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

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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*

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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	Length
	(5′→3′, T7 = TAATACGACTCACTATAGGG)	
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

three times.

2.6. Expression profiles of MsRyR mRNA in response to chlorantraniliprole

The 3rd instar larvae were selected and starved for 12 h before feeding experiment. Two sub-lethal concentration of 5 mg/L and 10 mg/L chlorantraniliprole were used to treat *M. separata* by intrathoracic injection as mentioned above. The solvent with *N*, *N*-dimethylformamide (DMF) was treated as the control group. Total RNA from *M. separata* was extracted after 24 h treatment, and the change of *MsRyR* mRNA levels was examined by the qRT-PCR method. *β-Actin* (accession number GQ856238) was used as housekeeping genes to correct for sample-to-sample variation.

2.7. RNAi in M. separate

The templates for transcription in vitro were prepared by PCR: a fragment of *MsRyR* (336 bp), and an enhanced green fluorescent protein gene *egfp* fragment (414 bp) were generated using specific primers (Table 1) conjugated with the T7 RNA polymerase promoter (taatacgactcactataggg). Then they were synthesized using the T7 Ribomax™ Express RNAi System (Promega) as described in the manual.

Forty late 2nd instar larvae per replicate were fed an artificial diet containing 100 μg dsRNA (MsRyR-, or egfp-dsRNA) in the 24-well plate; the diet was changed each day with fresh dsRNA continuously for 3 days. A diet with the same amount of ddH_2O (100 μl) was used as control. After 3 days, those larvae molted to the 3rd instar, and then total RNA was extracted from five living larvae each for control, *MsRyR*-, or *egfp*-dsRNA treated groups. Then approximately 30 larvae in the control, *MsRyR*-, or *egfp*-dsRNA treated groups were used for chlorantraniliprole bioassay test with a dose of 8 mg/L by microinjection [8]. Those larvae were transferred to a new 24-well plate that contained an artificial diet. One day after chlorantraniliprole application, the numbers of dead larvae were recorded. The experiment was replicated three times.

3. Results

3.1. Cloning and sequence analysis of MsRyR cDNA

Based on the transcriptome data of *M. separata*, nine primers were designed to amplify the ORF of *MsRyR* (Fig. 1). The assembled ORF of *MsRyR* was 15,372 bp of contiguous sequence and encoded 5124 amino acids. The sequence was submitted to NCBI with GenBank ID: MG712298.

3.2. Analysis of MsRyR amino acid sequence

Based on the ORF of *MsRyR*, the predicted protein MsRyR has a molecular weight (MW) of ~580 kDa and pI 4.45. The MsRyR amino acid sequence was analyzed for putative regulatory domains (Fig. 2). The NH2-terminal region of MsRyR contains an MIR motif (aa 100–206 and 216–400), named after three of the proteins that make up the motif: Mannosyltransferase, IP3R, and RyR; three SPRY (SPla and RyR) domains (669–808, 1097–1220, and 1557–1700) and two RyR and IP3R homology domains (448–646 and 2240–2470) were also found. Compared with other reported insect RyRs, including BmRyR from *B. mori*, PxRyR from *P. xylostella*, DmRyR from *D. melanogaster*, and AaRyR from *Aedes aegypti* (Fig. 3), the alignment showed there were six typical hydrophobic transmembrane (TM) domains in the COOH-terminal region of MsRyR, which were predicted using the TMHMM program at the positions 4456–4478 (TM1), 4647–4669 (TM2), 4733–4755 (TM3), 4875–4897 (TM4), 4923–4941 (TM5), and 5003–5022 (TM6) as displayed in Fig. 3. The sequence motif, GXRXGGGXGD in the pore-helix, which was identified between TM5 and TM6 and constitutes part of the pore-forming segments of the Ca^{2+} release channels, was highly conserved in MsRyR (4964–4984) and other insect RyRs (Fig. 3).

