Short Communication

Seasonal proportion change of ryanodine receptor mutation (G4946E) in diamondback moth populations

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This study examined the seasonal proportion change of ryanodine receptor mutation (G4946E) for Plutella xylostella populations using quantitative sequencing. Results showed that the proportions of G4946E generally increased from spring through summer, but then decreased in autumn. Furthermore, the proportions in late autumn were similar to those in early spring of the subsequent year. These results suggest that diamide effectiveness for P. xylostella control in the reference year can be evaluated based on a proportion survey in the prior year. © Pesticide Science Society of Japan

Keywords: chlorantraniliprole, diamide, flubendiamide, insecticide resistance, Plutella xylostella.

Electronic supplementary material: The online version of this article contains supplementary materials (Supplemental Table S1), which is available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

Diamides such as flubendiamide and chlorantraniliprole are especially active against lepidopteran pests, including the diamondback moth Plutella xylostella Linnaeus.1,2) Diamides bind selectively to the ryanodine receptor (RyR) in insect muscles and activate the release of calcium from internal stores in the sarcoplasmic reticulum, leading to feeding cessation, lethargy, muscle paralysis, and eventually death.1,2) Diamides show high insecticidal potency and low mammalian toxicity.3) Therefore, they have been widely used to control insect pests.

P. xylostella, a major pest of Brassica worldwide,4) has developed resistance to 95 compounds including diamides (IRAC, http://www.irac-online.org/). The development of resistance against diamides has been reported for P. xylostella in some countries including Thailand, the Philippines, China, and Japan.5–8) The diamide resistance of P. xylostella has been shown to be incompletely or almost completely recessive.7,9,10) The amino acid mutation (G4946E) in RyR has been shown to be a major factor related to target-insensitive diamide resistance in P. xylostella.5,9–11)

A method to estimate the proportions of G4946E in P. xylostella populations has been developed using quantitative sequencing (QS).12) Unlike conventional time-consuming bioassay methods, the QS-based method can estimate the proportion using insects collected in the field in a short time. In this study, using the QS-based method, we show the seasonal proportion change of G4946E in P. xylostella populations in the field.

Materials and Methods

1. Insects and study sites

Adult P. xylostella males were collected at cabbage fields (Site A and Site B) with the same area (56 m²) using pheromone-baited sticky traps set at the Kagawa Agricultural Experiment Station, Ayauta-Gun, Kagawa Prefecture, from March 15, 2015, to March 15, 2017. The traps (Sumitomo Chemical Co., Ltd., Osaka, Japan), which were comprised of a sticky paper plate and a white plastic roof, were set 50 cm above the ground. They were replaced every 5 days (from March 15, 2015, to September 20, 2015, and from April 25, 2016, to August 1, 2016) or 10 days (from September 20, 2015, to April 25, 2016, and from August 1, 2016, to March 15, 2017). The collected insects were used to estimate the G4946E proportion using the QS-based method.12)

Year-round cultivation of cabbages with almost the same planting schedules took place at both study sites. Site B was managed with no insecticide applications for pest control. At Site A, flubendiamide (Phoenix 20.0% WDG; Nihon Nohyaku Co., Ltd., Tokyo, Japan) (July 3, July 30, and August 14) and cyantraniliprole (Benevia 10.3% OD; DuPont, Tokyo, Japan) (April 5, May 2, June 14, and September 27) were applied respectively in 2015 and 2016. Both study sites were ca. 200 m away from each other.

Live insects were also collected at both study sites on May 17, 2016. The collected insects were maintained with radish seedlings at 25°C under a long photoperiod (16L:8D) and were subjected to bioassay as described below.

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2. Bioassay
Diamide insecticides, flubendiamide and chlorantraniliprole (Prevathon 5.0% FL; DuPont, Tokyo, Japan) were used for bioassay. Susceptibilities were assessed using newly molted third-instar larvae. The leaf-dipping bioassay method using *Brassica rapa* L. leaves was applied. In each bioassay, 5–7 concentrations of flubendiamide (0.02–2000 mg/L) and of chlorantraniliprole (0.005–500 mg/L) were tested. After 96 hr, the numbers of dead or moribund larvae and surviving larvae were recorded. Bioassay was conducted with at least three replicates. The LC$_{50}$ value was estimated using probit analysis.

3. Genomic DNA extraction
All adult males captured by the traps were removed from sticky plates using 96% *n*-hexane and were used for genomic DNA extraction. Genomic DNA was extracted individually using a Wizard genomic DNA purification kit (Promega K.K., Tokyo, Japan) and was dissolved in 40 µL of H$_2$O. An equal volume of genomic DNA (1 µL) was mixed. For the QS-based method described below, 1 µL of the resulting DNA mixture was used.

