Characterization of the Ryanodine Receptor Gene With a Unique 3′-UTR and Alternative Splice Site From the Oriental Fruit Moth

L. N. Sun, H. J. Zhang, L. F. Quan, W. T. Yan, Q. Yue,1 Y. Y. Li, and G. S. Qiu1

Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning 125100, People’s Republic of China (slncas@163.com; zhanghuaijiang@sina.com; 572925397@qq.com; ywtipcaas@163.com; yueqiang@caas.cn; lyy4455@163.com) and 1Corresponding author, e-mail: gszbpest@163.com

Received 13 October 2015; Accepted 27 November 2015

Abstract

The ryanodine receptor (RyR), the largest calcium channel protein, has been studied because of its key roles in calcium signaling in cells. Insect RyRs are molecular targets for novel diamide insecticides. The target has been focused widely because of the diamides with high activity against lepidopterous pests and safety for nontarget organisms. To study our understanding of effects of diamides on RyR, we cloned the RyR gene from the oriental fruit moth, Grapholita molesta, which is the most serious pest of stone and pome tree fruits throughout the world, to investigate the modulation of diamide insecticides on RyR mRNA expression in G. molesta (GmRyR). The full-length cDNAs of GmRyR contain a unique 3′-UTR with 625 bp and an open reading frame of 15,402 bp with a predicted protein consisting of 5,133 amino acids. GmRyR possessed a high level of overall amino acid homology with insect and vertebrate isoforms, with 77–92% and 45–47% identity, respectively. Furthermore, five alternative splice sites were identified in GmRyR. Diagnostic PCR showed that the inclusion frequency of one optional exon (f) differed between developmental stages, a finding only found in GmRyR. The lowest expression level of GmRyR mRNA was in larvae, the highest was in male pupae, and the relative expression level in male pupae was 25.67 times higher than that of larval pupae. The expression level of GmRyR in the male pupae was 8.70 times higher than in female pupae, and that in male adults was 5.70 times higher than female adults.

Key words: ryanodine receptor, Grapholita molesta, alternative splice site, expression

Diamide insecticides, a novel class of insecticides, were developed in the 1990s (Lahm et al. 2005, Teixeira and Andaloro 2012). Currently, the commercialized diamide insecticides include one phthalic diamide (flubendiamide) and two anthranilic diamides (chlorantraniliprole and cyantraniliprole). The former was discovered by Nihon Nohyaku and codeveloped with Bayer; the latter two were synthesized by DuPont and cocommercialized by DuPont and Syngenta. These insecticides have notably high activity against Lepidopteran species and are relatively safe to these insects’ natural enemies and mammals (Tohnishi 2005, Gopal and Mishra 2008, Dinter et al. 2009, Ioriatti et al. 2009, Tiwari and Stelinski 2012). The selectivity of diamide insecticides toward insects over mammals was determined to be due to the different isoforms of ryanodine receptors (RyRs), the target of diamides, in insects and mammals (Sattelle et al. 2008).

RyRs, the largest known calcium channel protein, are named after ryanodine, a plant alkaloid. The receptors are homomorphic tetramers with a large hydrophilic N-terminal domain and a small membrane-spanning C-terminal domain. Each monomer of ~550–580 kDa consists of ~5,000 amino acids. This channel is located on the endoplasmic reticulum of muscles, neurons, and many other cell types (Ogawa and Murayama 1998, Fill and Cordoba 2002, Hamilton 2005, Sattelle et al. 2008). RyRs mediate many cellular and physiological activities by regulating the release of Ca2+ from the lumen of the sarcoplasmic and endoplasmic reticulum to the cytosol of muscle and nonmuscle cells, causing such effects as neurotransmitter release, hormone secretion, gene expression, and muscle contraction (Ogawa and Murayama 1998). Three RyR isoforms have been indentified in mammals. RyR1 is primarily observed in skeletal muscle, RyR2 is expressed with high levels in cardiac muscle, and RyR3 is found in many tissues with low abundance but is relatively abundant in the brain and diaphragm. Two isoforms (RyR3 and RyR2) were found in fish, amphibians, and birds (Ottini et al. 1996, Ogawa et al. 1999). However, only one RyR isoform exists in such insects as Drosophila melanogaster, Plutella xylostella, Sogatella furcifera, Leptinotarsa decemlineata, Ostrinia furnacalis, Cnaphalocrocis medinalis, Carposcius sasakii, etc. (Xu et al. 2000; Sun et al. 2012; Wang et al. 2012a, 2013b;
Yang et al. 2014; Sun et al. 2015a,b). These insects RyRs have ~45–47% amino acid sequence identity with the three mammalian RyR isoforms (Sattelle et al. 2008).

Diamides are mainly used to control insects that attack food crops and vegetables (loriatti et al. 2009; Han et al. 2012; Roditakis et al. 2013; Campos et al. 2015). P. xylostella has shown high resistance to chlorantraniliprole and flubendiamide because of the high frequency of diamide use in southern China and certain countries in Southeast Asia (Troczyka et al. 2012; Wang et al. 2013a; Lin et al. 2013; Campos et al. 2015).

Materials and Methods

A laboratory colony of G. molesta was originally collected in 2012 from Xingcheng in Liaoning Province, China. Larvae were reared on immature apples without pesticides and an agar-free semiarti-
ficial diet in a laboratory for several generations under a photoperiod of 15:9 (L:D) h at 25 ± 1°C and 70–80% RH.