3.3. Phylogenetic relationships of the RyR family with other insects

The identities of amino acid sequences among several orders of insects are shown in Table 2. MsRyR shared the greatest identity with lepidopteran species *Helicoverpa armigera* RyR (HaRyR, 97.4%), and also had high identities with the RyRs of *Apis mellifera* (80.6%), *Tribolium castaneum* (80.5%), *Nilaparvata lugens* (79.6%), and *Drosophila melanogaster* (77.9%). However, the identities were only 45.1%, 42.6%, 44.8%, and 43.3% compared with *Caenorhabditis elegans* RyR, and *H. sapiens* RyRs 1–3, respectively.

A phylogenetic tree of ORF amino acid sequences of RyRs from the 33 species (Fig. 4) identified three distinct RyR clusters – mammal, insect, and nematode; the insect RyRs were separated from mammal and nematode RyRs but were more closely related to nematode RyRs. For insects, RyRs from different species in the same order, such as Diptera, Lepidoptera, Coleoptera, Hymenoptera and Hemiptera, were grouped together.

3.4. Toxicity of chlorantraniliprole to M. separata

The toxicity of chlorantraniliprole to *M. separata* was determined using the intrathoracic injection method [8]. According to the bioassay results, 8 ppm of chlorantraniliprole was used as the LC_{50} to treat *M. separata* (Table 3).

3.5. mRNA expression profiles and effect of chlorantraniliprole on expression of MsRyR

The mRNA expression level of *MsRyR* was analyzed using RT-qPCR at various developmental stages and from different body parts of *M. separata*. To test the amplification efficiency of the qRT-PCR primers for *Msβ-actin* and *MsRyR*, the slopes of linear regression equations were −3.4377 and −3.3849, respectively, and the amplification efficiency of the two qRT-PCR primers were 95.4% and 97.3%.

The developmental expression pattern revealed that *MsRyR* was expressed at all life stages, and the relative expression level of *MsRyR* mRNA was 1.0-, 1.4-, 3.3-, 4.2-, 1.0-, 1.8, 2.6-, and 1.0-fold higher in the 1st, 2nd, 3rd, 4th, 5th, and 6th instar larvae, pupae, and adults respectively (Fig. 5A), compared to that in the eggs. *MsRyR* mRNA was highly expressed in the head, while the abundance of *MsRyR* was lowest in the integument (Fig. 5B).

Samples were collected 24 h after treatment with chlorantraniliprole. The relative expression level of *MsRyR* mRNA was increased after exposure to insecticide (Fig. 5C). After 24 h of treatment with 5 ppm (LC_{25}), and 10 ppm (LC_{75}), expression levels of *MsRyR* mRNA were up-regulated 1.7- and 2.1-fold, respectively, compared to

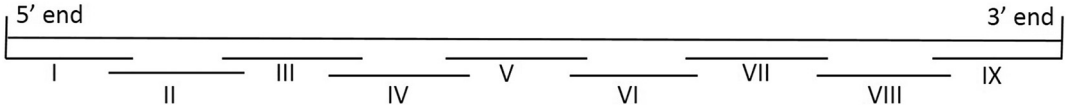

Fig. 1. PCR amplification of *M. separata* ryanodine receptor (*MsRyR*) cDNA. The complete black line (5′ end to 3′ end) indicates the full-length of *MsRyR* ORF. I to XI represent the nucleotide sequences of the overlapping cDNA clones. I-IX represent the nucleotide sequences amplified by primer sets (MsRyR1-9) of the overlapping cDNA clones.