4. Estimation of G4946E proportion
The proportion of G4946E was estimated according to the method reported by Sonoda *et al.* Briefly, PCR amplification of partial *RyR* from genomic DNA was conducted using primers 5′-tgataacgacggccagtacgctaccaagtgt-3′ and 5′-ccgctttatgcgtgacagact-3′. In the former primer, the M13–21 primer sequence was included in the 5′-end, as underlined. Quick Taq HS DyeMix (Toyobo Co., Ltd., Osaka, Japan) was used for PCR amplification. The PCR conditions were 1 cycle of 2 min at 98°C, 32 cycles of 10 sec at 98°C, 15 sec at 58°C, and 15 sec at 68°C, finishing with final extension of 68°C for 5 min. Amplified DNA fragments were sequenced directly using the M13–21 primer. The peak heights of nucleotides corresponding to G4946E were measured from the sequence chromatogram using software (PowerPoint 2016; Microsoft Japan, Tokyo, Japan). Then the nucleotide signal ratio was subjected to the quadratic equation ($y=87.11x^2+27.86x+4.98$) to estimate the proportion of G4946E. Signal ratios of 0 and 1.0 respectively denote the resulting proportions of 0% and 100% without incorporating them into the prediction equation.
Results and Discussion

A respective total of 2215 and 2716 male adults of *P. xylostella* were captured by the traps at Site A and Site B during the survey period (Fig. 1). In 2015, trap catches were highest at the end of May (Site B) and in early July (Site A and Site B). In 2016, trap catch peaks were observed in early June at both study sites. Trap catches decreased considerably in August. The decreased trap catches at both study sites persisted until the next spring.

In addition to G4946E, three mutations that might be involved in resistance to chlorantraniliprole (E1338D, Q4594L, and I4790M) were reported by Guo et al.\(^{11}\) They suggested that a combination of mutations, rather than a single mutation, might play an important role in the chlorantraniliprole resistance in *P. xylostella*. Our rough preliminary direct sequencing analyses showed no (Q4594L) or very limited (E1338D and I4790M) nucleotide substitutions in insects captured by the traps at both study sites (data not shown). Therefore, this study examined G4946E as a main factor conferring diamide resistance.

Results showed that the proportions of G4946E generally increased from spring to summer at both study sites and then decreased toward autumn in 2015 and 2016 (Fig. 1). The increase in proportion might be mainly due to selection with diamides. However, the cause of the decrease toward autumn is currently unclear, as reported in Hama\(^{15}\) for organophosphate resistance. Results also showed that the proportions of G4946E in late autumn were similar to those in early spring of the next year at both study sites (Fig. 1). Thus, seasonal proportion changes of G4946E were similar between Site A and Site B, possibly because of the migration of insects between both study sites. Reportedly, *P. xylostella* populations with higher sensitivities to flubendiamide had G4946E proportions less than 40\%.\(^{12}\) These results suggest that the effectiveness of diamides in *P. xylostella* control in early spring in the reference year can be evaluated based on the proportion survey conducted during the prior year, irrespective of insecticide practices.

Insects (F\(_1\) generation) collected at Site A in May 17, 2016, showed LC\(_{50}\) values of 192.7 mg/L and 77.0 mg/L against flubendiamide and chlorantraniliprole, respectively. The LC\(_{50}\) value of insects (F\(_2\) generation) collected at Site B in May 17, 2016 against chlorantraniliprole was 28.7 mg/L. The agriculturally recommended concentrations of flubendiamide and chlorantraniliprole for *P. xylostella* were 100 mg/L and 25 mg/L, respectively. Therefore, the populations at both study sites were defined as diamide resistant. In fact, the proportions of G4946E in insects trapped at Site A and Site B on May 16–20, 2016, were estimated to be 82.5% and 57.5%, respectively. However, the proportions of G4946E fluctuated seasonally; they were sometimes below 40% at both study sites (Fig. 1). These results suggest that effective control is possible, even for *P. xylostella* populations that have once been defined as diamide resistant based on temporal bioassay. However, proportions of G4946E lower than 40% were observed during limited periods in this study. Furthermore, the target developmental stage for diamides is mainly larva, not adult, in *P. xylostella*. For more reliable evaluation of diamide resistance using G4946E, the relation between the proportion of G4946E and the pest-control effect using diamides must be clarified using more field-collected samples.

Seasonal changes in insecticide-resistance levels of *P. xylostella* were reported for organophosphates and pyrethroids.\(^{15–17}\) Their bioassay data revealed that the resistance levels against organophosphates and pyrethroids were low in spring and early summer and high in autumn. Nevertheless, successive surveys of resistance levels have not been reported to date. This report is the first to describe seasonal changes of the insecticide resistance factor at a molecular level.

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