table construction

Newly hatched neonates from IPP-S, 4946E, 4790M and 4790K strains were randomly collected. Twenty individuals, representing one treatment, were placed on artificial diet (4 mL) in a plastic cup (diameter = 6.5 cm), with 14 or 15 simultaneous replications for each strain. All the cups were sealed by black gauze, and then placed into incubator with 25 ± 1 ℃, 60 ± 5% RH and 16h: 8h L: D. Larval development time and survivorship in each plastic cup were recorded at a fixed time each day. Pupae were weighed within 8 h of pupation and placed in a 2.0 mL eppendorf tube with perforated lid for emergence. Around three days later, emergence time of each pupa was recorded and their gender visually determined.

Twenty-four pairs of newly emerged male and female moths for each strain were used for evalution of fecundity. Each single pair was put into a transparent plastic cup (covered with gauze and provided 10% honey solution) for mating and oviposition. A piece of aluminum foil pre-treated with cabbage juice was placed into each cup and was replaced with a new one at a fixed time daily. The eggs were collected at an interval of 24 h for assessment of fecundity and

hatchability.

Statistical analysis was performed using method described by Wang and Wu.[35] All the obtained data were checked for normality using non-parametric Kolmogorov–Smirnov test ($p < 0.05$). Data conforming to a normal distribution (pupae body weight, emergence rate, female ratio, fecundity) were tested using one-tailed Student's *t*-test, while those with non-normally distributed (pupa duration, larvae duration, pupation rate, hatchability) were compared using one-tailed Mann-Whitney *U*-test. The net reproductive rate (R_0) was calculated as $R_0 = N_{n+1}/N_n$, where N_n is the neonate number of the parent generation and N_{n+1} is that of the next generation. The relative fitness of the introgressed resistant strain is calculated as the ratio of R_0 of the resistant strain to R_0 of the susceptible IPP-S strain.

3 RESULTS

3.1 Establishment of PxRyR G4946E mutation introgressed strain of *P. xylostella*

Ten single-pair families were established between FX-19 males (the second generation progeny of the field-collected individuals) and IPP-S females. After oviposition, the 10 male parents were genotyped and one (No.3) was homozygous for glutamic acid at PxRyR codon 4946 (Table 1). Nine single-pair families were established from these F_1 males and IPP-S females to generate backcross generation one (BC_1). Two further rounds of single-pair matings between male BC progeny and IPP-S females, followed by DNA-based genotyping assays to identify heterozygotes, produced BC_3 (Fig. 2). Then, 10 single-pair families from BC_3 siblings were established and genotyped following oviposition, revealing one pair were both $4946^{G/E}$ heterozygotes and their progeny (BC_3F_1) were reared. Finally, eight single-pair families from BC_3F_1 were established and following oviposition, both parents from one pair among the eight sets were genotyped as $4946^{E/E}$ homozygotes. The progeny of this pair were pooled to establish the 4946E strain. By genotyping 10 individuals randomly sampled before bioassays, the 4946E strain was confirmed to be homozygous for the mutant G4946E allele and the wild type 4790I allele.

3.2 Establishment of PxRyR I4790K and I4790M mutation introgressed strains of *P. xylostella*

Twenty-four single-pair crosses were constructed between BY-19 field-collected males and IPP-

S females. After oviposition, genotyping identified 16 males as mutant $4790^{K/K}$ homozygotes that were expected to produce $4790^{I/K}$ heterozygous progeny (Table 1). Then, 10 single-pair crosses between heterozygous male progeny ($4790^{I/K}$ from the No.10 set, Table 1) and IPP-S females were established to generate BC_1. Two further rounds of single-pair matings between male BC progeny and IPP-S females, followed by DNA-based genotype assays, produced BC_3 (Fig. 2). Subsequently, 15 single-pairs from BC3 siblings were established, and both parents from three sets of them were genotyped as $4790^{I/K}$, and their 240 progeny (BC_3F_1) were screened with the diagnostic dose of flubendiamide (0.15 μg/cm^2, expected to kill all susceptible larvae) and 45 larvae survived. Finally, 12 single-pair families between the survivals were established to generate BC_3F_2. By genotyping, both parents of the 12 pairs were mutant $4790^{K/K}$ homozygotes. BC_3F_2 progeny were pooled to generate the 4790K strain. Again, by genotyping 10 individuals randomly sampled before bioassays, the 4790K strain was confirmed to be homozygous for the mutant I4790K allele and homozygous for the wild type 4946G allele.

