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Nicotinic acetylcholine receptor subunit α6 associated with spinosad resistance in *Rhyzopertha dominica* (Coleoptera: Bostrichidae)

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ABSTRACT

The lesser grain borer, *Rhyzopertha dominica,* which is a primary pest of stored products, breaks up whole grains and makes them susceptible to secondary infestation by other pests. Insecticide application is the main control measure against this borer. A resistant strain of *R. dominica* against the insecticide, spinosad, was selected in the laboratory. The full-length cDNA of the target site of spinosad, nicotinic acetylcholine receptor subunit α6, from *R. dominica* (*Rdα6*) was cloned and analyzed using reverse transcription PCR and rapid amplification of cDNA ends. The complete 2133-bp cDNA contains the open reading frame of 1497 bp encoding a 498-amino-acid protein. There are four predicted transmembrane (TM) regions, and six extracellular ligand-binding sites at the N-terminus, upstream from the first TM in *Rdα6*. Three mutations have been found in the resistant strain compared with the susceptible one: (1) a 181-bp fragment truncated at the N-terminus, resulting in the appearance of a premature stop codon, (2) one missing bp at the position 997, causing a frame-shift mutation, and (3) an 87-bp fragment truncated in the TM2 region. In addition, real-time quantitative PCR was applied to detect the transcriptional expression of *Rdα6* in both the susceptible and resistant strains. The results indicated that the expression of *Rdα6* was significantly lower in then resistant strain than in susceptible one. In conclusion, mutation of *Rdα6* may cause *R. dominica* resistant to spinosad due to target site insensitivity.

1. Introduction

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), is a prominent pest in barns worldwide. It is a primary pest which breaks the hard husks of whole grains, rendering them susceptible to damage by secondary pests. It attacks many types of stored grains, including paddy rice, wheat, barley, soybean, maize, and sorghum and has become one of the most destructive pests [1]. The control of *R. dominica* mainly depends on chemical insecticides. Due to the long-term applications of insecticides, *R. dominica* has developed resistance to bioresmethrin, chlorpyrifos-methyl, deltamethrin, dichlorvos, fenitrothion, lindane, malathion, and phosphine [2]. Therefore, to control *R. dominica*, appropriate selection of alternative insecticides with different modes of action and awareness of environmental safety are necessary.

Spinosad, a fermentation product of bacteria with low toxicity to mammalians, has been applied as a potential alternative treatment for *R. dominica* despite its sensitivity to light [3]. Most barns are usually dim and are suitable for the application of spinosad. In addition, *R. dominica* has been reported to be susceptible to spinosad in wheat, rice, barley, sorghum, and maize [4–12]. Spinosad is not only highly insecticidal against living insects but is also suppressive against their progenies [7,10–12]. Furthermore, spinosad is not cross-resistant with other pesticides such as phosphine gas, deltamethrin, and organophosphates [13–16].

Rhyzopertha dominica is more susceptible to spinosad than are other stored-grain pests such as *Tribolium castaneum*, *Sitophilus oryzae*, and *Oryzaephilus surinamensis* [4,5], and resistance of *R. dominica* to spinosad has not yet been found. However, 27 insect species have been reported as resistant to spinosad [2], including ten species of Lepidoptera, seven species of Diptera, and two species of Coleoptera, which are *Tr. castaneum* and *Leptinotarsa decemlineata*. Two main mechanisms of spinosad resistance are target-site and metabolic resistances [17]. The primary target site is nicotinic acetylcholine receptor subunit α6 (nAChRα6) in the insect nervous system. In *Bactrocera dorsalis*, *Tuta absoluta*, *Frankliniella occidentalis*, *Thrips palmi*, and *Plutella xylostella*, spinosad resistance is associated with a target-site mutation [18–23]. However, both the target-site mutation and cytochrome P450 are associated with the spinosad resistance in *Th. palmi*. In *Musca domestica*, cytochrome P450 is overexpressed in the spinosad-resistant strain [24]. In addition, an increase in mixed-function oxidases activity is found in spinosad-resistant populations of *Tu. Absoluta* [25].

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Considering the application of spinosad to control *R. dominica* in stored products, the development of resistance to spinosad in the local populations of *R. dominica* was examined in this study. Furthermore, full-length cDNA of the spinosad target site, nAChRα6, was cloned from both susceptible and resistant strains to elucidate the mechanisms associated with spinosad resistance in *R. dominica*.

2. Materials and methods

2.1. Insects

Because spinosad has not been registered and applied to control *R. dominica* in Taiwan, the *R. dominica* colonies collected from barns in Shenkang Township, central Taiwan, were regarded as a susceptible strain and maintained in the laboratory without exposure to any pesticides for longer than 1 year. The resistant strain was selected from samples collected from barns in various locations in Taiwan. All strains were fed on wheat flakes and maintained in glass jars at 28 °C, 70% ± 5% relative humidity and in a 12:12 h photoperiod.

2.2. Insecticide

A commercial formulation of spinosad (800 g/kg, Entrust®) was provided by Dow AgroSciences LLC (Indianapolis, IN, United States). It was dissolved and diluted in water to attain desired concentrations.

