Appl Entomol Zool (2013) 48:507–513
DOI 10.1007/s13355-013-0212-4

ORIGINAL RESEARCH PAPER

Neonicotinoid resistance and cDNA sequences of nicotinic acetylcholine receptor subunits of the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae)

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Received: 16 January 2013 / Accepted: 30 August 2013 / Published online: 24 September 2013

Abstract The western flower thrips, *Frankliniella occidentalis* (Pergande), is difficult to control because of high insecticide resistance. In this study, susceptibility to major insecticides was examined in two Japanese strains (H-1 and KC) and a Chinese strain (BJ) using a leaf-dipping method. All three strains were resistant to permethrin and acetamiprid at agriculturally recommended doses. The median lethal concentration (LC_{50}) for acetamiprid was 1720 ppm in strain H-1, 4780 ppm in strain KC and >6680 ppm in strain BJ. In the presence of piperonyl butoxide, an inhibitor of cytochrome P450 monooxygenases, the LC_{50} for acetamiprid was 312 ppm in strain H-1, 837 ppm in strain KC and 1250 ppm in strain BJ. These results suggested that metabolism by cytochrome P450 monooxygenases is involved in acetamiprid resistance in these strains, though other factors also seem to play a role. Furthermore, cDNA cloning of the nicotinic acetylcholine receptor (nAChR) subunits was performed using degenerate primers, and the presence or absence of a point mutation in nAChR β1 was confirmed. The R81T mutation that had been reported in *Myzus persicae* (Sulzer) nAChR β1 was not found in *F. occidentalis* strains tested in this study.

Keywords *Frankliniella occidentalis* · Neonicotinoids · Nicotinic acetylcholine receptor · Piperonyl butoxide

Introduction

The western flower thrips, *Frankliniella occidentalis* (Pergande), is one of the most serious crop pests worldwide. *F. occidentalis* is a polyphagous pest against various vegetables, fruits and ornamental plants, causing extensive economic losses (Lewis 1997). In addition, *F. occidentalis* is known to transmit plant viruses such as tomato spotted wilt virus (Jones 2005; Whitfield et al. 2005).

F. occidentalis has developed resistance to major insecticides including organophosphates, carbamates and pyrethroids (Bielza 2008). Cytochrome P450 monooxygenases are often involved in insecticide resistance: for example, the toxicity of organophosphates (Espinosa et al. 2005; Zhao et al. 1995), carbamates (Espinosa et al. 2005; Jensen 2000; Zhao et al. 1995) and pyrethroids (Espinosa et al. 2005; Immaraju et al. 1992; Zhao et al. 1995) was increased in the presence of a cytochrome P450 inhibitor, piperonyl butoxide (PBO), suggesting that metabolism by cytochrome P450 monooxygenases is involved in the resistance to these chemicals. Recently, *F. occidentalis CYP6EB1* and *CYP6EC1* have been identified as the cytochrome P450 genes that are overexpressed in

Electronic supplementary material The online version of this article (doi:10.1007/s13355-013-0212-4) contains supplementary material, which is available to authorized users.

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acrinathrin-resistant populations (Cifuentes et al. 2012). Besides metabolism by cytochrome P450 monooxygenases, metabolism by esterases seems to be involved in resistance to organophosphates and carbamates (Jensen 2000; Zhao et al. 1994). An altered target site has been suggested to be responsible for spinosad resistance (Bielza et al. 2007), and a recent study revealed that a point mutation in the α6 subunit of the nicotinic acetylcholine receptor (nAChR) in *F. occidentalis* was associated with spinosad resistance (Puinean et al. 2013). However, despite these recent studies, most of the mechanism of insecticide resistance in *F. occidentalis* has not been clarified at the molecular level.

In the late 1970s, *F. occidentalis* began to spread globally from its original distribution in western North America (Gao et al. 2012; Kirk and Terry 2003). In Japan, serious damage to agricultural crops caused by *F. occidentalis* was first reported in the 1990s (Hayase and Fukuda 1991), and then *F. occidentalis* populations resistant to major insecticides were found in several areas (Katayama 1998). It is still possible that *F. occidentalis* populations could spread further through international trade in agricultural and horticultural products. Therefore, it is important to monitor insecticide susceptibility among several populations from different countries.

