Nicotinic acetylcholine receptor α6 subunit mutation (G275V) found in a spinosad-resistant strain of the flower thrips, *Frankliniella intonsa* (Thysanoptera: Thripidae)

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(Received February 1, 2018; Accepted April 11, 2018)

The flower thrips *Frankliniella intonsa* strain showing resistance to spinosad was established in the laboratory. The resistant strain showed an LC50 value of 1398.7 mg/L in a leaf dipping/contact assay. The LC50 value was ca. 280 times higher than that of the most susceptible strain. An insecticidal assay using synergists suggested no involvement of degradation enzymes, such as cytochrome P450, glutathione S-transferase, and carboxyl esterase, in the resistance. Glycine at amino acid position 275 of the nicotinic acetylcholine receptor (nAChR) α6 subunit was mutated to valine in the resistant strain. These results suggest that spinosad resistance in *F. intonsa* is conferred by the reduced sensitivity of nAChR. © Pesticide Science Society of Japan

Keywords: flower thrips, insecticide resistance, nAChR α6 subunit, target insensitivity, Thysanoptera.

**Introduction**

The flower thrips *Frankliniella intonsa* is recognized as a highly polyphagous pest affecting widely diverse ornamental and vegetable crops worldwide.1) Adults and nymphs of *F. intonsa* suck cell fluids from leaves, stems, flowers, and fruit surfaces, thereby causing silvery scars and leaf chlorosis. *F. intonsa* is also known as a vector of *Tomato spotted wilt virus*2) and *Impatiens necrotic spot virus*.3) In Japan, chemical control using insecticides has been applied to *F. intonsa* infestations. However, the reduced effectiveness of several groups of insecticides, including spinosad, against *F. intonsa* has been reported in some areas.4)

Spinosad, a broad-spectrum insecticide comprised of two macrocyclic lactones (spinosyn A and spinosyn D) produced by the microorganism *Saccharopolyspora spinosa* during fermentation,5,6) was introduced commercially to Japan in 1997. Spinosad interacts with the central nervous system and causes neuromuscular fatigue, thereby leading to tremors, paralysis, and ultimately death. The primary target of spinosad is the nicotinic acetylcholine receptor (nAChR).7) nAChR is comprised of five subunits, each containing four transmembrane domains and extracellular N-terminal domains that include the acetylcholine binding site. In Thysanoptera, target-insensitive spinosad resistance has been reported in *Frankliniella occidentalis*8) and *Thrips palmi*.9) In *F. occidentalis*, a point mutation in transmembrane region 3 of the nAChR α6 subunit (G275E) was shown to be involved in the resistance using the human nAChR α7 subunit with the analogous mutation (A275E).

Cytochrome P450s (CYP450s), an important degradation system for the metabolism of xenobiotics and endogenous compounds in insects,10–12) are another major mechanism of spinosad resistance. In Thysanoptera, the involvement of CYP450s in spinosad resistance has been reported in *T. palmi*.9)

To date, no mechanism conferring resistance to spinosad has been reported for *F. intonsa*. For this study, we cloned DNA fragments encoding the *F. intonsa* nAChR α6 subunit (Flα6) and examined the deduced amino acid sequences of the strains with different sensitivities to spinosad. Furthermore, the involvement of degradation enzymes such as CYP450, glutathione S-transferase (GST), and carboxylesterase (CE) in spinosad resistance was examined using the respective synergists.

**Materials and Methods**

1. **Insects and chemicals**

The KS1 and KS2 strains were collected respectively from strawberry plants *Fragaria×ananassa* at Tonosho Town, Kagawa Prefecture in 2015 and at Ayagawa Town, Kagawa Prefecture in 2016. Both strains were fed with fava bean *Vicia faba* sprouts. A strain of *F. intonsa* was collected on strawberry plants at Miyoshi Town, Tokushima Prefecture in 2016. Insects collected from Tokushima Prefecture exhibited reduced sensitivity to spinosad.
in a preliminary bioassay. Therefore, some insects were selected for more than eight generations using fava bean sprouts treated with spinosad (40 mg/L) to establish a spinosad-resistant TR strain. The remaining insects have been maintained on fava bean sprouts with no insecticide. The strain was designated as the TS strain for this study. Insects were maintained at 25°C under a long photoperiod (16L:8D).

Spinosad (Spinoace 20.0% SC; Dow AgroSciences LLC) and diethyl maleate (DEM; Nacalai Tesque Inc.) were used. Piperonyl butoxide (PBO) and S,S,S-tributyl phosphorotrithioate (DEF) were purchased from Wako Pure Chemical Industries Ltd.