The 4790M strain was developed from a F_0 male (Fig. 2) with a $4790^{M/K}$ corssed with an IPP-S $4790^{I/I}$ female. Thirteen single-pair families were then established from F_1 males and IPP-S females, and four of the males were genotyped as $4790^{I/M}$ heterozygotes. Subsequently, two further rounds of single-pair matings between $4790^{I/M}$ males and IPP-S females were performed to produce BC_3 (Fig. 2). BC_3 siblings were mated in single pairs (n = 15) and after oviposition, one pair were genotyped as $4790^{I/M}$. Their 144 progeny were treated with 0.15 μg/cm^2 flubendiamide and 30 larvae survived (BC_3F_1). Ten single-pair families between the 30 survivors were established to produce BC_3F_2. After oviposition, all the 20 parents of the 10 single-pair families were identified to be mutant $4790^{M/M}$ homozygotes. BC_3F_2 progeny were pooled to generate the 4790M strain. Prior to performing bioassays, 10 randomly selected individuals from the 4790M strain were gentotyped and confirmed homozygous for the I4790M allele and homozygous for the wild type 4946G allele.

3.3 Magnitude of diamide resistance in *P. xylostella* with different PxRyR mutations

To evaluate and compare the extent to which G4946E, I4790M or I4790K mutations contribute to diamide resistance, the toxicity of five chemicals were assessed against the susceptible IPP-

S strain and three introgressed strains (Table 2). When compared to the background IPP-S strain, the 4946E strain showed moderate resistance to chlorantraniliprole, cyantraniliprole and cyclaniliprole (39-fold to 54-fold) and high resistance to flubendiamide (365-fold) and tetraniliprole (739-fold). In contrast, the 4790M strain showed 16- to 57-fold resistance to all the five ingredients. Interestingly, the 4790K strain developed the highest resistance to all tested diamides with resistance ratios ranging from 1199- to > 2778-fold.

3.4 Inheritance of resistance to flubendiamide among three introgression strains

The toxicities of flubendiamide against the susceptible IPP-S strain, three genetically pure strains (4946E, 4790M and 4790K) and their F_1 progeny from reciprocal crosses were tested (Table 3). The LC_{50}s of F_1 progeny from reciprocal crosses (Resistant ♀ × IPP-S ♂, IPP-S ♀ × Resistant ♂) had no significant difference, with overlapping 95% fiducial limits. These data demonstrate the absence of sex-linked or maternal effects, and indicate flubendiamide resistance in the three mutation introgressed strains are autosomally inherited.

The log concentration-probit (LC-P) regression lines for the six F_1 progeny crosses were very close to that of the IPP-S strain (figure not shown). The dominance values (*D*) were ranged from < -0.85 to -0.43 for flubendiamide, suggesting an incompletely recessive mode of inheritance for flubendiamide resistance in the 4946E, 4790M and 4790K strains, respectively.

3.5 Complementation tests between strains

The responses of hybrid progeny from crosses between any two of the three introgressed resistant strains to flubendiamide were tested (Table 3). The progeny from 4790M males crossed with 4946E females, and vice versa, showed 119- and 120-fold resistance to flubendiamide respectively, compared to the background IPP-S strain. Similar patterns were also observed in hybrid progeny from 4790M and 4790K strains (146- and 115-fold). However, the hybrids from 4790K and 4946E strains, which were heterozygous at codon $4790^{(I/K)}$ and $4969^{(G/E)}$, exhibited more than 2000-fold resistance to this chemical. Overall, the resistance levels of hybrid progeny to flubendiamide were intermediate the responses of their parents.

3.6 Genetic linkage between three PxRyR mutations and flubendiamide resistance

The causal relationship between the introgressed PxRyR G4946E, I4790M or I4790K mutations and flubendiamide resistance was investigated by a set of genetic crosses (Fig. 3). For genetic

likage analysis of the G4946E allele and flubendiamide resistance, IPP-S adults were firstly reciprocally mass-crossed with moths from the introgressed 4946E strain to produce F_1 progeny. Male moths from the pooled F_1 progeny were mass-backcrossed to the female moths from the 4946E strain to generate backcross progeny. If resistance is monogenic, half the BC progeny were expected to be homozygous for G4946E and survive a discriminating dose of flubendiamide. When treated by the diagnostic concentration of flubendiamide (0.15 μg/cm^2), 52.3% larvae (38/72) survived after 48 h exposure (Table 4). All 30 survivors from the BC-treated group were G4946E mutant homozygotes (rr). In contrast, 30 BC-untreated individuals were genotyped as either heterozygotes (rs: 14) or mutant homozygotes (rr: 16). The data indicate the introgressed G4946E mutation of PxRyR is significantly linked (Fisher's exact test, $p < 0.0001$) with flubendiamide resistance in the 4946E introgressed strain.

Similarly, for the other two backcross sets, the survival rates in the BC-treated groups were 49.2% and 49.7% for I4790M and I4790K respectively. The survivors genotyped in BC-treated groups were also mutant homozygotes, and the larvae genotyped in BC-untreated groups around half homozygous mutants and half heterozygous mutants (Table 4). The results demonstrate that the introgressed I4790M and I4790K mutation are also significantly linked (Fisher's exact test, $p < 0.0001$) with observed resistance phenotype.