In this study, susceptibility to various insecticides was compared among three populations of *F. occidentalis*, i.e., Japanese (H-1 and KC) and Chinese (BJ) strains. All these populations were found to be resistant to neonicotinoids, and this resistance was examined further. The insecticidal activity of acetamiprid was enhanced in the presence of PBO, indicating that cytochrome P450 is involved. But even in the presence of PBO, these populations were resistant to acetamiprid, suggesting other factors to be involved in the resistance. Furthermore, cDNA sequences of nAChR have been analyzed, and the presence or absence of a known point mutation in nAChR β1 was examined.

Materials and methods

Collection and rearing of *F. occidentalis*

The H-1 strain of *F. occidentalis* was provided by Prof. T. Murai at Utsunomiya University. This strain was originally collected from gerbera flowers in Shimane Prefecture in Japan in 1994. The Kochi strain (KC) was collected in a citrus greenhouse in Kochi Prefecture in Japan in October 2012. The Beijing strain (BJ) was collected in a capsicum greenhouse in Beijing in September 2010.

These strains were fed with germinated broad bean seeds (Kokusai Pet Food, Kobe, Japan), and reared at 23 ± 1 °C under a long-day photoperiod (16L-8D) according to a reported method (Minakuchi et al. 2011; Murai and Loomans 2001).

Chemicals

Formulated acetamiprid (Mospilan 20 % SP, Nippon-Soda), permethrin (Adion 20 % EC, Sumitomo Chemical), spinosad (Spino-Ace 25 % WP, Nissan Chemical Industries, Ltd.) and fipronil (Prince 5 % SC, BASF Japan) were diluted with water in the presence of a spreading agent, Dine (Takemoto Oil & Fat), at 200 ppm. Imidacloprid (provided by Dr. H. Nishiwaki) was dissolved in ethanol to 2600 ppm and further diluted with water in the presence of 200 ppm Dine.

To calculate the LC_{50}, acetamiprid (Wako Pure Chemical Industries, Ltd.) was dissolved in ethanol to 44000 ppm, and this stock solution was diluted to 22.0–6600 ppm with water containing 200 ppm Dine. PBO (Tokyo Chemical Industry, Co., Ltd.) was dissolved in acetone to 10000 ppm and further diluted to 2000 ppm with ethanol.

Bioassays

Leaf disks of kidney beans (1 × 1 cm) were dipped in the test chemical solution for 1 min. After being dried at room temperature, each leaf disk was put on a 1 % agar dish (20 mm in diameter). Ten to 15 female adults anesthetized with CO_2 were placed on the leaf disk, and the dish was sealed with Parafilm. Bioassays were performed with at least three replicates. Mortality was assessed 2 days later. Corrected mortality was calculated according to Abbott's formula (Abbott 1925).

To assess synergistic effects of PBO, female adults were anesthetized with CO_2, dipped in ethanol or a 2000 ppm PBO solution for 10 s, and reared on leaf disks treated with acetamiprid solution as described above. Mortality was assessed 2 days later. The median lethal concentration, LC_{50}, was calculated by probit analysis with PriProbit version 1.63 (Sakuma 1998).

cDNA cloning

Total RNA was isolated from the whole body of the H-1 strain, and oligo-dT-primed reverse transcription was conducted as described previously (Minakuchi et al. 2011). To amplify cDNA encoding nAChR subunits, degenerate primers were designed based on the conserved amino acid sequences of other insects' nAChR proteins. PCR was performed with an annealing temperature of 46 °C. Primer sequences are listed in Supplementary Table 1.

To obtain full-length cDNA of each nAChR subunit, rapid amplification of cDNA ends (RACE) was conducted

as follows. cDNA pools were prepared from strain H-1 using a GeneRacer Kit (Life Technologies) as described previously (Minakuchi et al. 2011), and touchdown PCR and a secondary nested PCR were performed with Advantage 2 polymerase (Clontech) according to the manufacturer's instructions. Primer sequences are listed in Supplementary Table 1.