2. Insecticidal assay
An insecticidal assay was conducted according to previously reported methods.13,14 Kidney bean Phaseolus vulgaris leaves (about 3.5 cm × 2 cm) were dipped for 2 min in more than five concentrations of spinosad containing 0.02% of the spreading agent (Dain; Sumitomo Chemical Co., Ltd.). For the control test, kidney bean leaves were dipped in distilled water containing the spreading agent. The treated leaves were allowed to air-dry and were then inserted into the insecticide-treated plastic vials as described above.

3. Synergism test
For this study, PBO, DEM, and DEF, the respective inhibitors of CYP450, GST, and hydrolytic enzymes including CE, were used as synergists. The kidney bean leaves were dipped for 2 min in more than five concentrations of spinosad containing 0.02% of the spreading agent, synergists (PBO, 0.295 mM; DEM, 0.381 mM; DEF, 0.1% acetone). The LC50 value was estimated for each strain using probit analysis.15)

| Strain | Spinosad | Spinosad+PBO | Spinosad+DEM | Spinosad+DEF |
|--------|----------|--------------|--------------|--------------|
|        | n | LC50 (mg/L) (95% CL) | RR | n | LC50 (mg/L) (95% CL) | SR | n | LC50 (mg/L) (95% CL) | SR | n | LC50 (mg/L) (95% CL) | SR |
| KS1    | 246 | 6.6 (5.8–7.1) | 1.3 | 242 | 1.3 (1.0–1.8) | 5.1 | 235 | 7.2 (6.4–7.7) | 0.9 | 230 | 6.4 (5.0–7.3) | 1.0 |
| KS2    | 256 | 5.0 (6.9–8.1) | 1.0 | 240 | 1.1 (0.8–1.4) | 4.5 | 277 | 6.6 (5.1–7.7) | 0.8 | 278 | 5.5 (4.4–6.3) | 0.9 |
| TS     | 255 | 9.7 (8.9–10.1) | 1.9 | 234 | 2.8 (2.0–3.4) | 3.5 | 229 | 9.8 (9.1–10.7) | 1.0 | 242 | 8.3 (7.5–9.0) | 1.2 |
| TR     | 293 | 1398.7 (733.4–5826.5) | 279.7 | 243 | 586.0 (433.3–840.9) | 2.4 | 240 | 1221.8 (687.1–3987.2) | 1.1 | 241 | 1510.2 (872.6–4375.8) | 0.9 |

a) PBO: piperonyl butoxide; DEM: diethyl maleate; DEF: S,S,S-tributyl phosphorotrithioate. b) Resistance ratio (RR): LC50 of each strain/LC50 of the KS2 strain. c) Synergist ratio (SR): LC50 of spinosad alone/LC50 of spinosad+synergist. d) CL: confidence limit.

F. occidentalis. The synergist concentrations caused no mortality for F. intonsa. No other synergist concentrations were tested in this study. For the control test, kidney bean leaves were dipped in distilled water containing 0.02% of the spreading agent and 0.1% acetone. The treated leaves were allowed to air-dry and were then inserted into the insecticide-treated plastic vials as described above.

4. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was extracted from ca. 100 adults of each strain using Sepasol RNA I Super G (Nacalai Tesque Inc.). cDNA was constructed from 1 μg of total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd.). DNA fragments encoding the Fla6 gene were amplified from the KS1 strain using the primers 5′-ctctccatgaggagc-3′ and 5′-tcagctctagcttc-3′. The primers were designed to amplify a fragment encoding the nAChR β6 subunit of F. occidentalis (GenBank/EMBL/DDBJ accession no. HE965755). Amplified fragments were cloned into pGEM-T Easy Vector (Promega Corp.) and were subsequently sequenced. Based on the nucleotide sequences, the specific primers, Fi-α6-5′ (5′-gacctctgctgacattcagc-3′) and Fi-α6-3′ (5′-gggagcataaagctg-3′), were designed and were then used for PCR amplification, followed by cloning using pGEM-T Easy Vector.

The PCR conditions were 1 cycle of 3 min at 94°C followed by 40 cycles of 15 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C, and a final extension of 72°C for 7 min. In this study, Quick Taq HS DyeMix (Toyobo Co., Ltd.) was used for PCR amplification.