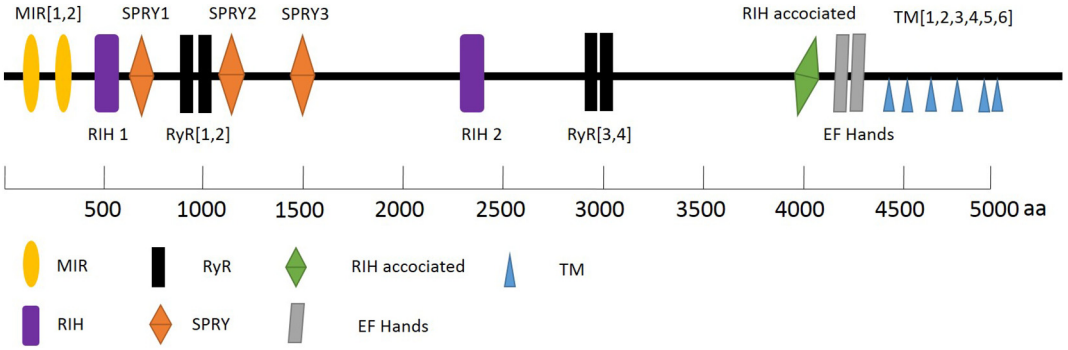

Fig. 2. Analysis of deduced MsRyR primary structure. The relative position of the conserved structural domains are predicted with InterPro (http://www.ebi.ac.uk/interpro/) and marked.

the level of the untreated control; there were no significant differences between the two dosages ($P = 0.370$).

3.6. Effect of dsMsRyR on MsRyR expression and chlorantraniliprole tolerance

After 3 days of continuous ingestion of dsMsRyR, the 2rd instar molted to 3rd instar. The mRNA levels of *MsRyR* in the treated larvae was significantly reduced to 40.1% (Fig. 6A) compared to that in corresponding controls. Three days of ingestion of dsRNA killed few larvae, then the living larvae that had been exposed to CK (ddH_2O), dsEgfp-, or dsMsRyR1-treated diets were using for further bioassay experiments. Approximately 30 larvae in each treatment were injected with chlorantraniliprole (LC_{50}). Larval mortality in the CK and dsEgfp treatment groups was 55.4% and 49.5%, respectively, both of which were much higher than that in the dsMsRyR1-treatment (33.1%, Fig. 6B).

4. Discussion

The insect RyR gene has been documented in several insect species including some lepidopterans [7,8,28]. However, even though chlorantraniliprole insecticide is widely applied, little is known about the structure and function of *M. separata* RyR [15,30]. Characterization and detailed analysis of the full-length *RyR* cDNA is fundamental for understanding the molecular mechanism of resistance.

We cloned the full-length ORF of RyR in *M. separata*, and BLAST and phylogenetic analyses indicated that the MsRyR amino acid sequence is homologous to different insect RyRs (especially *H. armigera*, Table 2), and different known domains were predicted (Fig. 2). The two MIR domains predicted at the N-terminal region exert a ligand transferase role [32]; a previous study showed no response of BmRyR to flubendiamide when the N-terminal sequence was deleted [33]. Moreover, other typical characteristics of RyRs were conserved in MsRyR, including two RIH domains that are intracellular calcium-release channel domains. Four RyR domains were identified, which are characteristic of the RyR structure and are conserved in all members of the intracellular Ca^{2+} release channel superfamily [34]. In addition, six transmembrane domains (Fig. 2) are present near the C-terminal amino acids between residues 4456 and 5022 in MsRyR. In studying the interaction of flubendiamide with the recombinant RyR, Kato et al. [33] found that the transmembrane domain plays an important role in the formation of an active site for flubendiamide. In the case of insect RyRs, five TM regions were mostly conserved in Diptera, Lepidoptera, Coleoptera, Hymenoptera, and Hemiptera, except TM1 with lower conservation [8].

Amino acid sequence alignment showed that MsRyR shares 85–92% similarity with other insect RyR homologs and 42.6–44.8% identity with mammalian homologs. Insect RyRs are genetically conserved and clearly segregated from three mammalian RyRs and one *C. elegans* RyR; insect RyRs also clustered together based on insect order (Fig. 4). Our results were consistent with other published data, illustrating that insect RyRs had low similarity with mammalian homologs and highlighting the potential of insect RyRs as insecticide targets.