3.7 Relative fitness comparison

To determine the potential fitness cost, life tables of the susceptible IPP-S and the introgressed resistant strains (4946E, 4790M and 4790K) were constructed (Table 5). Regarding development, significantly longer larval duration was observed in the 4946E strain. The male pupal duration of 4946E (4.03 ± 0.07 days) was also significantly longer than that of the susceptible IPP-S strain, whereas the female pupal duration of 4790M (3.64 ± 0.04 days) and pupal duration of 4790K were significantly shorter than the background reference strain. The pupae body weight of 4790M was significantly less than IPP-S. Moreover, compared with IPP-S (99.33 ± 0.01%), the pupation rate was significantly reduced in 4946E (92.33 ± 0.01%) and 4790M (95.00 ± 0.01%) strains. Female ratios of 4790K (39.31 ± 0.03%) was significantly lower than that of IPP-S strain (48.30 ± 0.04%). Interestingly, the hatchability of 4946E (74.38 ± 0.04%) was reduced, while that of 4790K (89.94 ± 0.02%) significantly increased.

Based on the net replacement rate (R_0), the relative fitness of the 4946E, 4790M and 4790K strains were estimated to 0.77, 0.93 and 0.92, respectively, compared with the background strain IPP-S (R_0 = 1.00). This indicates that there are relatively higher fitness costs associated with diamide resistance in 4946E than 4790M and 4790K strains of *P. xylostella* under laboratory conditions.

4 DISCUSSION

Diamide insecticides have been in wide use for more than 10 years, gaining economic and agricultural importance at a global scale.[36] However, extensive application of diamides against *P. xylostella* resulted in compromised field efficacy against this insect pest. Several mutations that occur in insect RyR have been identified, and their direct association with diamide resistance was confirmed in a number of studies.[29,36] However, resistance ratios have been generated by comparing field selected strains with laboratory references which can introduce strain specific bias and potentially mask the true extent of the diamide resistant phenotype. In the present study, we successfully introgressed the G4946E, I4790M, and I4790K alleles from field-derived populations into a common susceptible background strain. We then determined the contribution of each mutation to diamide resistance, confirmed a genetic association with flubendiamide resistance and calculated fitness of the introgressed strains.

The contribution of resistance to three diamides (chlorantraniliprole, cyantraniliprole, and flubendiamide) of the single G4946E has been evaluated by genome editing approach. With CRISPR/Cas9-mediated mutation knockin, an edited *S. exigua* G4946E strain showed high levels of resistance to flubendiamide (> 1000-fold), chlorantraniliprole (223-fold), and cyantraniliprole (336-fold).[27] In the current study, the acquired resistance levels to the three diamides (365-fold to flubendiamide, 52- and 54-fold to the other two) were relatively consistant with the above results. Namely, the G4946E allele knockin or introgression results in the resistance pattern as flubendiamide > chlorantraniliprole ≈ cyantraniliprole. Of course, the resistance ratios for G4946E mutation strains exhibited significant variance in *S. exigua* and *P. xylostella*. This might be due to different interaction intensity between the receptor site(s) of SeRyR or PxRyR and diamides.

When the naturally-occurring I4790M mutation was knocked into *P. xylostella,* the

obtained strain exhibited moderate resistance to flubendiamide (40.5-fold) and low resistance to chlorantraniliprole and cyantraniliprole (6.0- and 7.7-fold, respectively).[28] In this study, the I4790M introgressed strain of *P. xylostella* also showed moderate levels of resistance (16- to 57-fold) to the three chemicals. Similarly, the introgressed I4790M *S. exigua* strain had 21- to 25-fold resistance to to flubendiamide, chlorantraniliprole and cyantraniliprole.[22] In contrast, the current study revealed that the I4790K mutation confers very high levels of resistance (> 1000-fold) to all five commercial diamides. The I4790K mutation was shown to cause high levels of diamide resistance in field populations of *P. xylostella* from Japan[17] and the mutation has subsequently been identified in Australia.[37] Here we report the discovery of the same I4790K mutation for the first time among field populations in China. In view of the extreme high levels of diamide resistance attributed to the I4790K mutation, and the recent detection of this allele in multiple countries, ongoing monitoring of field populations will be necessary to tract the prevalence of I4790K among geographical regions that routinely use diamides to control populations of *P. xylostella*.

Because the newly developed diamides tetraniliprole and cyclaniliprole act on insect RyR, we hypothesized that cross resistance may occur between these two compounds and previous insecticides (chlorantraniliprole, flubendiamide, and cyantraniliprole). Here, we showed that the G4946E, I4790M, and I4790K mutations of PxRyR confer moderate to high levels of resistance to all five diamides in *P. xylostella*. Thus, target-site mutation-based cross resistance was clearly observed in this study. This finding implies that cross resistance among diamides does occur against field populations of *P. xylostella* carrying these specific RyR mutations.