Total RNA was extracted from larvae, pupa, and adults according to the instructions in the RNAprep pure Tissue Kit (TIANGEN, China). The RNA pellet was dissolved in ddH2O. First-strand cDNA was synthesized from 1 μl total RNA (650 μg/ml) using the Takara cDNA Synthesis Kit (Takara, China) following the manufacturer’s instructions. Fourteen fragments were amplified to obtain the full-length cDNA of GmRyR (Fig. 1).

Degenerate primers were designed by the method of Sun et al (2012, 2015a,b); specific primers were designed based on the obtained sequences (Table 1). The 5′ and 3′ ends were amplified with nested PCR, with adaptor primers provided by the SMARTer RACE cDNA Amplification Kit (Clontech, Japan). Amplification of each fragment was performed with the following steps: an initial denatur-
ing step at 94°C for 1 min followed by 30 cycles of 98°C for 10 s, 48–65°C (determined by the Tm of primers) for 30 s, and 72°C for 1–3 min (depending on the length of the amplified fragments), end-
ing with an additional polymerization step at 72°C for 5 min. The PCR products of all fragments were purified and subcloned into the pMD19-T Simple Vector (Takara). JM1109 (E. coli) competent cells transformed by the recombinant plasmids produced earlier were inoculated. The positive recombinant clones were then sequenced by BGI (Beijing, China).

Nucleotide sequences of full-length GmRyR cDNA were assembled by overlapping the 14 amplified fragments. GmRyR characterization was conducted using the same methods as performed in P. xylostella, Spodoptera exigua, and Ca. sasakii (Sun et al. 2012, 2015a,b).

The relative expression abundances of five GmRyR samples during three developmental stages (larvae, pupae, and adults) were measured using quantitative real-time PCR. Total RNA was extracted from whole bodies of 10 insects of each sample using the same method as earlier. First-strand cDNA was synthesized from 1,000ng RNA using the High Capacity cDNA Reverse Transcription Kit (ABI, USA). Real-time qPCR for the RyR gene and GAPDH gene as an endogenous control from G. molesta were carried out in 20 μl reaction volumes containing 1 μl cDNA (200ng/ μl), 10 μl SYBR Premix Ex Taq (KAPA, USA), 0.4 μl each of forward and reverse primers (10 μM) (Table 1), and 8.2 μl ddH2O using the ABI 7500. Real-time PCR System (Applied Biosystems) in the same amplification condition: 95°C for 3 min followed by 40 cycles at 95°C for 3 s and 60°C for 20 s. Three biological replicates per sample were examined. The amplification efficiency of RyR and GAPDH were estimated by using $E = (10^{-1/\text{slope}}) - 1$, where the slope was derived from the plot of the cycle threshold (Ct) value versus the log of the serially diluted template concentration, and computed to be 0.9584 and 0.9742, respectively. The data analysis method for quantification of the transcript level of GmRyR was computed according the 2$^{\Delta \text{ACt}}$ method (Schmittgen and Livak 2008). The GmRyR expression data were shown as means ± SD. A statistical analysis was performed by Duncan’s multiple range test for signif-

Results

The full-length cDNA of GmRyR (GenBank KM034750) is 16,299 bp long, as analyzed by overlaying all of the amplified sequenced fragments. The cDNA contains a 272 bp 5′-UTR, a unique 625 bp 3′-UTR with a 29-bp polyA tail, and an open reading frame (ORF) of 15,402 bp. From the ORF, a protein of 5,133 amino acid residues with molecular weight of 580.00 kDa is encoded with a predicted isoelectric point of 5.40.

GmRyR showed amino acid identities with CsRyR, SrRyR, BmRyR, O/RyR, PxRyR, SfRyR, and DmRyR of 92, 93, 91, 93, 91, 79, and 79%, respectively. However, the amino acid identities

[Fig. 1. PCR amplification and cloning of GmRyR cDNA.]
of GmRyR with Oryctolagus cuniculus RyR1–3 was only 44–46%, and the amino acid identity of GmRyR with CeRyR was only 46% as well. To investigate the evolutionary relationships between GmRyR and 32 other RyR isoforms from 25 species, a phylogenetic analysis was performed using ClustalW and MEGA 6.0 based on the ORF amino acid sequences with high bootstrapping support in 1,000 replications (Fig. 2). The phylogenetic tree showed that insect RyRs are well segregated from invertebrate and vertebrate RyRs. GmRyR was clustered with GrRyR from the peach fruit moth, suggesting these two genes are closely related. A number of nucleotide differences were observed between these two overlapping clones. A total of 29 nucleotide differences resulted in 26 amino acid polymorphisms (Table 2). These polymorphisms were located both in the N- and C-terminal regions of GmRyR and may represent different alleles or few errors committed during the PCR procedure.