Temporal and spatial expression analysis showed that MsRyR is widely expressed in all tissues and development stages. *MsRyR* mRNA was most abundant in the head, in accordance with data from other lepidopteran insects, such as *S. exigua* [35], *H. armigera* [7], *P. xylostella* [12], and *C. suppressalis* [8]. This result indicated that RyR has a regulatory function and is mainly distributed between the junctions of the muscular and nervous systems. Through the analysis of temporal expression, we found that *MsRyR* mRNA was highly expressed in the L2 larvae and was lower in eggs and the L6 instar. This phenomenon is similar to but not the exactly the same as in *C. suppressalis*, *P. xylostella*, and *D. melanogaster* [8,12,36]. However, not all insect RyRs shared the same expression pattern as *M. separata*. OfRyR is highly expressed in adults in *O. furnacalis* [37], and in *H. armigera*, expression of *HaRyR* was significantly lower in eggs and exhibited no differences among third instar larvae, pupae, and adults [7]. In summary, temporal and spatial transcript profiles of RyR varied among insect species.

Lepidopteran RyRs are very sensitive to diamide insecticide [8]. Our research revealed that *MsRyR* could be up-regulated by chlorantraniliprole (Fig. 5C). This situation was similar to some other insects: the RyR gene in *C. suppressalis* and *P. xylostella* could be induced by the low-concentration treatments of chlorantraniliprole [8,35], while higher doses of cyantraniliprole, which is another novel diamide insecticide, notably up-regulated the expression levels of the BtRyR gene [38]. To date, no label rates of chlorantraniliprole resistance have been observed in the field for any noctuid pest [25]. Previous studies conducted in different insects showed that RyR G4946E mutation is associated with major functional changes that result in insensitivity to diamide insecticide [17–20]. The RyR-associated target-site resistance to diamide insecticides has not yet been reported in *M. separata*. The resistance-related residue G^{4905} site in MsRyR was not replaced in *M. separata* in the present study as shown in Fig. 3. Therefore, further work could be conducted to detect the relationship between the mutation of this site and chlorantraniliprole resistance in the *M. separata*.

To further confirm the function of RyR, we tested the effect of chlorantraniliprole sensitivity after an RNAi experiment targeting *MsRyR*. We found that dietary ingestion of dsMsRyR significantly reduced the mRNA level of *MsRyR* in the treated 3rd instar by 58.9%, and greatly decreased chlorantraniliprole-induced mortality. Consistent with our result, knockdown of RyR in *Sogatella furcifera* [39] and *Leptinotarsa decemlineata* [40] decreased chlorantraniliprole-induced mortality effects. The deletion mutation of the RyR gene *unc-68* in *C. elegans* led to low efficacy of ryanodine-induced paralytic effects [41,42]. Another study in *P. xylostella* showed that *PxRyR* was down-regulated in chlorantraniliprole-resistant populations [43]. However, we did not find any negative effects of dsMsRyR on larval growth during the 3 days of ingestion, and further work is needed to test the negative effects of

