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Molecular cloning and characterization of GABA receptor and GluCl subunits in the western flower thrips, *Frankliniella occidentalis*

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ABSTRACT

To understand the role of target site insensitivity in abamectin resistance in the western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), cDNAs encoding gamma-aminobutyric acid receptor subunit (*FoRdl*) and glutamate-gated chloride channel (*FoGluCl*) were cloned from WFT, and both single nucleotide polymorphisms (SNPs) and mRNA expression levels of *FoRdl* and *FoGluCl* were detected in a susceptible strain (ABA-S) and a laboratory selected strain (ABA-R) displaying 45.5-fold resistance to abamectin. Multiple cDNA sequence alignment revealed three alternative splicing variants of *FoRdl* and two alternative splicing variants of *FoGluCl* generated by alternative splicing of exon 3. While sequence comparison of *FoRdl* and *FoGluCl* in ABA-S and ABA-R strains identified no resistance-associated mutations, the expression level of *FoGluCl* in ABA-R strain was 2.63-fold higher than that in ABA-S strain. Thus, our preliminary results provide the evidence that the increased mRNA expression of *FoGluCl* could be an important factor in *FoGluCl*-mediated target site insensitivity in WFT.

1. Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), is one of the most important insect pests worldwide (Gao et al., 2012). The high polyphagy and fecundity, short generation time, together with haplo-diploid reproductive system enable WFT to cause serious feeding damage to fruits, vegetables and many crops (Gao et al., 2012; Kirk and Terry, 2003). In addition, as an important virus vector, WFT can transmit several plant viruses, such as *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) (Pappu et al., 2009; Webster et al., 2011). The use of insecticides is the primary strategy employed to control WFT, however, due to the biological attributes of WFT and the abuse of insecticides, WFT has developed resistance to a range of insecticide classes, including pyrethroids, neonicotinoids, carbamates, organophosphates, spinosad and abamectin (Gao et al., 2012; Bielza, 2008; Cloyd, 2016; Li et al., 2016; Wang et al., 2016a).

Abamectin (avermectin B1) belongs to the avermectin subfamily of macrocyclic lactones, produced by fermentation of the soil-dwelling microorganism *Streptomyces avermitilis* (Lasota and Dybas, 1991). Abamectin exerts broad spectrum of activity against pests including WFT, and several studies have reported that WFT has developed resistance to abamectin around the world (Dagli and Tunc, 2007; Herron and James, 2007; Immaraju et al., 1992; Wang et al., 2014; Zhao et al., 2013). In a previous study, we reported that the enhanced oxidative metabolism mediated by cytochrome P450 monooxygenases (P450s) was a major mechanism for abamectin resistance in ABA-R strain of WFT (Chen et al., 2011). However, due to the fact that the piperonyl butoxide (PBO) could not completely bring the abamectin resistance to a susceptible level, additional mechanisms, such as target site insensitivity, might also be involved in abamectin resistance.

In insect nervous system, both excitatory and inhibitory synaptic transmission are mediated by members of the Cys-loop ligand-gated ion channel (Cys-loop LGIC) superfamily, such as nicotinic acetylcholine receptors (nAChRs), gamma-aminobutyric acid (GABA) receptors and glutamate-gated chloride channels (GluCls) (Jones and Sattelle, 2007). Several lines of evidence have suggested that GluCls were the primary targets of macrocyclic lactones, while binding studies suggested that macrocyclic lactones also act on the insect GABA receptor subunit, Rdl (resistance to dieldrin) (Cully et al., 1994; Nakao et al., 2015; Wolstenholme, 2012; Wolstenholme and Rogers, 2005). In the present study, cDNAs encoding Rdl (*FoRdl*) and GluCl (*FoGluCl*) were cloned from WFT. Single nucleotide polymorphisms (SNPs) were detected in abamectin susceptible (ABA-S) and resistant (ABA-R) strains of WFT. The mRNA expression levels of these two target genes in ABA-S and ABA-R were also analyzed.

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Table 1
Primers used for RT-PCR, RACE and qPCR.

Primer name	Sequence (5′ to 3′)*	Description
192.GABAF1	TGGGTNCCNGAYACNTTYTT(WVPDTFF)	Rdl RT-PCR
193.GABAF2	TAYTTYCAYATHGCNACNAC(YFHIATT)	
474.GLURF1	AAYGARAARGARGGNCAYTT(NEKEGHF)	GluCl RT-PCR
476.GLURR2	CARCANGGDATRTADATYTG (QIYIPCC)	
212.C4.1R2	TTGCTGGTGGTTGCGATGTGAA	Rdl 5′RACE
469.C4.1R6	CTCGGGCTGGTGTAGCATTTCGGTTC	
213.C4.1F1	CTGGCTGAACCGAAATGCTA	Rdl 3′RACE
214.C4.1F2	GCTAATGTCCTCAACTAACGCTG	
506.GLUCLR1	GGATGAGGTAGTATGAGAACTCCCGC	GluCl 5′RACE
504.GLUCLF2	CTATGGGTGGACAACCAACGACTT	GluCl 3′RACE
471.Q2F1	ATGGGCAAAAGAGTAAGGCC	Rdl RT-PCR for SNP analysis
473.W45R1	CTACTTGTCCTCCTGCAACA	
781.GuClF1	TACGACGCTAGAATACGACC	GluCl RT-PCR for SNP analysis
783.GuClR1	AAGTTTACTGGTCCTCGGCC	
814.GABAF	CATCTACCTGGGCACCTGTT	Rdl qPCR
815.GABAR	GTCGTGAACTATGAAGCGCA	
812.GuClF	CAGTATTCGCATTTCGCTCA	GluCl qPCR
813.GuClR	AACCGAGGAAGATGGAGGTT	
706.FoR2_actinF	CGGTCAGGTCATCACCATTG	β-actin qPCR
707.FoR2_actinR	TCGTCTCGTGTATTCCGCAC	

* Corresponding amino acid sequences for degenerate primers were shown in parentheses.