### Table 1. Primers used in cloning G. molesta RyR cDNA

| Name | Primer name | Primer sequence (5'–3') | Description (length bp) |
|------|-------------|-------------------------|-------------------------|
| S1   | F1          | TTYCAYGTRACNCAYTGGTC     | RT-PCR product S1 (824) |
|     | R1          | TGYTTYTCYTCGTGTYTCCAT    |                         |
| S2   | F2          | TTCCGTGAACTCTGCGGAGA     | RT-PCR product S2 (1,431)|
|     | R2          | GCAGGGAGATCTTGGCTGGA     |                         |
| S3   | F3          | TCGATGGCCCTCCTCCTCT      | RT-PCR product S3 (1,604)|
|     | R3          | CATCTGGTCCTGACCCATT      |                         |
| S4   | F4          | GTAYAACAARGAYCARCCCAT    | RT-PCR product S4 (1,482)|
|     | R4          | TCAACATTCTCCGACAAATTCAT  |                         |
| S5   | F5          | MRDCCRCAYCARTGGGCTAG     | RT-PCR product S5 (842) |
|     | R5          | GCRCYYTCVGGCATTGTTCCC   |                         |
| S6   | F6          | CGAAAATCCTTGCTGCTC       | RT-PCR product S6 (2,383)|
|     | R6          | TACTCTCCGACAGCGGTGACA    |                         |
| S7   | F7          | CGHGARGGCKTGTCMAGATT    | RT-PCR product S7 (854) |
|     | R7          | TGGTCTTGGTGAGGCGAGT     |                         |
| S8   | F8          | ATGGGACACACCTGCGACAT    | RT-PCR product S8 (1,394)|
|     | R8          | TACCTGGACACCCATTG       |                         |
| S9   | F9          | TCGATACTTTGCGGATGTCG    | RT-PCR product S9 (1,586)|
|     | R9          | ATGGGACACACCTGCGACAT    |                         |
| S10  | F10         | ATMCAYGARCAAGARATGGGA   | RT-PCR product S10 (824) |
|     | R10         | CTTCTNARCATNGHARCATCAT  |                         |
| S11  | F11         | AGTTTGTCCAAAGCTTACTC    | RT-PCR product S11 (2,142) |
|     | R11         | CTTCTCCTGAGCCGCAAATAG   |                         |
| S12  | F12         | TGGGACAARTRTGGYRAAGA    | RT-PCR product S12 (716) |
|     | R12         | AATGARACTTTGGGATGTCG    |                         |
| 3'end| 3'OF        | CTGGATGACATGATGGGAA      | RT-PCR product 3’RACE (1,143) |
|     | 3'IF        | AAGGAGGACAGGGCCAGAAGCA  |                         |
| 5'end| 5'OR        | AGATTCGACAGAGCCATCCAGG  | RT-PCR product 5’RACE (977) |
|     | 5'IR        | GGCCTTGCAATTAATGACCTC   |                         |
| RyR  | RyR-F       | GCTTACCCGAGGAGGAGTGGA   | qRT-PCR (97)            |
|     | RyR-R       | CTGTGTTGCTGCTTCTGCTTCT  |                         |
| GAPDH| GF          | GCCGCACTACAGCCGCAATGCA  | qRT-PCR (109)           |
|     | GR          | CGCGGATGAGACTGACGGACAG  |                         |
| ASP-a| PaF         | GACGCGAGCAGATGATGTT    | diagnostic PCR for the presence of exon a |
|     | PaR         | AATTTCCTTTGCGGCTCTCG    |                         |
| ASA-a| AaF         | TCCGAGACCCGGTAAAGGCA   | diagnostic PCR for the absence of exon a |
|     | AaR         | CCGTCTGATGTGTTCTGATG    |                         |
| AS-b | bF          | TACAGGCGTATGACAGTGGC    | diagnostic PCR for exon b |
|     | bR          | TCAGCATCTGGTCTTACAGG    |                         |
| AS-c | cF          | CACCGGCGGTTGCGAGGAAAAGT| diagnostic PCR for exon c |
|     | cR          | TCCTATCTGTGGGTTACAGG    |                         |
| ASP-d| PfF         | GCCCGATGACAGCGATCGA     | diagnostic PCR for the presence of exon d |
|     | PdR         | AAGGGGATGACCTGATGAGCA   |                         |
| ASA-d| AdF         | AGTGTCACAGACGAACTCCA   | diagnostic PCR for the presence of exon d |
|     | AdR         | AAGGGGATGACCTGATGAGCA   |                         |
| ASP-e| PeF         | CAGATGTCACAGCGAGATCC    | diagnostic PCR for the presence of exon e |
|     | PeR         | GCCGAGATGAGTCTGACTGGA   |                         |
| ASA-e| AeF         | GCTGCTTGCGACGGATTTCA    | diagnostic PCR for the absence of exon e |
|     | AeR         | GCTGCTTGCGACGGATTTCA    |                         |
| ASP-f| PfF         | TACGCCTATGCTCCCTGCTT    | diagnostic PCR for the absence of exon f |
|     | PdR         | AGCTCCGATTTATGGGACCA    |                         |
| ASA-f| AfF         | TCTTACAGCAACACTGCTGTT  | diagnostic PCR for the absence of exon f |
|     | AfR         | CCTTCTGTCGCCAGATGTTCTCT |                         |
Fig. 2. Phylogenetic tree of GmRyR and 32 other RyR isoforms. The corresponding GenBank accession numbers are as follows: Heliothis virescens (HvRyR) ADE98118.1; Helicoverpa armigera (HaRyR) AIA23855.1; Sp. exigua (SrRyR) AFC36359.1; B. mori (BmRyR) DJ085056.1; O. furnacalis (OfRyR) AGH68757.1; C. medinalis (CmRyR) AFI80904.1; Pieris rapae (PrRyR) AGI62938.1; G. molesta (GmRyR) KM034750; Ca. sasakii (CsRyR) AHN16453.1; P. xylostella (PxRyR) AEI91094.1; L. decemlineata (LdRyR) AHW99830.1; Bemisia tabaci (BtRyR) AFK84957.1; Nilaparvata lugens (NlRyR) AIA23857.1; S. furcifera (SrRyR) AIA23859.1; Laodelphax striatella (LsRyR) AIA23858.1; Aedes aegypti (AaRyR) XP_001657320.1; D. melanogaster (DmRyR) AAM71083.1; Trichurus trichiura (TTrRyR) CDW52896.1; Caenorhabditis elegans (CeRyR) BAA03099.1; Strongyloides ratti (SrRyR) CEF62113.1; Homo sapiens (HsRyR2) NP_001026.2; Or. cuniculus (OcRyR2) NP_001076226.1; Mus musculus (MmRyR2) NP_076357.2; H. sapiens (HsRyR1) NP_000531.2; Or. cuniculus (OcRyR1) NP_001095188.1; M. musculus (MmRyR1) NP_033135.2; Meleagris gallopavo (MgRyRb) ABY50125.1; Rana catesbeiana (RcRyRb) BAA04647.2; Me. gallopavo (MgRyRb) ABY50126.1; M. musculus (MmRyR3) NP_808320.2; H. sapiens (HsRyR3) CAA04798.1; Or. cuniculus (OcRyR3) NP_001076231.1. The neighbor-joining tree was generated in MEGA 6.0 with 1,000 bootstrap replicates.
Discussion