The toxicity results from this study can be summarized as (i) resistance levels mediated by PxRyR mutations can be ranked in the order I4790K > G4946E > I4790M, which enriches the previous evidence (G4946E > I4790M) from *in vivo* assays. (ii) each introgression strain exhibited the highest level of resistance to flubendiamide among the five tested diamides, indicating that 4946G and 4790I are higher affinity binding sites for flubendiamide than for the other four diamides. (iii) resistance levels of hybrid progeny from crosses between any two of the three introgressed strains to flubendiamide fell in between the corresponding RRs of their parents. Our study not only systematically assessed the relative contribution of major RyR mutations to diamides-resistant phenotypes in *P. xylostella*, but also provides an important

reference for diamide resistance assessment and management in other arthropods.

Genetic linkage analysis can help to confirm the relationship between target-site mutations and insecticide resistance phenotypes. Aiming to eliminate potential off-target effects, this test is now commonly used in the field of insecticide resistance gene editing. For example, knockout of the cadherin gene in *Helicoverpa armigera*, *CYP9M10* gene in *Culex quinquefasciatus*, *nAChRα6* in *P. xylostella*, *S. exigua* and *H. armigera*, ATP-binding cassette (ABC) transporter *C2* in *Ostrinia furnacalis*, and knock-in of the PxRyR I4790M mutation in *P. xylostella* were each linked with the corresponding resistance phenotype.[28,38-43] In the current study, we found that the observed flubendiamide resistance in three introgressed resistant strains was genetically linked with G4946E, I4790M, and I4790K mutations, respectively. Using introgression, we therefore confirmed that these diamide resistance phenotypes are mediated by three independent PxRyR mutations.

Inheritance studies of diamide resistance over the past decade have demonstrated that insect resistance mediated by RyR mutation is autosomal and incompletely recessive.[36] Flubendiamide resistance in a *P. xylostella* population from the Philippines (mediated by G4946E) was monogenic and recessive.[25] A recent gene editing study performed in *P. xylostella* showed that, when I4790M is the only mechanism, flubendiamide resistance is incompletely recessive.[28] Flubendiamide resistance observed in our three introgressed strains was inherited in a recessive mode, with *D* values ranging from < -0.85 to -0.43. Consistent with these findings, a diamide-resistant *T. absoluta* strain carrying G4946E and I4790M mutations, a G4946E mutation knockin strain of *S. exigua*, and an I4790M introgressed strain of *S. exigua* were also found to be incompletely recessive.[19,22,27] Management strategies can delay the development of this recessive resistance trait more readily than if it were a dominant or incompletely dominant trait. Several resistance management solutions, such as minimizing diamide applications and use of a rotation program that involves insecticides with different mode of action, prove useful with continuous practice.

Crossing introgression strains 4790K and 4946E produced heterozygotes with one wild type allele and one resistance allele at each of these codon positions. We unexpectedly observed very high levels of resistance to flubendiamide, which surpassed LC_{50} levels of 4946E homozygotes and was relatively similar to that of 4790K homozygotes (>2,000 fold resistance).

Insects have only one gene encoding ryanodine receptors and insect RyRs are homomeric tetramers[44]. It is suggested that the PxRyR teteramers that form with both $4790^{I}/4946^{E}$ and $4790^{K}/4946^{G}$ subunits may be sufficient for conformational change that prevents flubendiamide interaction.

Insecticide resistance evolution in insects often has an overall fitness cost, when compared to susceptible counterpart. Significant deleterious effects on growth, survival rates, fecundity, or hatchability have been demonstrated in *P. xylostella* strains with resistance to pyridalyl, abamectin, fufenozide, metaflumizone, and tebufenozide.[35,45-48] Negative genetic trade-offs have been identified in *P. xylostella* as a consequence of diamide selection. Larval development time, pupation and emergence rate, and fertility were strongly affected in a cyantraniliprole-selected laboratory strain.[49] When insecticide selection pressure was absent, a field-evolved chlorantraniliprole-resistant *P. xylostella* strain showed significantly reduced larval weight and fecundity.[30] However, some positive effects (e.g. egg hatchability and longevity) were observed in *P. xylostella* when the insects were treated with a sublethal concentration of chlorantraniliprole.[30,50] However, no study on the fitness costs of diamide resistance mediated by RyR mutations gave unambiguous results. In this study, we found the effects on the fitness component of different mutations differed. For example, G4946E introgression resulted in longer female pupa duration, while I4790K reduced the parameter in both males and females. Interestingly, significantly higher hatchability was observed in the I4790K introgressed resistant strain. Despite this, it is likely that the impact on physiological functions of G4946E is significantly larger than those of the I4790M/K mutations. We speculate that the fitness costs resulting from PxRyR mutations might be higher in the field than in the lab, and that the I4790K mutation will increase prevalence in the field.

Our findings indicate that a single PxRyR mutation (G4946E, I4790M or I4790K) can cause moderate to high levels of resistance to diamides in *P. xylostella*, and the resulting flubendiamide resistance is inherited in a recessive mode. Furthermore, we found negative physiological traits could emerge without sustainable diamide selection in the introgressed resistant strains. The information from this study will help to optimize strategies for diamide resistance management based on molecular screening in the field.