These PCR products were purified, cloned into the pGEM-T Easy vector (Promega), and sequenced. The DNA sequence data have been deposited in DDBJ/EMBL-Bank/GenBank International Nucleotide Sequence Database under the accession numbers AB748920-AB748926 and AB826436.

Results

Susceptibility of three strains of *F. occidentalis* to various insecticides

The mortality of strains H-1, KC and BJ exposed to various insecticides was evaluated (Table 1). For all strains, mortality was high at 63.0 ppm of fipronil or 310 ppm of spinosad. Thus, they were susceptible to fipronil and spinosad at agriculturally recommended concentrations. By contrast, the mortality with permethrin, acetamiprid and imidacloprid was low even at high concentrations: for example, the mortality at 2000 ppm of permethrin was only 2 % for H-1, 0 % for KC and 8 % for BJ (Table 1). Thus, these strains are resistant to permethrin and neonicotinoids.

The insecticidal activity of neonicotinoids was further analyzed to calculate LC_{50} values. We were not able to determine the LC_{50} for imidacloprid since we could not prepare imidacloprid solutions at higher concentrations because of low solubility. As listed in Table 2, the LC_{50} of acetamiprid was 1720 ppm for strain H-1, 4780 ppm for strain KC and >6680 ppm for BJ. We could not determine the LC_{50} of the BJ strain for acetamiprid since we could not prepare acetamiprid solutions at higher concentrations. In the presence of PBO, the LC_{50} of acetamiprid was 312 ppm for H-1, 837 ppm for strain KC and 1250 ppm for BJ. Thus, PBO had a synergistic effect, but these strains were resistant to acetamiprid even in the presence of PBO.

Table 1 Mortality of the Japanese (H-1 and KC) and Chinese (BJ) strains of *F. occidentalis* against various types of insecticides

Chemicals	Concentration (ppm)	Mortality (%)		
		H-1	KC	BJ
Phenylpyrazole				
Fipronil	63.0	100	88	73
Spinosyns				
Spinosad	310	97	88	97
Pyrethroids				
Permethrin	2000	2	0	8
Neonicotinoids				
Acetamiprid	2000	42	9	6
Imidacloprid	1500	4	ND	5

ND not determined

Table 2 LC_{50} of acetamiprid and synergism by PBO in H-1 and BJ strains of *F. occidentalis*

Strains	Chemicals	LC_{50} (ppm)	95 % CL[a]	Synergism ratio[b]
H-1	Acetamiprid	1720	624–5350	
	Acetamiprid + PBO	312	118–713	5.5
KC	Acetamiprid	4780	2780–10000	
	Acetamiprid + PBO	837	194–1470	5.7
BJ	Acetamiprid	>6680[c]	–	
	Acetamiprid + PBO	1250	512–3120	>5.3

[a] 95 % confidence limit

[b] LC_{50} of acetamiprid/LC_{50} of acetamiprid + PBO

[c] The mortality at 6680 ppm was 15 %

cDNA sequences of nAChR subunits

RT-PCR with degenerate primers followed by 5′- and 3′-RACE yielded several cDNA sequences from the H-1 strain. Blastx searches revealed that these sequences are homologous to the nAChR α1, α3, α4, α6, α8 and β1 subunits of other insect species (Table 3). We were not able to obtain full-length cDNA for the α1, α3 and α4 since RACE PCR was not successful. Two cDNA fragments of α4, designated FonAChR α4 isoforms 1 and 2, shared a 119-bp 5′-region, but differed in the 3′-region. Two cDNA fragments of α8, designated FonAChR α8 isoform 1 (2169 bp) and isoform 2 (2896 bp), shared a 1477-bp 5′-region, but again differed in the 3′-region. The cDNA fragment of nAChR α6 that we had obtained differed from a reported nAChR α6 sequence (HE965755) (Puinean et al. 2013) in the 5′-region. These are likely to be generated by an alternative splicing.