Ryanodine was isolated from extracts of the stem and root of *Ryania* Speciosa Vahl and exhibited high insecticidal activity to insects and mammals in 1948 (Rogers et al. 1948). A series of experiments found that ryanodine led to muscle contraction by regulating Ca2+ release. Thus, RyR was named after this alkaloid (Pesah et al. 1985). However, ryanodine was banned from use in pest control because of its toxicity to mammals. Although insect RyRs have been used as targets of insecticides since ryanodine was isolated, little research about RyRs was reported until the development of diamide insecticides. The diamide insecticides activate the calcium release channel that sensitizes to ryanodine in insect and show high selectivity toward Lepidopteran insect pests over mammalian (Cordova et al. 2006). The diamides could evoke typical symptoms for insect including poisoning, body contraction, feeding cessation, paralysis, and subsequent mortality. Because diamides are nonreactive in mammals, RyR isoforms from insects have been popular diamide targets, with increasing interest from the pesticide field since the 2000s. Recently, full-length RyRs from insects were cloned, namely PvRyR, GmRyR, OfRyR, BdRyR, SfRyR, LdRyR, and SeRyR (Sun et al. 2012a, 2012b, 2013; Wang et al. 2012a, 2013b, 2014; Yuan et al. 2014; Yang et al. 2014). The unique 625 bp 3’-UTR found in these species was different from other RyRs that reported, as it is the longest 3’-UTR found to date. The deduced 5,133 amino acid sequence of *GmRyR* showed higher amino acid identity (91–93%) with reported Lepidopteran insect RyRs compared other insect RyRs (78–79%). Furthermore, Lepidopteran insect RyRs share only 44–47% amino acid identities with mammalian isoforms. Therefore, the selectivity of diamide insecticides toward insect RyRs was suggested to be due to differences in RyR types between insects and mammals (Wang et al. 2012b). The spectrum of activity for diamide is wide, with flubendiamide targeting mostly Lepidoptera, while chlorantraniliprole and cyantraniliprole are effective in controlling Homoptera, Coleoptera, Diptera, and Thysanoptera species in addition to Lepidoptera (Teixeira and Andaloro 2013).

A multiple alignment of the C-terminal amino acid sequence from *GmRyR* with other reported RyRs, including BmRyR, PxRyR, OrRyR, HvRyR, DmRyR, and OcRyR1 (*Os. cuniculus* RyR), showed that the TM regions had high identities among the insect RyR isoforms aligned. The fragment motif, GVRAGGGGID, sitting at residues 4,983–4,994 between TM5 and TM6 is known to form part of the pore-forming segments of the RyR Ca2+ release channel (Zhao et al. 1999). This motif from *GmRyR* shared complete identity with other all RyR isoforms. Moreover, the residue that enables Ca2+ sensitivity in the C-terminal domain of rabbit RyR1 (E4032) was also detected in *GmRyR* (E4170). Residues corresponding to H4997, R4913, and D4917 in OcRyR1, which were shown to play an important role in the activity and conductance of the RyR Ca2+ release channel, were also conserved in *GmRyR* (I4992, R5008, and D5012) (Zorzato et al. 1990).

Three binding regions have been predicted to be critical to diamide insecticide sensitivity. One region lies near the N-terminus of RyRs, suggesting the possibility of inhibiting Ca2+ release by binding to this region.
Flubendiamide was identified to incorporate into the TM domain (amino acids 4,111–5,084) of BmRyR (Kato et al. 2009). Residues 188–295 of GmRyR share 95% identity and are equivalent with the 183–290 region of BmRyR; they both share 47–50% identity with mammalian RyRs. However, there were three sites in two regions with significant differences among species for the equivalent residues 188–295 of GmRyR (Table 3). AaRyR and DmRyR have a different residue in region I, three nematode species have three different residues in region II, and HsRyR1, MmRyR1, OcRyR1, and MgRyRa were different from other vertebrate RyRs. Table 3 indicated that region I residues were EV in Lepidoptera insects except for the peach fruit moth CsRyR, where they were EI. Region I residues were highly variable in other species. Region II residues contain eight amino acids, which have significant differences in all insects, except Noctuidae. Moreover, seven residues