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Table 1. Ryanodine receptor genotypes of individual larvae from two field populations of *P. xylostella*

FX-19 population (G_2)			BY-19 population (G_0)		
No.	4790aa	4946aa	No.	4790aa	4946aa
1	I/I	G/E	1	K/K	G/G
2	I/I	G/G	2	I/K	G/G
3 [a]	I/I	E/E	3	K/K	G/G
4	I/I	G/G	4	K/K	G/G
5	I/I	G/E	5	I/K	G/E
6	I/I	G/E	6	I/K	G/G
7	I/I	G/E	7 [a]	K/M	G/G
8	I/I	G/G	8	K/K	G/G
9	I/M	G/E	9	K/K	G/G
10	I/M	G/E	10 [a]	K/K	G/G
			11	K/K	G/G
			12	K/K	G/G
			13	I/K	G/G
			14	I/I	E/E
			15	K/K	G/G
			16	K/K	G/G
			17	K/K	G/G
			18	K/K	G/G
			19	K/K	G/G
			20	I/I	G/E
			21	K/K	G/G
			22	K/K	G/G
			23	K/K	G/G
			24	I/K	G/G
Mutated allele frequency			Mutated allele frequency		
4790M	0.10		4790M	0.02	
4790K			4790K	0.79	
4946E		0.40	4946E		0.08
Pooled	0.10	0.40	Pooled	0.81	0.08

[a] The single-pair families selected to establish the three near-isogenic strains (4946E, 4790M and 4790K).

Table 2. Toxicity of diamides against the susceptible *P. xylostella* reference strain IPP-S and introgressed strains 4946E, 4790M or 4790K

Strain	Insecticide	N [a]	Slope ± SE	LC_{50} (μg/cm^2)	95% Fiducial limits	RR [b]
IPP-S	Chlorantraniliprole	192	1.98 ± 0.31	0.027	0.022 - 0.034	
	Cyantraniliprole	240	3.14 ± 0.37	0.030	0.015 - 0.051	
	Flubendiamide	288	1.65 ± 0.18	0.018	0.014 - 0.022	
	Tetraniliprole	240	2.43 ± 0.28	0.008	0.006 - 0.010	
	Cyclaniliprole	288	1.75 ± 0.21	0.006	0.005 - 0.008	
4946E	Chlorantraniliprole	240	5.11 ± 0.61	1.450	1.072 - 1.838	54
	Cyantraniliprole	288	4.07 ± 0.40	1.559	1.167 - 1.997	52
	Flubendiamide	288	1.99 ± 0.26	6.575	5.440 - 7.927	365
	Tetraniliprole	240	3.38 ± 0.35	5.915	3.294 - 8.713	739
	Cyclaniliprole	288	1.90 ± 0.21	0.233	0.184 - 0.288	39
4790M	Chlorantraniliprole	240	2.46 ± 0.26	0.568	0.370 - 0.991	21
	Cyantraniliprole	288	2.69 ± 0.28	0.477	0.313 - 0.699	16
	Flubendiamide	288	3.16 ± 0.35	1.028	0.910 - 1.172	57
	Tetraniliprole	336	1.67 ± 0.16	0.183	0.147 - 0.234	23
	Cyclaniliprole	336	2.46 ± 0.21	0.093	0.078 - 0.110	16
4790K	Chlorantraniliprole	240	5.47 ± 0.86	43.73	40.51 - 48.68	1620
	Cyantraniliprole	288	4.54 ± 0.47	52.86	41.37 - 69.28	1762
	Flubendiamide [c]	192	0.95 ± 0.34	> 50	NA	> 2778
	Tetraniliprole	288	4.11 ± 0.48	23.22	21.20 - 25.56	2902
	Cyclaniliprole	240	2.94 ± 0.32	7.192	6.128 - 8.520	1199

[a] Numbers of larvae used in bioassay, including untreated controls.

[b] RR (Resistance ratio) = LC_{50} of an introgression strain / LC_{50} of IPP-S.

[c] LC_{50} of flubendiamide against 4790K strain could not be determined because of an insufficient dose response (only 27% mortality at 50 μg/cm^2 of flubendiamide treatment).

Table 3. Toxicity of flubendiamide against *P. xylostella* larvae from a susceptible strain (IPP-S), three resistant strains (4946E, 4790M, and 4790K), and the F_1 progeny from crosses between strains