Detection of the R81T mutation in nAChR β1

In the peach-potato aphid *Myzus persicae*, it was revealed that a single point mutation (R81T) in the loop D of nAChR β1 is responsible for neonicotinoid resistance (Bass et al. 2011). At present, this is the only example of a point mutation found in a field-evolved neonicotinoid-resistant population. We confirmed the presence or absence of the R81T mutation in nAChR β1. The amino acid sequence of nAChR β1 was highly conserved including the putative loops comprising the ligand-binding domain (Fig. 1). The

Table 3 PCR-amplified nAChR cDNAs from *F. occidetnalis*

PCR-amplified nAChR cDNA (accession no.)	Length (bp)	Most similar nAChR as determined by blastx (accession no.)	*E* value
FonAChRα1 (AB748920)	358	*Nilaparvata lugens* nAChRα1 (ACM88003, ACO55078)	1E-80
FonAChRα3 (AB748921)	380	*Apis florea* nAChR α-like (XP_003696385)	2E-68
FonAChRα4 isoform 1 (AB748922)	486	*Nasonia vitripennis* nAChRα4 (NP_001234904, ACY82687)	1E-113
FonAChRα4 isoform 2 (AB748923)	267	*Ctenocephalides felis* nAChRα4 (ABB43003)	1E-60
FonAChRα8 isoform 1 (AB748924)	2169	*Periplaneta americana* nAChRα8 (AFA28130)	0.0
FonAChRα8 isoform 2 (AB748925)	2896	*Periplaneta americana* nAChRα8 (AFA28130)	0.0
FonAChRβ1 (AB748926)	1443	*Nilaparvata lugens* nAChRβ1 (ACJ07013)	0.0

R81T mutation in loop D was not found in three *F. occidentalis* strains in this study.

Discussion

The resistance of thrips to insecticides is a serious problem worldwide. In this study, susceptibility to insecticides was compared among three strains of *F. occidentalis* from Japan and China.

First, we examined the susceptibility of *F. occidentalis* to several commercially available insecticides. The H-1, KC and BJ strains were resistant to permethrin (Table 1). This is not surprising because pyrethroid resistance of *F. occidentalis* has been reported in Japan ever since the species was first identified in the 1990s (Katayama 1998). It seems that *F. occidentalis* developed pyrethroid resistance in North America before spreading worldwide (Immaraju et al. 1992; Katayama 1998). The H-1 and BJ strains also showed resistance to acetamiprid and imidacloprid (Table 1). We analyzed neonicotinoid resistance further. As shown in Table 2, measurements of LC_{50} revealed that strain BJ was the most resistant to acetamiprid among three strains in this study.

Neonicotinoid resistance has been reported in *F. occidentalis* (Zhao et al. 1995), but its molecular mechanism is unknown. We found that PBO had synergistic effects on the H-1, KC and BJ strains (Table 2), suggesting that enhanced cytochrome P450 activity is involved in acetamiprid resistance. In general, neonicotinoid resistance in insects is attributable to enhanced detoxification and an altered nAChR. In other insect species including the silverleaf whitefly *Bemisia tabaci* (Gennadius) and the brown planthopper *Nilaparvata lugens* (Stål), neonicotinoid resistance was mainly attributed to enhanced detoxification by P450 monooxygenases (Karunker et al. 2008; Nauen et al. 2002; Wen et al. 2009). Importantly, all the *F. occidentalis* strains that we tested were resistant to acetamiprid even in the presence of PBO. This suggests that acetamiprid resistance is partly attributable to enhanced cytochrome P450 activity, though other factors such as metabolism by detoxifying enzymes and/or altered target sites may also be involved.