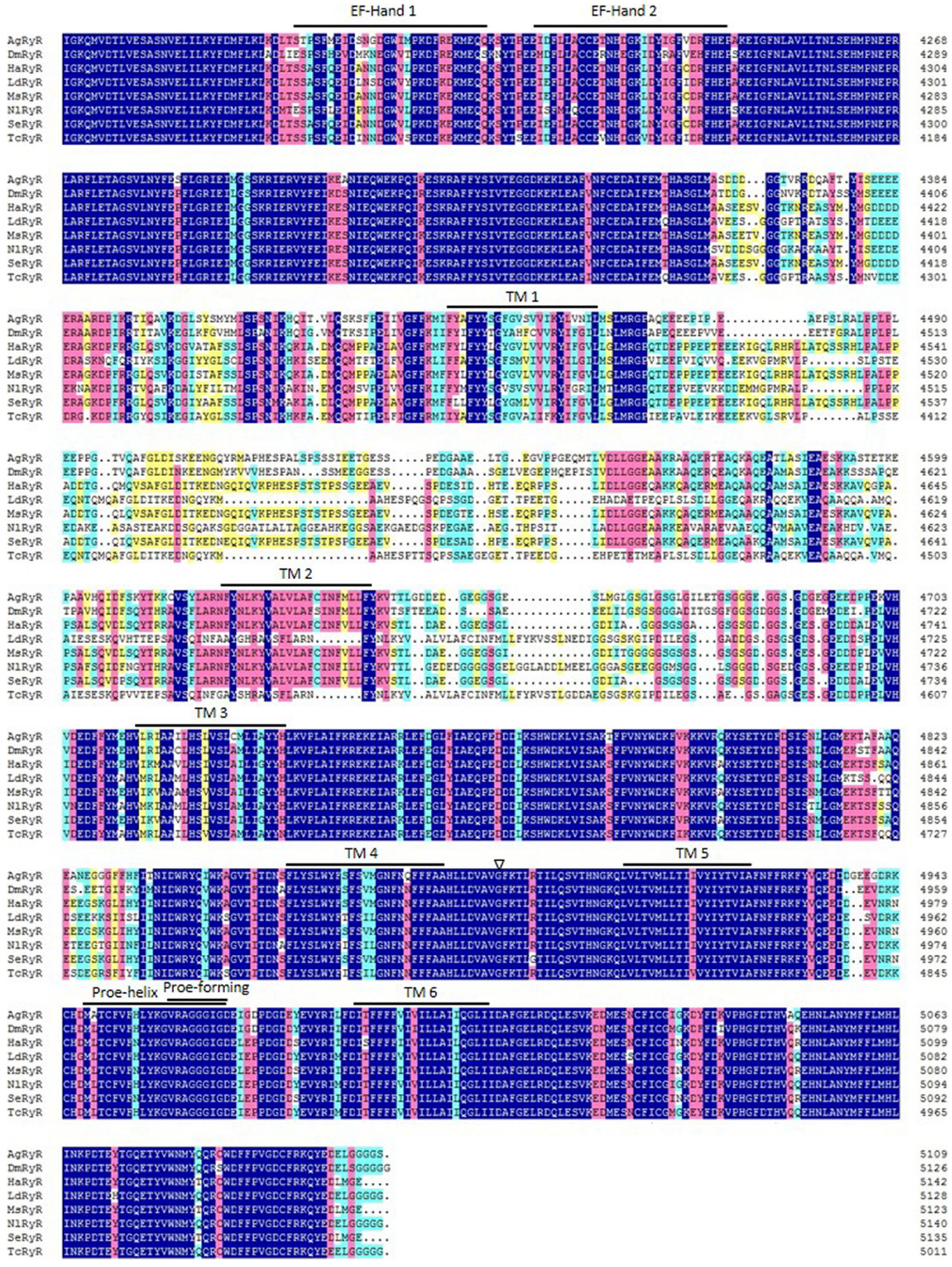

Fig. 3. Alignment of COOH-terminal region of the insect RyR isoforms. Identical amino acids are shown in blue boxes and similar amino acids are highlighted in pink boxes. Two EF-hand motifs, six transmembrane domains (TM1–6) and the pore-helix including the pore-forming segment between TM5 and TM6 are highlighted with black lines. The amino acid G4905 was marked with triangle, and G4905 was the mutation position of resistance in RyR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Comparison of ryanodine receptor protein sequences from different organisms.