Strain/cross	N [a]	Slope ± SE	LC_{50} (μg/cm^2)	95% Fiducial limits	RR [b]	*D* [c]
IPP-S	288	1.65 ± 0.18	0.018	0.014 -0.022		
4946E	288	1.99 ± 0.26	6.575	5.439-7.927	365	
4790M	288	3.16 ± 0.35	1.028	0.910-1.172	57	
4790K [d]	192	0.95 ± 0.34	> 50	NA	> 2778	
Mode of inheritance tests						
4946E♀×IPP-S♂	288	2.65 ± 0.26	0.035	0.029-0.041	1.9	-0.77
4946E♂×IPP-S♀	288	2.01 ± 0.20	0.049	0.037-0.055	2.7	-0.65
Pooled	576	2.26 ± 0.16	0.040	0.035-0.045	2.2	-0.73
4790M♀×IPP-S♂	288	2.78 ± 0.26	0.057	0.048-0.067	3.2	-0.43
4790M♂×IPP-S♀	288	2.85 ± 0.28	0.049	0.041-0.058	2.7	-0.50
Pooled	576	2.79 ± 0.19	0.053	0.047-0.059	2.9	-0.47
4790K♀×IPP-S♂	288	2.74 ± 0.27	0.033	0.028-0.039	1.8	< -0.85
4790K♂×IPP-S♀	288	2.39 ± 0.24	0.032	0.026-0.038	1.7	< -0.85
Pooled	576	2.55 ± 0.18	0.033	0.028-0.037	1.8	< -0.85
Complementation tests						
4790M♂×4946E♀	336	3.89 ± 0.36	2.138	1.919 - 2.368	119	
4790M♀×4946E♂	384	3.66 ± 0.35	2.156	1.924 - 2.397	120	
4790M♂×4790K♀	288	3.49 ± 0.35	2.634	2.276 - 3.048	146	
4790M♀×4790K♂	288	2.47 ± 0.26	2.067	1.692 - 2.485	115	
4790K♂×4946E♀	288	7.29 ± 0.78	37.16	32.83 - 43.54	2064	
4790K♀×4946E♂	384	4.85 ± 0.46	41.52	35.38 - 50.97	2307	

[a] Numbers of larvae used in bioassay, including the control.

[b] RR (Resistance ratio) = LC_{50} (an introgressed resistant strain or F_1 progeny) / LC_{50} (IPP-S).

[c] The degree of dominance (*D*) ranges from -1 (completely recessive) to +1 (completely dominant).

[d] LC_{50} of flubendiamide against 4790K strain could not be determined because of an insufficient dose response (only 27% mortality at 50 μg/cm^2 of flubendiamide treatment).

Table 4. Genetic association between the introgressed resistant alleles and flubendiamide resistance phenotype in three resistant strains of *P. xylostella*

Strain	Survival [a]	Number of individuals for each genotype [b]			
		Total	*ss*	*rs*	*rr*
IPP-S		30	30	0	0
4946E		30	0	0	30
BC-untreated group		30	0	14	16
BC-treated group	52.3% (38/72)	30	0	0	30
IPP-S		30	30	0	0
4790M		30	0	0	30
BC-untreated group		30	0	10	20
BC-treated group	49.2% (92/187)	30	0	0	30
IPP-S		30	30	0	0
4790K		30	0	0	30
BC-untreated group		30	0	18	12
BC-treated group	49.7% (91/183)	30	0	0	30

[a] The survival rate of the backcross progeny (BC) treated with diagnostic concentration of flubendiamide (0.15 μg/cm^2).

[b] *ss* represent homozygous for wild-type allele at amino acid either 4946 or 4790 position of PxRyR , while *rs* means heterozygote bearing $4946^{G/E}$, $4790^{I/M}$ or $4790^{I/K}$, and *rr* represent homozygote carrying $4946^{E/E}$, $4790^{M/M}$ or $4790^{K/K}$ allele, respectively.

Table 5. Comparison of fitness components (mean ± SE) and relative fitness in the susceptible IPP-S strain and three mutation introgressed strains of *P. xylostella*

Fitness component [a]	IPP-S	4946E	4790M	4790K
Number of neonates for tests	300	300	280	300
Larvae duration (days)				
♂	7.08 ± 0.05	8.31 ± 0.13*	7.07 ± 0.05	7.10 ± 0.07
♀	7.08 ± 0.05	8.31 ± 0.13*	7.08 ± 0.06	7.10 ± 0.07
Pupa duration (days)				
♂	3.83 ± 0.04	4.03 ± 0.07*	3.76 ± 0.05	3.54 ± 0.06*
♀	3.68 ± 0.03	3.78 ± 0.05	3.46 ± 0.04*	3.32 ± 0.05*
Pupae body weight (mg)				
♂	5.82 ± 0.07	5.88 ± 0.06	5.64 ± 0.04*	5.88 ± 0.04
♀	7.14 ± 0.09	7.18 ± 0.12	6.92 ± 0.07*	6.94 ± 0.10
Pupation rate (%)	99.33 ± 0.01	92.33 ± 0.01*	95.00 ± 0.01*	98.57 ± 0.01
Emergence rate (%)	84.53 ± 0.03	82.29 ± 0.03	79.02 ± 0.02	83.74 ± 0.03
Female ratio (%)	48.30 ± 0.04	43.49 ± 0.05	49.14 ± 0.04	39.31 ± 0.03*
Fecundity (eggs by each female)	121.9 ± 4.43	126.5 ± 5.86	126.4 ± 7.13	127.2 ± 6.24
Hatchability (%)	82.06 ± 0.02	74.38 ± 0.04*	81.09 ± 0.03	89.94 ± 0.02*
Predicted neonate number of next generation	12170	9327	10587	11136
Net reproductive rate (R_0) [b]	40.57	31.09	37.81	37.12
Relative fitness	1.00	0.77	0.93	0.92

[a] Significantly different from IPP-S at $p < 0.05$ level according to one-tailed Student's *t*-test or Mann-Whitney *U*-test.