(residues 183–290 from Bombyx mori) and two are located in the C-terminal TM domain (residues 4,610–4,655 from Drosophila and residue 4,946 from P. xylostella) (Kato et al. 2009, Troczka et al. 2012, Tao et al. 2013). Flubendiamide was identified to incorporate into the TM domain (amino acids 4,111–5,084) of BmRyR (Kato et al. 2009). Residues 188–295 of GmRyR share 95% identity and are equivalent with the 183–290 region of BmRyR; they both share 47–50% identity with mammalian RyRs. However, there were three sites in two regions with significant differences among species for the equivalent residues 188–295 of GmRyR (Table 3). AaRyR and DmRyR have a different residue in region I, three nematode species have three different residues in region II, and HsRyR1, MmRyR1, OcRyR1, and MgRyRa were different from other vertebrate RyRs. Table 3 indicated that region I residues were EV in Lepidoptera insects except for the peach fruit moth CsRyR, where they were EI. Region I residues were highly variable in other species. Region II residues contain eight amino acids, which have significant differences in all insects, except Noctuidae. Moreover, seven residues

(residues 183–290 from Bombyx mori) and two are located in the C-terminal TM domain (residues 4,610–4,655 from Drosophila and residue 4,946 from P. xylostella) (Kato et al. 2009, Troczka et al. 2012, Tao et al. 2013). Flubendiamide was identified to incorporate into the TM domain (amino acids 4,111–5,084) of BmRyR (Kato et al. 2009). Residues 188–295 of GmRyR share 95% identity and are equivalent with the 183–290 region of BmRyR; they both share 47–50% identity with mammalian RyRs. However, there were three sites in two regions with significant differences among species for the equivalent residues 188–295 of GmRyR (Table 3). AaRyR and DmRyR have a different residue in region I, three nematode species have three different residues in region II, and HsRyR1, MmRyR1, OcRyR1, and MgRyRa were different from other vertebrate RyRs. Table 3 indicated that region I residues were EV in Lepidoptera insects except for the peach fruit moth CsRyR, where they were EI. Region I residues were highly variable in other species. Region II residues contain eight amino acids, which have significant differences in all insects, except Noctuidae. Moreover, seven residues
(N4922, N4924, N4935, L4950, L4981, N5013, and T5064) are unique to RyR isoforms from Lepidopteran insects (Wang et al. 2012a). Wang et al. (2012a) and Cui et al. (2013) speculated that these residues might contribute to the differences in Ca$^{2+}$ release channel properties between Lepidopteran and non-Lepidopteran insects RyRs (Wang et al. 2012a, Cui et al. 2013). However, we have different thoughts from Wang and Cui due to the insecticidal spectrum of diamides. Four special residues (D4452, P4504, V4647, and I4758) in residues 4,146–5,133 of GmRyR corresponding to residues 4,111–5,084 of BmRyR are shown in Table 2. Each residue is different among insects RyRs, nematode RyRs, and invertebrate RyRs. We think that these four residues might be involved in Ca$^{2+}$ release from channels among Lepidoptera, non-Lepidoptera, nematode, and invertebrate RyRs. Isolation of microsomal membranes from insect muscles suggest that flubendiamide and chlorantraniliprole target a site localized in the pore of the insect RyR complex distinct from the ryanodine-binding site, suggesting that diamides can bind to several sites in RyR at the same time.

The putative adenine ring binding domain Y[GAST]VG[KTQSN] was found at two locations (residues 1,145–1,148 [YSGS] and 5,098–5,101 [YTGQ]) in GmRyR. Two possible nucleotide-binding sites, identified on the basis of the consensus GXGXXG motif (Wierenga and Hol 1983), were located at positions 3,999–4,004 (GVGLEG) and 4,717–4,722 (GSGEGS) in the GmRyR sequence. A potential calmodulin-binding site in GmRyR was recognized in residues 3,745–3,774, corresponding to residues 3,614–3,643 in OcRyR1 (Xiong et al. 1998). In addition, the binding sites of adenine nucleotide have no particularities compared with other Lepidopteran RyRs, such as O. furnacalis, C. medinalis, and P. xylostella (Wang et al. 2012a, Sun et al. 2012, Cui et al. 2013).

The presence of each putative alternative splice variant and expression of GmRyR mRNA in five discrete mRNA pools (larva, female pupa, male pupa, female adult, and male adult) were determined (Fig. 4). The alternative splice segments were different from CmRyR, OfRyR, BdRyR, and NlRyR (Wang et al. 2012, 2013; Cui et al. 2013; Yuan et al. 2014). Five alternative splice segments were located between amino acid residues 89–93 (a), 1,141–1,173 (b/c), 1,487–1,507 (d), 2,949–2,954 (e), and 3,699–3,706 (f). Interestingly, only the mutually exclusive exons (b/c) were found in CmRyR, OfRyR, BdRyR, and NlRyR and were developmentally regulated (Wang et al. 2012a, 2014; Cui et al. 2013; Yuan et al. 2014). Furthermore, alternative splicing of residues (Ala 3481-Gln3485) in RyR1 was thought to be developmentally regulated (Kimura et al. 2009). The BLAST results showed that residues 89–93 were present in CsRyR, PxRyR, HaRyR, HvRyR, and OfRyR. The analysis of alternatively spliced regions revealed that region b/c and d were located in the central part of the predicted SPRY domain.