2. Materials and methods

2.1. Insect strains

The laboratory selected ABA-R strain with 45.5-fold resistance to abamectin was derived from a susceptible strain (ABA-S) after 15 selection cycles with abamectin during 18 generations (Chen et al., 2011). About 20 2nd-instar nymphs of ABA-S or ABA-R were collected and stored at −80 °C until use for total RNA extraction. At least three repetitions were prepared for a given sample.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs were extracted from the collected samples using an SV total RNA isolation system (Promega, Madison, WI), according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the Primescript™ First-Strand cDNA Synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Degenerate primer pairs were designed against the amino acid residues that were conserved between insects (Table 1). PCR was performed in a 25 μL reaction volume containing 20–50 ng cDNA, 0.8 μM of each degenerate primer, 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 1.25 U Ex Taq™ polymerase and 2.5 μL Ex Taq™ buffer (Takara, Dalian, China). A touchdown PCR program was used, which consisted of 1 cycle at 94 °C for 5 min, 12 cycles at 94 °C for 30 s, 52–41 °C (decreasing by −1 °C/cycle) for 30 s, and 72 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 10 min.

2.3. Rapid amplification of cDNA ends (RACE)

To complete the cDNA sequence of *FoRdl* and *FoGluCl*, 5′ -RACE and 3′ -RACE reactions were performed using 5′-full RACE core set and 3′-full RACE core set (Takara, Dalian, China) respectively, following the manufacturer's instructions. Gene specific primers (GSP) used for the 5′ - and 3′ -RACE are listed in Table 1.

2.4. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was employed for cDNA template synthesis using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions. The SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa, Dalian, China) and gene specific primers were used for the gene expression determining (Table 1). The housekeeping gene β-actin was used as an internal control (Cifuentes et al., 2012). A 20 μL PCR reaction volume contains 10 μL SYBR Premix EX Taq™ II (2×), 2 μL diluted cDNA template with a concentration of 200 ng/μL, 0.4 μL ROX Reference Dye II (50×) and 0.4 μM of each primer. The reaction mixtures were performed on a Bio-Rad CFX 96 Real-time PCR system with a condition of 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and a melting curve analysis was performed at the end. The relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method and normalized to β-actin in the same sample (Pfaffl, 2001). Means and standard errors were obtained from the average of three independent sample sets.

2.5. Cloning and sequence analysis

RT-PCR and RACE products were subcloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced. The sequence alignment was performed using CLUSTALW with the default settings (Thompson et al., 1994). The aligned sequences were used to construct the phylogenetic tree in MEGA 5 with a bootstrapping of 1000 iterations. The molecular mass and isoelectric point were determined using ExPASy online service (http://web.expasy.org/compute_pi/). The cDNA sequences of *FoRdl* and *FoGluCl* have been deposited in the GenBank and the accession numbers were MH249047 and MH249048, respectively.

2.6. Data analysis

Statistical analysis was performed by one-way ANOVA (SPSS version 10.0, SPSS Inc., Chicago, USA) with at least three repeats, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. cDNA cloning and characterization of FoRdl and FoGluCl

The 4070 bp full length cDNA of *FoRdl* contains a 48-bp 5′-untranslated region (UTR), 1401 bp of ORF encoding a 466-amino acid residue protein with a calculated molecular mass of 52.34 kDa and an isoelectric point (pI) of 8.79, and a 2621-bp 3′-UTR (Fig. 1). Despite repeated attempts to amplify the 5′ end using conventional RACE strategy, we only obtained 1931 bp partial cDNA of *FoGluCl*, which encodes 437 amino acid residues and contains a 620-bp 3′-UTR. The common features of insect Cys-loop LGIC subunits, such as six ligand binding loops (Loop A-F) and four conserved transmembrane regions (TM1–4), were conserved in both FoRdl and FoGluCl (Fig. 1). An amino acid sequence alignment shows that FoRdl was 64.7% and 84.0% identical in pairwise comparisons with the *Rdl* amino acid sequences in *Drosophila melanogaster* and *Tribolium castaneum*, respectively. The deduced FoGluCl amino acid sequence showed 78.0% and 78.3% identities to GluCl from *D. melanogaster* and *T. castaneum*, respectively. Phylogenetic analysis reveals that Rdl proteins form a sister group relationship with Lcch3 subunits, and FoRdl and FoGluCl were closely related to blattaria and hymenoptera homologues, respectively (Fig. 2).

3.2. Alternative splicing

The alignment of multiple cDNA clone sequences revealed three alternative splicing variants of FoRdl (FoRdl-A, FoRdl-B and FoRdl-C) and two alternative splicing variants of FoGluCl (FoGluCl-A and FoGluCl-B) in WFT (Fig. 3). Comparison of the cDNA sequences with corresponding genomic sequences derived from the whole genome shotgun database (WGS) assembly of WFT deposited at GenBank revealed that both FoRdl and FoGluCl variants were generated by