[b] Relative fitness = R_0 (a mutation introgression strain) / R_0 (IPP-S).

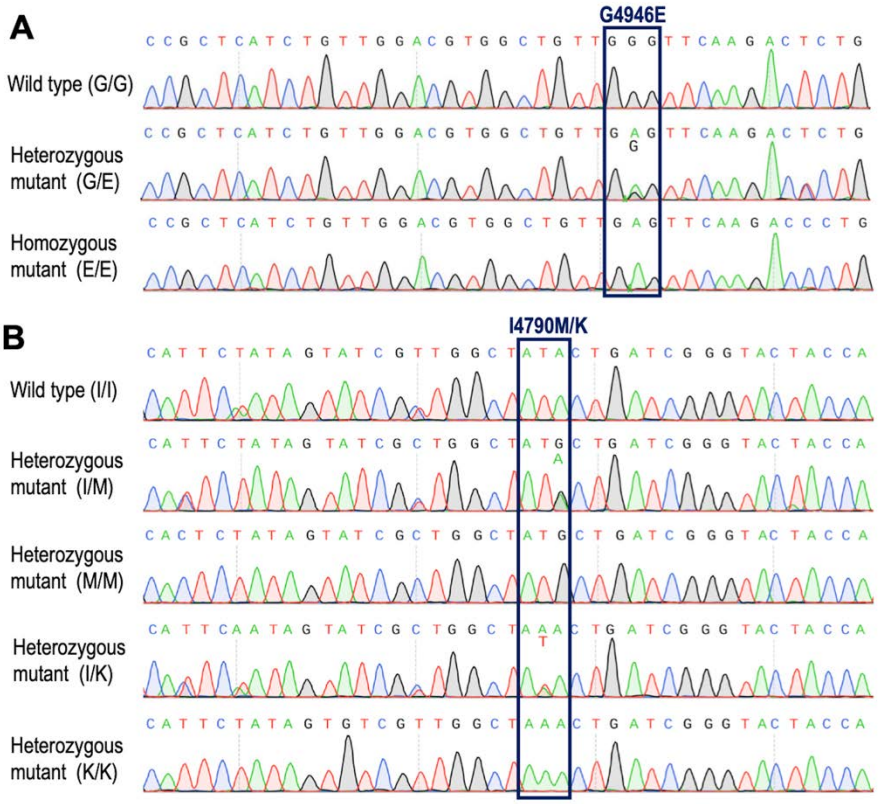

Figure 1. Representative chromatograms produced from sequencing PCR amplicons enabled unambiguous genotyping of individual carrying the G4946E (A) or I4790M/K (B) mutations of the *P. xyloxtella* ryanodine receptor.