	M. separata	*H. armigera*	*A. mellifera*	*N. lugens*	*D. melanogaster*	*T. castaneum*	*C. elegans*	*H. sapiens* RyR1	*H. sapiens* RyR2	*H. sapiens* RyR3
M. separata		97.4	80.6	79.6	77.9	80.5	45.1	42.6	44.8	43.3
H. armigera			80.5	79.3	77.7	80.0	44.8	42.4	44.6	43.2
A. mellifera				82.6	77.9	81.4	45.3	42.3	44.7	43.2
N. lugens					77.4	80.2	44.9	42.4	44.8	43.3
D. melanogaster						76.4	44.6	42.0	44.1	42.8
T. castaneum							44.9	41.7	44.0	42.7
C. elegans								37.4	39.4	37.6
H. sapiens RyR1									64.0	64.3
H. sapiens RyR2										67.4
H. sapiens RyR3										

The corresponding GenBank IDs are *Nilaparvata lugens* (NlRyR), AGW82429.1; *Apis mellifera* (AmRyR), XP_006569096.1; *Mythimna separata* (MsRyR), this study; *Drosophila melanogaster* (DmRyR), NP_001246210.1; *Helicoverpa armigera* (HaRyR), AHB33498.1; *Tribolium castaneum* (TcRyR), XP_008191330.1; *Caenorhabditis elegans* (CeRyR), BAA08309.1; *Homo sapiens* RyR1 (HsRyR1), NP_000531.2; *H. sapiens* RyR2 (HsRyR2), NP_001026.2; *H. sapiens* RyR3 (HsRyR3), NP_001027.3.