5. Nucleotide sequencing
The plasmid DNA used for nucleotide sequencing was purified using a Plasmid Mini Extraction Kit (Bioneer Corp.). The nucleotide sequence was determined using a dye terminator cycle sequencing kit (Applied Biosystems) and a DNA sequencer (3500 Genetic Analyzer; Applied Biosystems). Seven, five, seven, and seven clones were sequenced, respectively, for KS1, KS2, TS, and
Fig. 1. Alignments of 22 haplotypes of deduced amino acid sequences in the nAChR α6 subunit genes isolated from the KS1 (haplotypes 1, 3, 4, 5, 7, and 9), KS2 (haplotypes 1, 2, 6, and 8), TS (haplotypes 7 and 10–15), and TR (haplotypes 16–22) strains of Frankliniella intonsa. The transmembrane domains (M1, M2, and M3) and the loop C (lpC) of the N-terminal extracellular domain are indicated by dashed lines. Amino acid position 275 (numbered according to the nAChR α6 subunit of F. occidentalis) is boxed. Putative alternative exons 8a/8b are shaded.
TR strains. Nucleotide and deduced amino acid sequences were analyzed using Genetyx ver. 13 (Genetyx Corp.).

Direct sequencing of Fla6 fragments amplified from cDNA with Fi-a6-5’ and Fi-a6-3’ was conducted using the primer Fi-a6-seq (5’-tgtgatgcagttcaccatg-3’).

Results and Discussion

The KS1, KS2, TS, and TR strains respectively showed LC_{50} values of 6.6 mg/L, 5.0 mg/L, 9.7 mg/L, and 1398.7 mg/L (Table 1). The resistance level of the TR strain was estimated to be 279.7 times higher than that of the most susceptible KS2 strain.

The G275E mutation in the nAChR α6 subunit was shown to be involved in spinosad resistance in F. occidentalis using the human nAChR α7 subunit and Xenopus oocyte.[9] The G275E mutation in the nAChR α6 subunit was also reported in spinosad-resistant T. palmi.[9] In this study, the DNA fragments encoding the Fla6 gene were amplified from the KS1, KS2, TS, and TR strains using RT-PCR reactions with primers Fi-a6-5’ and Fi-a6-3’. The amplified DNA fragments (ca. 0.5 kb) were cloned and sequenced (accession nos. LC363510–LC363528 and LC373565–LC373570). Seven, five, seven, and seven clones respectively sequenced for KS1, KS2, TS, and TR strains displayed the highest amino acid identity (88–94%) with the nAChR α6 subunit in F. occidentalis,[9] demonstrating that the amplified DNA fragments encode the nAChR α6 subunit (data not shown). The deduced amino acid sequences were categorized into 22 haplotypes (Fig. 1). All haplotypes derived from the KS1 (haplotypes 1, 3, 4, 5, 7, and 9), KS2 (haplotypes 1, 2, 6, and 8), and TS (haplotypes 7 and 10–15) strains encoded Gly at amino acid position 275 (numbered according to the nAChR α6 gene at position equivalent to exons 8a/8b in the nAChR α6 gene) (Fig. 1). The other haplotypes encoded the putative exon 8b (Fig. 1). The other haplotypes encoded the putative exon 8a (Fig. 1).

Direct sequencing of the amplified Fla6 gene fragments revealed that the respective frequencies for G275 and V275 might be 100% for the KS1 and KS2 and TR strains (data not shown). No detectable nucleotide peak corresponding to V275 was detected in the TS strain, which has been maintained without spinosad selection (data not shown). Insects with V275 might be eliminated, possibly because of fitness costs for spinosad resistance. The functional significance of the G275V mutation, relative to the G275E mutation found in F. occidentalis[8] and T. palmi,[9] remains to be elucidated in the future.

In this study, the involvement of three major groups of enzymes including CYP450, GST, and CE in the spinosad resistance of F. intonsa was examined using the respective synergists. The LC_{50} values of the four strains were decreased slightly (2.4-fold to 5.1-fold) with PBO treatment (Table 1). The results suggest that F. intonsa developed an innate CYP-mediated degradation system against spinosad. No synergistic effect was observed for DEM or DEF (Table 1). These results suggest that degradation enzymes are not responsible for spinosad resistance in F. intonsa. In F. occidentalis, enhanced metabolic detoxification by such degradation enzymes was not responsible for spinosad resistance.[16,17] Little or no involvement of the degradation enzymes in spinosad resistance was reported in other insects, including P. xylostella[19] and M. domestica.[19] However, resistance to spinosad in S. exigua,[20] H. armigera,[21] S. litura,[22] and T. palmi[17] was associated with CYP450-mediated detoxification.

Consequently, the results of this study suggest that resistance to spinosad in F. intonsa is conferred by target insensitivity, possibly caused by the G275V mutation in Fla6. In F. occidentalis, spinosad-resistant strains without G275E were found in the USA and China.[20] Furthermore, mis-spliced or truncated transcripts of the nAChR α6 subunit have been correlated with spinosad resistance in P. xylostella and B. dorsalis.[24–27] Further analyses of nucleotide sequences and transcripts of the Fla6 gene and those in y-aminobutyric acid receptors, a putative secondary target site of spinosad, must be conducted to elucidate the spinosad resistance mechanisms in F. intonsa.

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