### Table 3. Differences of RyRs in the N-terminal and TM domains

| Anino acid position* | Lepidoptera | Coleoptera | Homoptera | Diptera | Nematode | Vertebrate |
|----------------------|-------------|------------|-----------|---------|-----------|-----------|
| 114                  | L           | Q          | L         | N       | N         | A         |
| 129                  | Q           | L          | Q         | N       | V         | T         |
| 151                  | L           | Q          | E         | N       | V         | L         |
| 188–189              | EV          | TW         | TW        | V       | VS        | TW        |
| 240–247              | TW          | STEGGQ     | E         | VS      | VS        | Q         |
| 4,452                | D           | P           | V         | E       | VS        | VS        |
| 4,504                | P           | V           | V         | E       | VS        | VS        |
| 4,647                | V           | I           | M         | E       | VS        | VS        |
| 4,758                | I           | M           | M         | E       | VS        | VS        |

*The number of amino acid M in the initial ORF from GmRyR represents 1.
and region e was located between the third and fourth RyR domains. The alternatively spliced region f was only in pupae and adults from GmelinRyR. Splice variants of RyR2 have been recognized for over a decade but their functions are as undefined to date. Moreover, Knight and Flexner (2007) confirmed that males were more sensitive to chlorantraniliprole-related disruption of mating than females. Thus, further studies are needed to elucidate the characterization and functions of each GmelinRyR splice variant.

There are some differences in RyR expression between invertebrates and vertebrates. RNA from eggs of G. molesta was not extracted because the eggs were not successfully collected. Real-time quantitative PCR showed that the expression level of GmelinRyR mRNA was quite different in larvae, pupae, and adults, particularly in pupae and adults of different sexes. The results showed the lowest expression level in larvae and the highest in male pupae. Wang et al. (2014) reported that NfRyR mRNA expression levels in macropterous female adults were significantly higher than that in brachypterous female adults as well as macropterous and brachypterous male adults. Guo et al. (2012) reported that the expression abundance of the RyR in the second-instar larvae and adults were considerably higher than those of the prepupae and pupae in P. xylostella. Wang et al. (2012b) showed that OxRyR expression level in larvae was higher than in pupae. The expression levels of OfRyR were gradually upregulated with increasing age (Cui et al., 2013), and different expression levels of RyR were shown in BdRyR (Yuan et al., 2014). In summary, although we cloned and characterized the expression levels of GmelinRyR, its properties and functions remain to be studied.

Acknowledgments

This research was conducted under the support of the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP), the Fruter Industry Technology System-Innovation Teams in Liaoning Province (LNGSCTXY-13/14), and the PhD Start-up Fund of the Natural Science Foundation of Liaoning Province (20141157).

References Cited

Campos, M. R., T. B. M. Silva, W. M. Silva, J. E. Silva, and H. A. A. Siqueira. 2015. Susceptibility of Tuta absoluta (Lepidoptera: Gelechiidae) Brazilian populations to ryaneodine receptor modulators. Pest Manag. Sci. 71: 537–544.

Cordova, D., E. A. Benner, M. D. Sacher, J. J. Rauth, J. S. Sopa, G. P. Lahn, T. P. Selby, T. M. Stevenson, L. Flexner, S. Gutteridge, et al. 2006. Anthranilic diamides: a new class of insecticides with a novel mode of action, ryaneodine receptor activation. Pest. Biochem. Physiol. 84: 196–214.

Cui, L., D. B. Yang, X. J. Yan, C. H. Rui, Z. Y. Wang, and H. Z. Yuan. 2013. Molecular cloning, characterization and expression profiling of a ryaneodine receptor gene in Asian Corn Borer, Ostrinia furnacalis (Gueneé). PLoS One 8: e75825.

Dunfer, A., K. E. Brugger, N. M. Frost, and M. D. Woodward. 2009. Chlorantraniliprole (Rynaxypyr): a novel DuPont™ insecticide with low toxicity and low risk for honey bees (Apis mellifera) and bumble bees (Bombus terrestris) providing excellent tools for uses in integrated pest management, pp. 984–996. In Proceedings, Hazards of Pesticides to Bees 10th International Symposium of the ICP-Bees Protection Group, 8–10 October 2008, Bucharest.

Fill, M., and J. A. Copella. 2002. Ryaneodine receptor calcium release channels. Physiol. Rev. 82: 893–922.

Guo, L., B. Z. Tang, W. Dong, P. Liang, and X. W. Gao. 2012. Cloning, characterization and expression profiling of the cDNA encoding the ryaneodine receptor in diamondback moth, Plutella xylostella (L.) (Lepidoptera: Plutellidae). Pest Manag. Sci. 68: 1605–1614.

Guo, L., P. Liang, X. G. Zhou, and X. W. Gao. 2014a. Novel mutations and mutation combinations of ryaneodine receptor in a chlorantraniliprole resistant population of Plutella xylostella (L.). Sci. Rep. 4: 6924.

Guo, L., Y. Wang, X. G. Zhou, Z. Y. Li, S. Z. Liu, P. Liang, and X. W. Gao. 2014b. Functional analysis of a point mutation in the ryaneodine receptor of Plutella xylostella (L.) associated with resistance to chlorantraniliprole. Pest Manag. Sci. 70: 1083–1089.

Gopal, M., and E. Mishra. 2008. Analytical method for estimation of a new insecticide flubendiamide and its safety evaluation for usage in rice crop. Bull. Environ. Contam. Toxicol. 81: 360–364.

Hamiton, S. L. 2005. Ryaneodine receptors. Cell Calcium 38: 253–260.