2.3. Selection of spinosad-resistant strain

The spinosad-resistant strain was selected using a modified grain application method described elsewhere [16]. Briefly, extra-fine talc powder (Merck Chemicals, Darmstadt, Germany) was coated with desired concentrations of spinosad in a 250 ml flask. Wheat flakes were added into the flask and coated completely by the spinosad-treated powder. Adult *R. dominica* were placed into the flask. After 24 h, dead insects were removed and survivors were placed into a new glass jar with non-spinosad-treated wheat flakes. After another 24 h, the LC_{50} of the surviving *R. dominica* to spinosad was determined using the modified grain application method again using seven concentrations. Mortality data were analyzed by probit analysis [26] using a computer program [27]. The program provided the lethal concentration with 95% fiducial limits of LC_{50} and the linearity of dose-mortality and determined slope and chi-square value of each strain tested. The surviving *R. dominica* was left in the jar for another 2 months. After this time, a new generation had developed in the jar. All of the older generation survived *R. dominica* and these new adults in the jar were used for the next selection. The concentration of spinosad used for resistance selection was the LC_{50} from the previous selection process.

2.4. cDNA cloning of nAChRα6 using reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

Total RNA of the susceptible *R. dominica* adults was extracted using TRIZOL® Reagent (Invitrogne, Carlsbad, CA, United States) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA using the GeneRacer™ Kit (Invitrogen) following the manufacturer's instructions. To obtain the cDNA fragments of *R. dominica* nAChRα6 (*Rdα6*), PCR was performed with degenerate primers, Rdα6F2 and Rdα6R (Table 1), which were designed based on conserved regions of the nAChRα6 sequence from five species including: *P. xylostella* [18], *Bombyx mori* [28], *Tr. castaneum* [29], *Apis mellifera* [30], and *Anopheles gambiae* [31]. PCR cycling parameters were 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, and finally 72 °C for 7 min. The 769-bp PCR product was purified using the QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, United States), cloned into pCR®II-TOPO® vector (Invitrogen), and transformed into One Shot® TOP10 competent cells (Invitrogen). The cDNA fragment was sequenced using an ABI PRISM Big Dye Terminator Cycle sequencing Core kit with AmpliTaq DNA polymerase (ABI, Foster City, CA, United States), and then the sequences were obtained from the Tri-I Biotech (Taipei, Taiwan). Protein blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches indicated that the derived amino acid sequence of the 769-bp PCR product had high identity to *Tr.* castaneum nAChRα6.

Table 1
Sequences of primers used in this study.

Primer name	Primer sequence
Rdα6F2	YAAYAGTGCKGAYGARGGWTTYGACGG
Rdα6R	RTCRTCRTCKATRTCYARHACRTTBGC
Rdα6F2R08215′P	GATATCAGTACACACGGCACTATGAGG
Rdα6F2R08215′NP	GATCGTATTCTTCTTGCCAGGCATTCC
Rdα6F2R08213′P	GTACTGATATCTTCGATGGCGCTGCTC
Rdα6F2R08213′NP	GATACCAACTACGTCAGATGCAGTTCC
fRdα6F	GATCGCAGCTGAAATGGCCATAGTAGG
fRdα6R	GCAGTAGCGGTACAGTGCAGTTAAATG
Rda6qPCR05	GAGTCGGAATCCTTGGAAGTGAAGTTC
Rda6qPCR06	GTACATAAGAACGTCAGGCTTCCACAG
Rd-18s qPCR-01	CGAGACTCTGGCCTGCTAAC
Rd-18s qPCR-02	CCGCCTGTCCCTCTAAGAA

The 5′ and 3′ ends of the putative *Rdα6* cDNA were synthesized using the GeneRacer™ kit (Invitrogen). Specific primers were designed based on the sequence of the 769-bp fragment. The pair of primers for 5′ RACE PCR were Rdα6F2R08215′P (Table 1) and GeneRacer™ 5′ primer (Invitrogen). PCR cycling parameters were 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, and finally 72 °C for 10 min. The pair of primers for nested PCR were Rdα6F2R08215′NP (Table 1) and GeneRacer™ nested 5′ primer (Invitrogen). PCR cycling parameters were 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The initial 3′ RACE PCR was performed with sense primer Rdα6F2R08213′P (Table 1) and antisense primer GeneRacer™ 3′ primer (Invitrogen). Nested 3′ RACE PCR was conducted with sense primer Rdα6F2R08213′NP (Table 1) and antisense primer GeneRacer™ nested 3′ primer (Invitrogen). The initial PCR cycling parameters were 94 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for 10 min. The nested PCR parameters were the same as that for 5′ RACE. The PCR products were purified, cloned, and sequenced as described previously. The 5′ and 3′ RACE PCR products encompassed the full-length nAChRα6 cDNA. To obtain the entire open reading frame of nAChRα6 in one clone, two specific primers, fRdα6F and fRdα6R (Table 1), were designed in the 5′ and 3′ untranslated regions. PCR cycling parameters were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and finally 72 °C for 10 min. The PCR product was purified, cloned, and sequenced as described previously.

2.5. Sequence and phylogenetic analyses

Predictions of a signal peptide and transmembrane regions were performed using SignalP 4.1 Server [32] and TMpred [33], respectively.

The complete nAChRα6 protein sequence from the GenBank were obtained for phylogenetic analyses with *Rdα6*. Amino acid sequences were aligned using the Muscle Multiple Alignment option in SeaView4 [34]. Phylogenetic relationship analysis was conducted using neighbor-joining (NJ). The substitution model p-distance was chosen to construct the NJ tree using the MEGA 6.0 software [35]. Two nAChRα6 genes from hemimetabolous insects, *F. occidentalis* and *Periplaneta americana*, were used as the outgroup. One thousand replications of bootstrapping analyses were applied.

To compare the *Rdα6* sequences between susceptible and resistant strains, the full-length cDNA was cloned and sequenced as described previously using primers fRdα6F and fRdα6R from both strains.