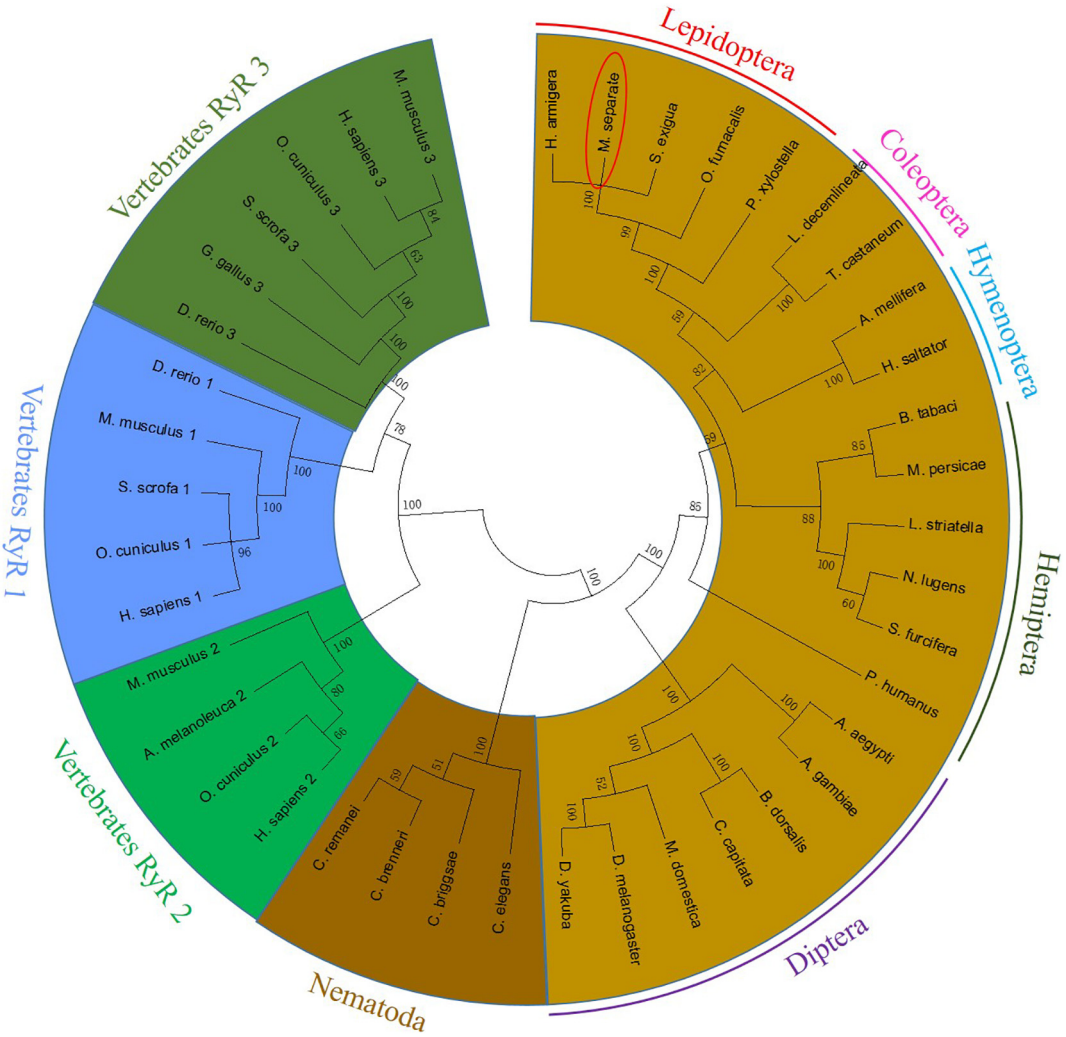

Fig. 4. The phylogenetic tree of deduced MsRyR compared with that of other known RyRs of different species. RyR proteins originate from *Helicoverpa armigera* (AHB33498.1), *Spodoptera exigua* (AIA23854.1), *Ostrinia furnacalis* (AGH68757), *Plutella xylostella* (AFW97408), *Leptinotarsa decemlineata* (AHW99830.1), *Tribolium castaneum* (EEZ99829), *Apis mellifera* (XP_392217.5), *Harpegnathos saltator* (EFN78897.1), *Bemisia tabaci* (AFK84957.1), *Myzus persicae* (AJA41114.1), *Laodelphax striatella* (AFK84959), *Nilaparvata lugens* (AGW82429), *Sogatella furcifera* (AHW99829.1), *Pediculus humanus* (XP_002424547.1), *Aedes aegypti* (XP_001657320), *Anopheles gambiae* (XP_318561), *Bactrocera dorsalis* (AHY02115.1), *Ceratitis capitata* (XP_012158402.1), *Musca domestica* (XP_011296547.1), *Drosophila melanogaster* (NP_476992), *D. yakuba* (XP_002089690.1), *Caenorhabditis elegans* (BAA08309), *C. briggsae* (XP_002637345.1), *C. brenneri* (EGT47004.1), *C. remanei* (EFP05547.1), *Homo sapiens* (RyR1, EAW56797.1; RyR2, EAW70071.1; RyR3, NP_001027.3), *Oryctolagus cuniculus* (RyR1, NP_001095188.1; RyR2, NP_001076226.1; RyR3, NP_001076231.1), *Sus scrofa* (RyR1, NP_001001534.1; RyR3, XP_013833720.1); *Mus musculus* (RyR1, AAP29981.1; RyR2, NP_076357.2; RyR3, NP_808320.2); *Danio rerio* (RyR1, NP_001096041.1; RyR3, XP_009293048.1); *Gallus gallus* (RyR3, NP_996757.2).

Table 3
Bioassay of chlorantraniliprole on *M. separata*.

Insecticide	Number	Slope ± SE	LC_{50} (mg/L)	LC_{50} (95% FL) (ppm)	X^2 (df)	P
Chlorantraniliprole	300	−0.948 ± 1.013	8.626	6.483–11.477	2.208 (3)	0.137