Han, W.-S., F. Zhang, F. Y. Shen, M. Liu, C. C. Ren, and X. W. Gao. 2012. Residual toxicity and sublethal effects of chlorantraniliprole on Plutella xylostella (Lepidoptera: Plutellidae). Pest Manag. Sci. 68: 1184–1190.

Ioriatti, C., G. Anfora, G. Angeli, V. Mazzoni, and F. Trona. 2009. Effects of chlorantraniliprole on eggs and larvae of Lobesia botrana (Denis & Schiffermuller) (Lepidoptera: Tortricidae). Pest Manag. Sci. 65: 717–722.

Jones, M. M., J. R. Robertson, and R. A. Weiznierz. 2010. Susceptibility of oriental fruit moth (Lepidoptera: Tortricidae) larvae to selected reduced-risk insecticides. J. Econ. Entomol. 103: 1815–1820.

Kato, K., S. Kyonaka, Y. Sawaguchi, M. Tohnishi, T. Masaki, N. Yasokawa, Y. Mizuno, E. Mori, K. Inoue, I. Hamachi, et al. 2009. Molecular characterization of flubendiamide sensitivity in the lepidopterous ryaneodine receptor Ca2+ release channel. Biochemistry 48: 10342–10352.

Kimura, T., J. D. Lucek, P. J. Harvey, S. M. Pace, N. Ikemoto, and M. G. Casarotto. 2009. Alternative splicing of RyR1 alters the efficacy of skeletal EC coupling. Cell Calcium 45: 264–274.

Knight, A. L., and L. Flexner. 2007. Disruption of mating in coding moth (Lepidoptera: Tortricidae) by chlorantraniliprole, an anthranilic diamide insecticide. Pest Manag. Sci. 63: 180–189.

Lahn, G. P., T. P. Selby, H. F. Huredenberger, T. M. Stevenson, B. J. Myers, G. Sebeyouno, B. K. Smith, L. Flexner, C. E. Clark, and D. Cordov. 2005. Insecticidal anthranilic diamides: a new class of potent ryaneodine receptor activators. Bioorg. Med. Chem. Lett. 15: 4898–4906.

Lin, Q. S., F. L. Jin, Z. D. Hu, H. Y. Chen, F. Yin, Z. Y. Li, X. L. Dong, D. Y. Zhang, S. X. Ren, and X. Feng. 2013. Transcriptome analysis of chlorantraniliprole resistance development in the diamondback moth Plutella xylostella. PLoS One 8: e72314.

Ogawa, Y., and T. Murayama. 1998. The structure and function of ryaneodine receptors, pp. 5–22. In R. Sitasesapan and A. J. Williams (eds.), Characteristics of Ryaneodine Receptor Type 3 Isoform (RyR3) and Its Homologues, Imperial College Press, London.

Ogawa, Y., T. Murayama, and N. Kurebayashi. 1999. Comparison of properties of Ca2+ release channels between rabbit and frog skeletal muscles. Mol. Cell. Biochem. 190: 191–201.

Ottini, L., G. Marziali, A. Conti, A. Charlesworth, and V. Sorrentino. 1996. Alpha and beta isoforms of ryaneodine receptor from chicken skeletal muscle are the homologues of mammalian RyR1 and RyR3. Biochem. J. 315: 207–216.

Pessah, I. N., A. L. Waterhouse, and J. E. Casida. 1985. The calcium ryaneodine receptor complex of skeletal and cardiac muscle. Biochem. Biophys. Res. Commun. 128: 449–456.

Roditakis, E., C. Skarmoutsou, M. Staurakaki, R. M. Martinez-Aguirre, L. Garcia-Vidal, P. Bielza, K. Haddi, C. Rapisarda, J. L. Rison, A. Bassi, et al. 2013. Determination of baseline susceptibility of European populations of Tuta absoluta (Meyrick) to indoxacarb and chlorantraniliprole using a novel dip bioassay method. Pest Manag. Sci. 69: 217–227.

Rogers, E. F., F. R. Koniuszy, J. Shavel, and K. Folkers. 1948. Chlorantraniliprole (Ryania speciosa) providing excellent tools for uses in integrated pest management, pp. 984–996. In Proceedings, Hazards of Pesticides to Bees 10th International Symposium of the ICP-Bees Protection Group, 8–10 October 2008, Bucharest.

Sattelle, D. B., D. Cordova, and T. R. Cheek. 2008. Insect ryaneodine receptors: molecular targets for novel pest control chemicals. Invert. Neurosci. 8: e75825.

Schmitzgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3: 1101–1108.

Sun, L. N., L. Cui, C. H. Rui, X. J. Yan, D. B. Yang, and H. Z. Yuan. 2012. Modulation of the expression of ryaneodine receptor from Plutella xylostella as a result of diadime insecticide application. Gene 511:265–273.
Sun, L. N., G. S. Qiu, L. Cui, C. S. Ma, and H. Z. Yuan. 2015a. Molecular characterization of a ryanodine receptor gene from Spodoptera exigua and its upregulation by chlorantraniliprole. Pest. Biochem. Physiol. 123: 56–63.

Sun, L. N., H. J. Zhang, W. T. Yan, C. S. Ma, and G. S. Qiu. 2015b. Molecular cloning and expression profiling of a ryanodine receptor gene in the peach fruit moth (Carposina sasakii). Scientia Agricultura Sinica 48: 1971–1981.

