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Silence of ryanodine receptor gene decreases susceptibility to chlorantraniliprole in the oriental armyworm, *Mythimna separata* Walker

Jin-Da Wang[a,*,1], Li-Fei Chen[a,1], Ya-Ru Wang[a], Hua-Ying Fu[a], Ahmad Ali[a], Da Xiao[b], Ran Wang[b,**], San-Ji Gao[a]

[a] National Engineering Research Center of Sugarcane, Fujian Agricultural and Forestry University, Fuzhou 350002, China
[b] Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China

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ABSTRACT

The ryanodine receptors of insects are the main target sites of diamide insecticides, which show highly selective insecticidal activity relative to toxicity in mammals and provide a novel option for managing lepidopteran pests. The oriental armyworm, *Mythimna separata* (Walker), is a destructive pest of agricultural crops, and great efforts have been undertaken to control this pest including repeated insecticide applications. In this study, full-length cDNA of a ryanodine receptor gene from *M. separata* (*MsRyR*) was cloned and characterized. The cDNA of *MsRyR* had a 15,372 bp open reading frame and encoded 5124 amino acids (GenBank ID: MG712298). *MsRyR* shares 78–97% identity with RyR isoforms of other insects, and < 50% identities with *Homo sapiens* RyRs 1–3. Temporal and spatial expression analysis detected *MsRyR* at all developmental stages and in all tissues. The highest relative levels of *MsRyR* were detected in the second instar and head. Exposure to chlorantraniliprole after 24 h significantly increased the expression levels of whole body *MsRyR* mRNA. In addition, dietary ingestion of dsMsRyR significantly reduced the mRNA level of *MsRyR* and greatly decreased chlorantraniliprole-induced mortality. Our results revealed that the *MsRyR* could be the molecular target of chlorantraniliprole, and provided the basis for further understanding the resistance mechanism of chlorantraniliprole.

1. Introduction

The ryanodine receptors (RyRs), a type of Ca^{2+} release channel, play an important role in intracellular Ca^{2+} signalling transmission [1,2] and further regulate various life processes such as muscle contraction, release of neurotransmitters, hormone secretion, and fertilization [3]. RyRs are the largest ion channels currently known. Mammals have three types of RyRs – RyR1, RyR2, and RyR3 [4,5]. Two RyR isoforms (RyRA and RyRB) have been identified and characterized in fish and birds; these two RyR isoforms are highly related to mammalian RyR1 and RyR3. In contrast, only one isoform of RyR has been detected in insects and nematodes [6–8]. Due to regions with a high level of structural divergence between mammalian and insect isoforms, RyRs could serve as potential targets for insecticides. Chlorantraniliproles belong to the class of diamides that target RyRs, and have been commercially developed [9,10]. However, extensive and intensive application of this agent has caused rapid development of insecticide resistance and led to serious food safety concerns [11]. Several studies have revealed the existence of chlorantraniliprole resistance in different insects [12–14], and monitoring of *M. separata* in the area around Beijing showed a high level of resistance to chlorantraniliprole [15]. Understanding the molecular function of RyR is a key step in studying resistance mechanisms to chlorantraniliprole.

The study of RyR coding sequences may provide key information for understanding the molecular basis for high selectivity of diamide insecticides and of resistance mechanisms. Currently, the underlying mechanisms of diamide resistance are thought to be due to target-site mutations located in the transmembrane domain of the insect RyRs [16]. The amino acid substitution G4946E in the C-terminal region of RyR was widely confirmed in the diamide-resistant populations of different insects [17–20]. Further studies conducted in *Plutella xylostella* flight muscle membrane preparations [21] and recombinant RyR variants stably expressed in Sf9 cells [22] demonstrated functional evidence of the G4946E mutation for diamide resistance. This functional mutation is also confirmed by genome modification in the model insect *Drosophila melanogaster* [23,24] and in the non-model insect *Spodoptera*

* Corresponding author at: Fujian Agricultural and Forestry University, Fuzhou 350002, China.
** Corresponding author.
E-mail addresses: jdwang@fafu.edu.cn (J.-D. Wang), rwang1105@126.com (R. Wang).
[1] Both authors contributed equally to this work.

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exigua [25].

The oriental armyworm, *Mythimna separata* Walker is a devastating pest of cereals in eastern Asia. It has been documented to infest species from 100 plant families, affecting > 300 kinds of food and industrial crops, such as corn, rice, and sugarcane [26,27]. In addition, *M. separata* is a migratory pest with outbreaks in specific years and landing places that often result in substantial economic damage to crops [28,29]. Therefore, it is often the target of repeated insecticide applications. However, long-term use of chemical insecticides has caused serious environmental problem and has also led to rapid development of insecticide resistance [30]. In order to better understanding of the action mode and the resistance mechanism of the novel diamide insecticides, a full-length RyR cDNA (named MsRyR) from *M. separata* was cloned and characterized and the mRNA expression pattern of *MsRyR* was profiled. The second goal of our study was to evaluate the influence of MsRyR-dsRNA on susceptibility of *M. separata* larvae to chlorantraniliprole and confirm the molecular target of chlorantraniliprole in *M. separate*.

2. Materials and methods

2.1. Insects

Mythimna separata was originally purchased from Henan Jiyuan Baiyun Industry Company (Jiyuan, Henan, China), and the colony was maintained in the laboratory for 10 years without exposure to insecticides. The insects were maintained on an artificial diet in the laboratory at 26 ± 1 °C, with 70% relative humidity and a 16:8 (L:D) photoperiod.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from developmental stages (eggs, 1st to 6th instar larvae, pupae and adults), and tissues (integument, foregut, midgut, hindgut, and head) from 6th-instar larvae with the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then the remaining genomic DNA was removed, and the first-strand cDNA was synthesized from 1 μg total RNA using the Prime Script™ 1st Strand cDNA Synthesis Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) for reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR).