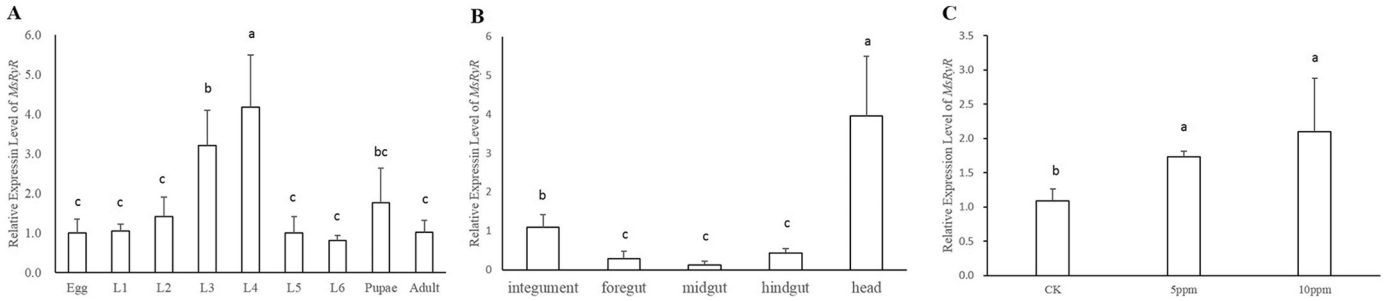

Fig. 5. Relative expression level of *MsRyR* mRNA under different situations. (A) Temporal expression levels of *MsRyR* mRNA. L1, L2, L3, L4, L5 and L6 indicate the 1st, 2nd, 3rd, 4th, 5th and 6th instar larvae, respectively. (B) Spatial expression levels of *MsRyR* mRNA in 5th instar larvae. (C) Relative expression level of *MsRyR* in larvae treated with chlorantraniliprole. Larvae treated with DMF were used as control. Different lowercase letters (a, b and c) indicate significant difference ($P < 0.05$) based on one way ANOVA followed by Tukey's HSD for multiple comparisons. The error bars represent the means ± SE of three replicates.

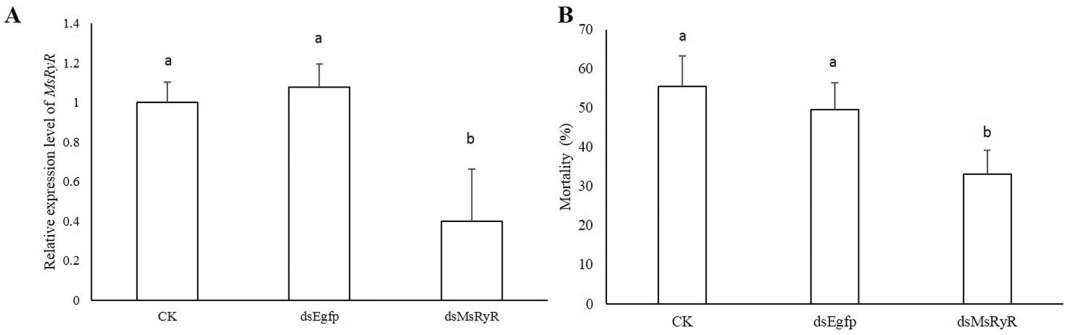

Fig. 6. Effects of dietary ingestion of dsMsRyR on *M. separata*. (A) Relative expression level of *MsRyR*. (B). The mortalities of 3rd instar larvae treated with chlorantraniliprole. The mortality was evaluated 24 h after insecticide treatment. The values represent averages with vertical bars indicating SE; bars topped with the same lowercase or uppercase letters are not statistically significantly different at $P = 0.05$.

dsMsRyR.

In the current study, we cloned and characterized the *RyR* gene from the devastating crop pest *M. separata*. We also confirmed the expression profiles of *MsRyR* in different developmental stages and various body parts of adults. Additionally, *MsRyR* could be induced by chlorantraniliprole and knockdown of *MsRyR* resulted in insensitivity to chlorantraniliprole. Further work should be done to reveal the structure and function of insect RyRs, and to provide a basis for understanding the remarkable selectivity for Lepidoptera and the resistance mechanisms for this novel diamide insecticide.

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