nAChR is known to be a primary target of neonicotinoids. Here we obtained cDNA sequences encoding putative nAChR α1, α3, α4, α6, α8 and β1 subunits from *F. occidentalis*. In insects, the nAChR complex can be heteropentamers consisting of two α and three β subunits, or homopentamers of only α subunits (Fayyazuddin et al. 2006; Thany et al. 2006). Many subunits have been identified in individual insect species: for example, there are 10 subunit genes in *Drosophila melanogaster* (Meigen) (Sattelle et al. 2005) and 12 genes in the silkworm *Bombyx mori* (Linnaeus) (Shao et al. 2007). Studies are in progress to obtain cDNA sequences of other subunits from *F. occidentalis*. Two isoforms, probably generated by alternative splicing, were obtained for nAChR α4 and α8, respectively. The C-terminal part of the putative *F. occidentalis* nAChR β1 that we cloned showed low homology to nAChR β1 sequences from other insects (Fig. 1). We assume there to be other splicing variants of *F. occidentalis* nAChR β1. In insects, posttranscriptional processing of nAChR such as alternative splicing and A-to-I mRNA editing is observed frequently, which increases the structural diversity of each subunit (Jones et al. 2007). For example, 18 transcripts have been found for nAChR α6 from the red flour beetle *Tribolium castaneum* (Herbst), a result of alternative and optional exon usage (Rinkevich and Scott 2009).

F. occidentalis has developed resistance to various insecticides worldwide, and susceptible *F. occidentalis* strains are not available. Therefore, it would be difficult to elucidate the molecular mechanism of neonicotinoid resistance by comparing various strains. Therefore, we confirmed the presence or absence of a point mutation that had been reported in other insect species. Only one example of target-site changes that confer neonicotinoid resistance is the R81T mutation in loop D of *M. persicae* nAChR β1: this mutation was found in a field-evolved neonicotinoid-resistant population and is involved in reduced sensitivity to neonicotinoids (Bass et al. 2011). In