2.3. Cloning and sequence analysis

The open reading frame (ORF) of the RyR gene was predicted by utilizing the tool ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) according to our transcriptome data (unpublished). Then, ten specific primers were designed to amply the ORFs (Table 1) from *M. separata*. The cDNA fragments were further assembled and aligned with DNAMAN (DNAMAN 5.2.2, Lynnon BioSoft). The isoelectric point and molecular weight of deduced protein sequences were analyzed using the ExPASy Proteomics Server (http://cn.expasy.org/tools/pi_tool.html). The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the signal peptides and transmembrane helices [25]. The ScanProsite tool (http://prosite.expasy.org/scanprosite/) and InterPro (http://www.ebi.ac.uk/interpro/) [26] were applied to predict the putative motifs and domains, which were also aligned with other published RyRs. MEGA 6.0 was used to construct the phylogenetic tree using the maximum likelihood method and the bootstrap values were calculated based on 1000 replications.

The signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The matured RyR protein sequences from *B. tabaci*, and other Hemipteran pest species were aligned using ClustalX 1.83 and a phylogenetic tree was constructed in MEGA5.1 using the neighbor-joining method with 1000-fold bootstrap resampling. Transmembrane domains were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The regions of putative motifs were predicted by ExPASy ScanProsite (http://prosite.expasy.org/scanprosite/) or alignment to other published RyRs.

Table 1
Primers used for RT-PCR, and qRT-PCR.

Primer name	Primer sequence (5′ → 3′, T7 = TAATACGACTCACTATAGGG)	Length
MsRyR1	F: AAGATGGCGGAAGCAGAGG R: TGATGGCGGCAAGCAACT	1609
MsRyR2	F: TGCTTGCCGCCATCATCA R: GACTCTGTGCTACCGCTGTA	1861
MsRyR3	F: AGCAGAAGCAAGCGGACTT R: CACAGAGCATGGACCAACCT	1974
MsRyR4	F: TCCGACATCCGAGGTTGGT R: GCAGTCCACAGCGAGATAGG	1975
MsRyR5	F: TACCTATCTCGCTGTGGACTG R: TCTGTTCCGTGTCGCTCTG	1996
MsRyR6	F: TGGCATCCATCCGCAACTA R: CCTCTGGCACATTGTTCCTC	1628
MsRyR7	F: CGTGACAATGGTAACAGCAGAA R: GCAGCCGCAGCAGATAATC	1931
MsRyR8	F: CGCACTCTTCAGGTTCATACAA R: TTGCTTCTTGGCTTGCTCAC	1765
MsRyR9	F: TGAGGAGGCGGAGGTATCA R: TGTCTGTGGCTAGAAGGTTGT	1714
dsMsRyR	F: T7 + TGTCTTGGCAGCACTATTTG R: T7 + ATGTTACAAGCCCGATGTCT	280
dsEgfp	F: AAGTTCAGCGTGTCCGGC R: CACCTTGATGCCGTTCTTC	414
qMsRyR	F: CAAGAGAAGGATGACCAGA R: GCACGATGACAGTAGAGT	97
β-Actin	F: CGATTCCGTTGCCCTGAGG R: CATGATCGAGTTGTAGGTGGTCT	87

2.4. qRT-PCR analysis of MsRyR expression profiles

The relative transcription levels of *MsRyR* in different developmental stages (eggs, 1st to 6th instar larvae, pupae and adults) and tissues (integument, foregut, midgut, hindgut and head) from 6th-instar larvae were examined using qRT-PCR. qRT-PCR was performed using gene-specific primers and SYBR Premix EX Taq™ (TaKaRa, Dalian, China) in three biological replicates with different samples on the ABI 7500 system (Applied Biosystems, CA, USA) with 20 μl reactions containing 1.0 μl cDNA (200 ng/μl), 10 μl SYBR Premix Ex Taq™, 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM) (Table 1), 0.4 μl Rox Reference Dye II, and 7.8 μl nuclease free water. Thermal cycling conditions were: 95 °C for 30s, 40 cycles of 95 °C for 5 s, 62 °C for 34 s. After the cycling protocol, a melting curve analysis from 60 °C to 95 °C was applied to all reactions to verify a single PCR product. The amplification efficiency was estimated by using the equation, $E = 10^{-1}/$slope, where the slope was derived from the plot of cycle threshold (Ct) value verse five serially diluted template concentration. Quantification of transcript level of the *MsRyR* was conducted using the $2_{-\triangle\triangle Ct}$ method [31] and *β-actin* was used as housekeeping gene to correct for sample-to-sample variation. All statistical analyses on the expression of *MsRyR* in different stages and various body parts were analyzed using one-way ANOVA followed by Tukey' HSD for multiple comparisons in SPSS 18.0.

2.5. Bioassay

Twenty-five 3rd instar larvae were selected and starved for 12 h before intrathoracic injection with 2 μl chlorantraniliprole solution of various doses (1 mg/L, 5 mg/L, 8 mg/L, and 10 mg/L) using *N*, *N*-dimethylformamide (DMF) as the carrier solvent as describe before [8]. After 24 h, the mortality rate was recorded and corresponding LC_{50} was calculated by SPSS 18.0 Probit analysis. The experiment was replicated

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