Tao, Y., S. Gutteridge, E. A. Benner, L. H. Wu, D. F. Rhoades, M. D. Sacher, M. A. Rivera, J. Desaeger, and D. Cordova. 2013. Identification of a critical region in the Drosophila ryanodine receptor that confers sensitivity to dia-mide insecticides. Insect Biochem. Mol. Biol. 43: 820–828.

Teixeira, L. A., and G. T. Andaloro. 2012. Diazinon resistance in the oriental fruit moth (Carposina sasakii). PLoS One 7: e36623.

Tiwari, S., and L. L. Stelinski. 2013. Molecular characterization of a ryanodine receptor gene in the oriental fruit fly, Bactrocera dorsalis (Hendel). PLoS One 9: e95199.

Tohnishi, M., H. Nakao, T. Furuya, A. Seo, H. Kodama, K. Tsubata, S. Fujioka, H. Kodama, T. Hirooka, and T. Nishimatsu. 2005. Flubendiamide, a novel insecticide highly active against Lepidopterous insect pests. J. Pest. Sci. 30: 354–360.

Trocza, B., C. T. Zimmer, J. Elias, C. Schorn, C. Bass, T. G. E. Davies, L. M. Field, M. S. Williamson, R. Slater, R. Nauen. 2012. Resistance to diazinon in brown planthopper, Nilaparvata lugens (Stål). Pest Manag. Sci. 69: 1066–1072.

Tohnishi, M., H. Nakao, T. Furuya, A. Seo, H. Kodama, K. Tsubata, S. Fujioka, H. Kodama, T. Hirooka, and T. Nishimatsu. 2005. Flubendiamide, a novel insecticide highly active against Lepidopterous insect pests. J. Pest. Sci. 30: 354–360.

Wang, J., Y. P. Liu, J. K. Gao, Z. Xie, L. Huang, W. Wang, and J. Wang. 2013a. Molecular cloning and mRNA expression of a ryanodine receptor gene in the cotton bollworm, Helicoverpa armigera. Pest. Biochem. Physiol. 107: 327–333.

Wang, J., Z. Xie, J. K. Gao, Y. P. Liu, W. L. Wang, L. Huang, and J. J. Wang. 2014. Molecular cloning and characterization of a ryanodine receptor gene in brown planthopper (BPH), Nilaparvata lugens (Stäl). Pest Manag. Sci. 70: 790–797.

Wang, J. J., Y. Q. Li, Z. J. Han, Y. L. Zhu, Z. J. Xie, J. Wang, Y. P. Liu, and X. C. Li. 2012a. Molecular characterization of a ryanodine receptor gene in the rice leaffolder, Cnaphalocrocis medinalis (Gueneé). PLoS One 7: e36623.

Wang, X. L., S. W. Wu, Y. H. Yang, and Y. D. Wu. 2012b. Molecular cloning, characterization and mRNA expression of a ryanodine receptor gene from diamondback moth, Plutella xylostella. Pest. Biochem. Physiol. 102: 204–212.

Wang, X. X., S. K. Khakame, C. Ye, Y. H. Yang, and Y. D. Wu. 2013b. Characterisation of field-evolved resistance to chlorantraniliprole in the diamondbackmoth, Plutella xylostella, from China. Pest Manag. Sci. 69: 661–665.

Wierenga, R. H., and W. G. J. Hol. 1983. Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. Nature 302: 842–844.

Xiong, H., X. Y. Feng, L. Gao, L. Xu, D. A. Pasek, J. H. Seok, and G. Meissner. 1998. Identification of a novel EF-hand Ca2+ binding domain in lobster skeletal muscle ryanodine receptor/Ca2+ release channel. Biochemistry 37: 4804–4814.

Xu, X., M. B. Bhat, M. Nishi, H. Takeshima, and J. Ma. 2000. Molecular cloning of cDNA encoding a Drosophila ryanodine receptor and functional studies of the carboxyl-terminal calcium release channel. Biophys. J. 78: 1270–1281.

Yan, H. H., C. B. Xue, G. Y. Li, X. L. Zhao, X. Z. Che, and L. L. Wang. 2014. Flubendiamide resistance and Bi-PASA detection of ryanodine receptor G4946E mutation in the diamondback moth (Plutella xylostella L.). Pest. Biochem. Physiol. 115: 73–77.

Yang, Y., P. J. Wan, X. X. Hu, and G. Q. Li. 2014. RNAi mediated knockdown of the ryanodine receptor gene decreases chlorantraniliprole susceptibility in Sogatella furcifera. Pest. Biochem. Physiol. 108: 58–65.

Yuan, G. R., W. Z. Shi, W. J. Yang, X. Z. Jiang, W. Dou, and J. J. Wang. 2014. Molecular characteristics, mRNA expression, and alternative splicing of a ryanodine receptor gene in the oriental fruit fly, Bactrocera dorsalis (Hendel). PLoS One 9: e95199.

Zhao, M. C., P. Li, X. L. Li, R. J. Winkfein, and S. W. Chen. 1999. Molecular identification of the ryanodine receptor pore-forming segment. J. Biol. Chem. 274: 25971–25974.

Zheng, Y., X. Peng, G. Liu, H. Pan, S. Dorn, and M. Chen. 2013. High genetic diversity and structured populations of the oriental fruit moth in its range of origin. PLoS One 8: e78476. doi:10.1371/journal.pone.0078476

Zorzato, F., J. Fujii, K. Otso, M. Phillips, N. M. Green, F. A. Lai, G. Meissner, and D. H. MacInennan. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. 265: 2244–2256.