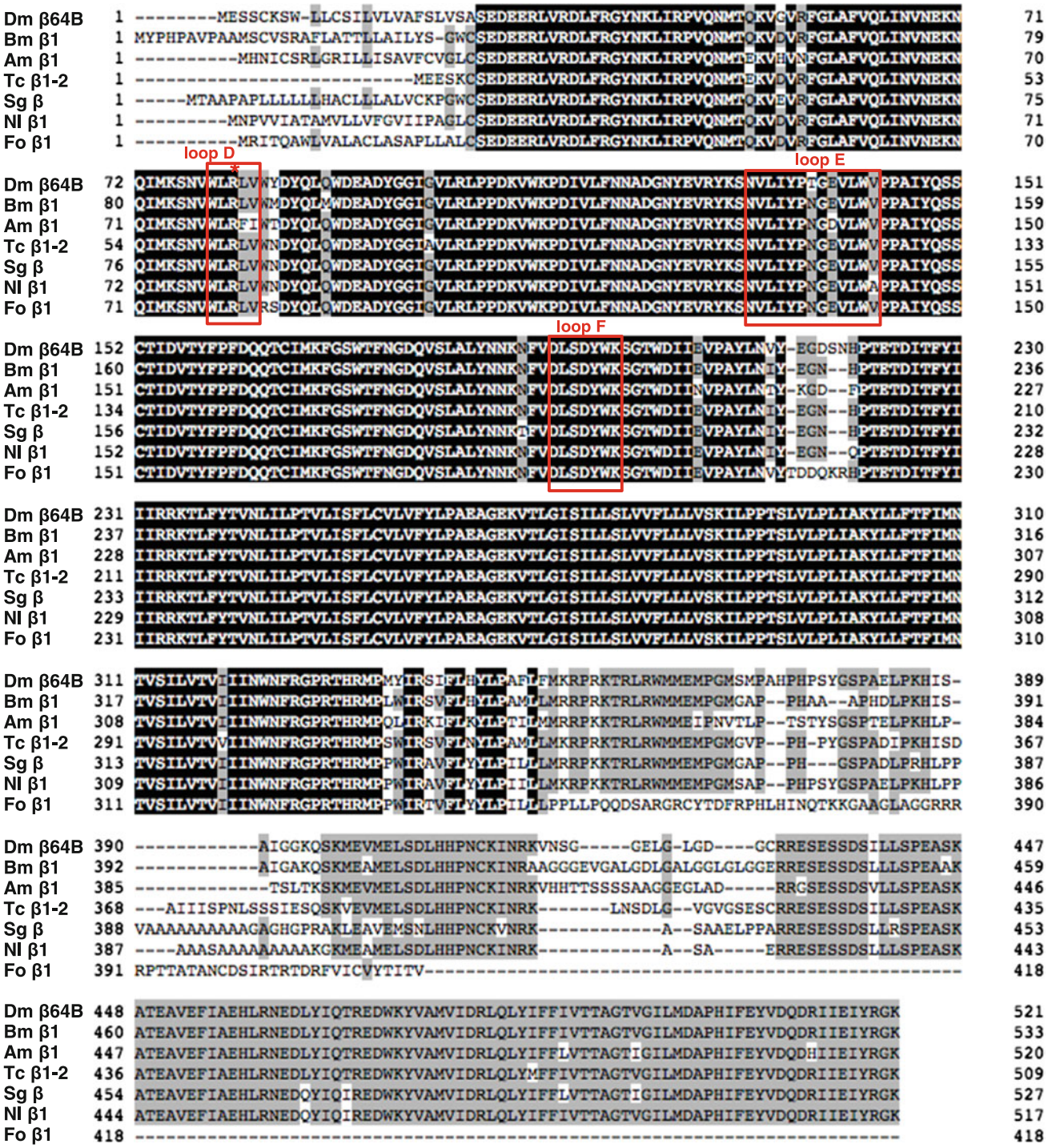

Fig. 1 Alignment of protein sequences of AChR β1 subunits and related sequences. Dm β64B, *Drosophila melanogaster* nAChR β64B isoform A (DDBJ/EMBL-Bank/GenBank accession no.: NP_523927); Bm β1, *Bombyx mori* nAChR β1 (ABV72692); Am β1, *Apis mellifera* nAChR β1 (NP_001073028); Tc β1-2, *Tribolium castaneum* nAChR β1 isoform 2 (NP_001156000); Sg β, *Schistocerca gregaria* nAChR β (ABA39253); Nl β1, *Nilaparvata lugens* nAChR β1 (ACJ07013); Fo β1, *F. occidentalis* nAChR β1 (this study). Amino acid residues shared by four to six species are indicated by *shaded letters*, and those common to seven species are shown with *white letters* over a *black* background. The R81T mutation site that has been reported in *Myzus persicae* is indicated with an *asterisk*. Putative loops comprising the ligand-binding domain are indicated in *boxes*

this study, the R81T mutation in loop D was not found in three strains. Other unknown structural factors may affect neonicotinoid sensitivity of the receptor.

Besides mutations in the genomic DNA, target-site changes could be caused by RNA editing (Hoopengardner et al. 2003). Interestingly, A-to-I RNA editing that results in non-synonymous changes in nAChR β1 of *N. lugens* happened at different frequencies between the susceptible and resistant strains, indicating that RNA editing may cause target-site changes and affect insecticide resistance (Yao et al. 2009). To elucidate the molecular mechanism of insecticide resistance in more detail, RNA editing sites in *F. occidentalis* nAChR need to be investigated in future studies.

In conclusion, we examined the susceptibility of three *F. occidentalis* strains from China and Japan and found that these are resistant to neonicotinoids. We showed that metabolism by cytochrome P450 monooxygenases is involved in neonicotinoid resistance in these strains, though other factors also seem to play a role. The R81T mutation in loop D of nAChR β1 that had been reported in *M. persicae* was not found in *F. occidentalis* strains tested in this study. Elucidation of the mechanism of neonicotinoid resistance awaits sequencing of all nAChR subunits and their functional studies.

Acknowledgments We thank Prof. T. Murai (Utsunomiya University, Japan) for providing strain H-1 of *F. occidentalis*, Mr. M. Shimomoto (Kochi Prefecture, Japan) for providing strain KC, Dr. H. Nishiwaki (Ehime University, Japan) for providing imidacloprid, and Mr. T. Sano and Ms. F. Ishii (Nagoya University) for assistance with insect rearing. This study was supported by a Grant-in-aid for Scientific Research (no. 21780046) from the Japan Society for the Promotion of Science (JSPS) and the JSPS-National Natural Science Foundation of China (NSFC) Joint Research Project.

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