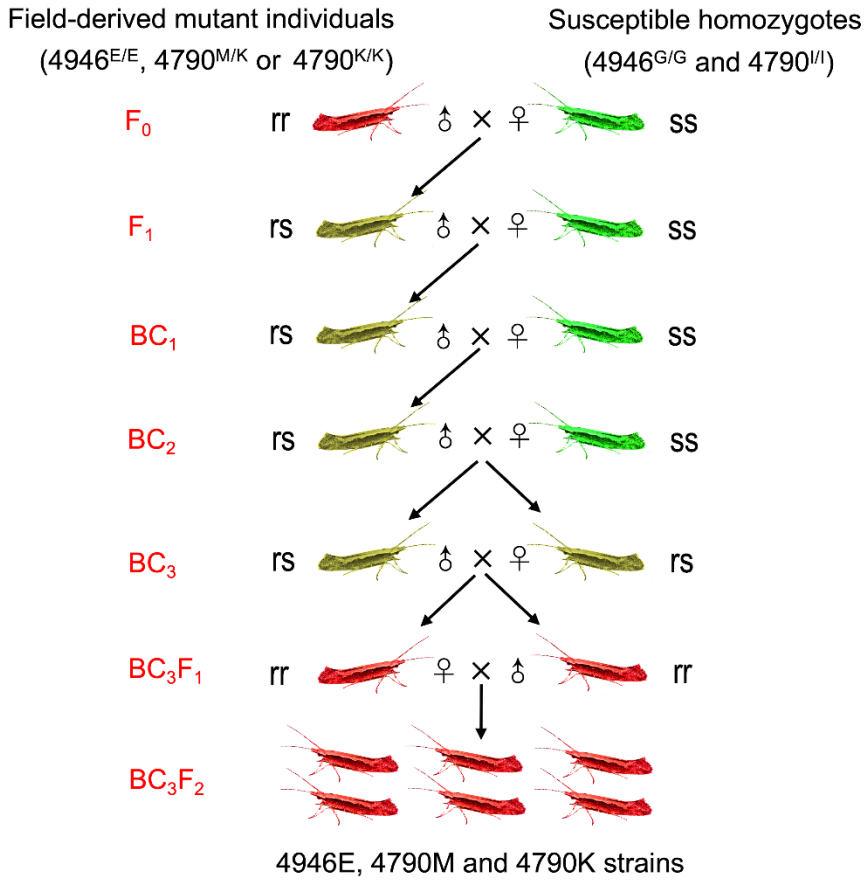

Figure 2. Introgression of *P. xylostella* field-derived diamide resistant alleles (G4946E, I4790M or I4790K) into the susceptible refernce strain IPP-S. "ss" represents susceptible homozygote ($4946^{G/G}$ and $4790^{I/I}$), while "rs" refers to heterozygotes bearing one resistant and one susceptible allele ($4946^{G/E}$, $4790^{I/K}$ or $4790^{I/M}$) and "rr" represents mutants with two resistance alleles at the same codon ($4946^{E/E}$, $4790^{M/M}$, $4790^{K/K}$ or $4790^{M/K}$).

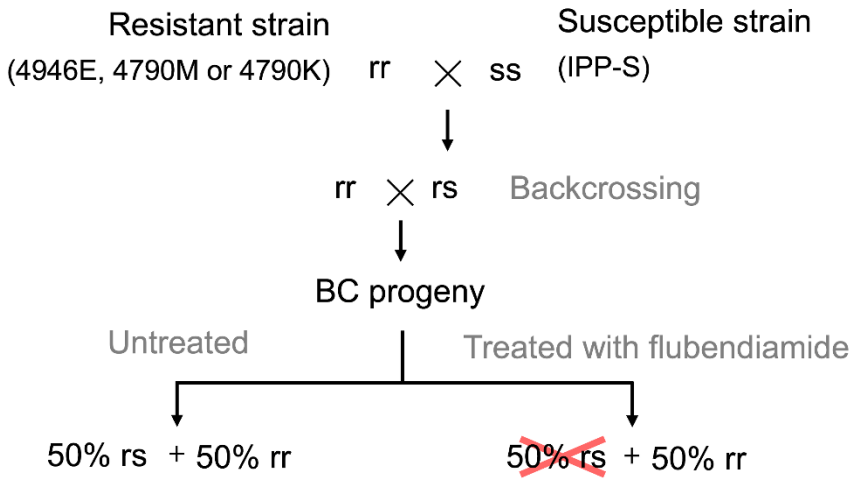

Figure 3. The crossing design for generating backcross progeny used for linkage analysis of flubendiamide resistance in the introgressed 4946E, 4790M or 4790K strains of *P. xylostella*. ss, rr, rs represent wild-type, homozygous mutant and heterozygous mutant insects, respectively. Expected ratios of heterozygous and homozygous progeny are indicated.

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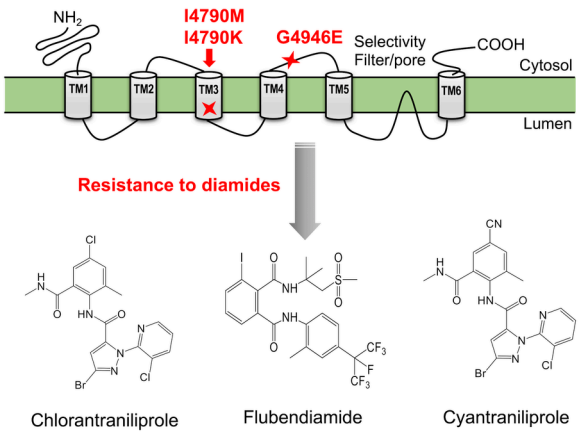
Introgression of three separate RyR amino acid substitutions into a common genetic background confer different levels of resistance to five diamide insecticides in *Plutella xylostella*.



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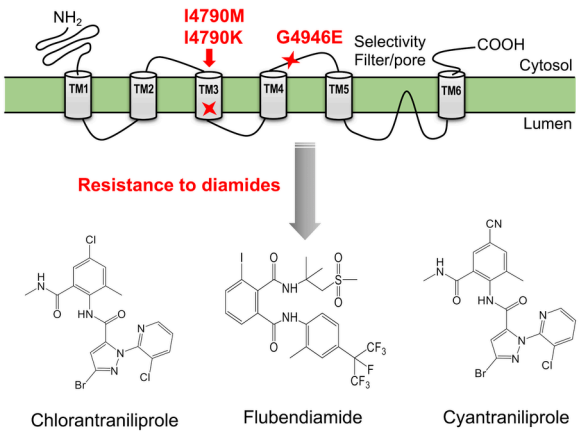

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