Pesticide Biochemistry and Physiology 123 (2015) 56–63

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Molecular characterization of a ryanodine receptor gene from *Spodoptera exigua* and its upregulation by chlorantraniliprole

Lina Sun [a,b], Guisheng Qiu [a,b], Li Cui [c], Chunsen Ma [a,c], Huizhu Yuan [c,*]

[a] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Beijing 100193, China
[b] Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning Province 125100, China
[c] Laboratory of Integrated Pest Management in Crops, Ministry of Agriculture; Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

ARTICLE INFO

Article history:
Received 3 July 2014
Accepted 3 March 2015
Available online 18 March 2015

Keywords:
Ryanodine receptor
Spodoptera exigua
Expression
Chlorantraniliprole

ABSTRACT

Chlorantraniliprole is a novel diamide insecticide that targets the insect ryanodine receptor, a Ca^{2+} release channel. *Spodoptera exigua* is a significant insect pest, and chlorantraniliprole is the most popular diamide insecticide used against this pest. To better understand the effects of diamides on RyR expression and $[Ca^{2+}]$, we isolated the *Se*RyR cDNA and investigated changes in *Se*RyR expression as a result of the application of chlorantraniliprole. The full-length cDNAs of *Se*RyR contain an open reading frame (ORF) of 15,357 bp with a predicted protein consisting of 5118 amino acids. *Se*RyR shares 77–92% identity with other insect RyR isoforms and 45–47% identity with vertebrate RyR isoforms. Furthermore, the relative expression abundances of RyR mRNA extracted from *S. exigua* fat body cells after 24 h of culture in 0.1, 1, 10, 100 nM, 1 μM and 100 μM of chlorantraniliprole changed 1.04-, 0.89-, 1.83-, 2.58-, 4.03- and 3.12-fold compared to blank control, respectively. The regression equation for the relative expression levels of *Se*RyR after 24 h as a function of the chlorantraniliprole concentration was $Y = 0.6455 + 0.8188LgX$, $R^2 = 0.97093$ for the cell line IOZCAS-Spex-II. These results outline the effects of chlorantraniliprole on the expression of *Se*RyR and provide a basis for the discovery of a compound that may exhibit selective insect activity.

1. Introduction

The Ryanodine receptor (RyR) is the largest known ligand-gated calcium channel, with a molecular mass of ~2.3 MDa. This channel controls the release of calcium from intracellular stores and regulates a variety of cellular processes, such as muscle contraction, gene transcription, neurotransmitter release, hormone secretion, and cell proliferation [1,2]. The RyRs are homomeric tetramers that have been intensively studied because their point mutations are responsible for some human diseases [3,4]. Mammals express three isoforms of RyR protein, while insects only express one [5]. These RyRs are mainly located in the sarcoplasmic reticulum of muscle and the endoplasmic reticulum of neurons, epithelial cells and many other cell types [5–8]. The localization of the three RyR in mammals depends on the tissue. RyR1 is the dominant isoform found in skeletal muscle, and RyR2 has been detected at high levels in cardiac muscle. RyR3 protein is expressed in many tissues, including the diaphragm and brain.

Flubendiamide, the first insecticide to target insect RyR, was discovered by Nihon Nohyaku and co-developed with Bayer [9]. Thereafter, chlorantraniliprole and cyantraniliprole were developed by DuPont Crop Protection [10]. Cordova et al. reported that anthranilamide stimulates the release of RyR-mediated Ca^{2+} stores in *Periplaneta americana* embryonic neurons, while voltage-gated Ca^{2+} channels remain unaffected [11]. Diamides show novel and intrinsic target-site selectivity for insects over mammals. Therefore, the Insecticide Resistance Action Committee (IRAC) (www.irac-online.org) classified them into a new mode of action group (Group 28): the insecticidal RyR modulators. Anthranilic diamides exhibit high activity against lepidopteran larvae by evoking typical symptoms, which culminate in complete contraction paralysis and subsequent mortality; these agents are potent against coleopteran and hemipteran insects [10–13]. Diamide insecticides have been extensively used in China. Unfortunately, *Plutella xylostella* has rapidly developed resistance to diamides because of the overuse of flubendiamide and chlorantraniliprole in Southern China and Thailand [14,15]. To clarify the molecular mechanisms of diamide insecticide activity or resistance to lepidopterans, the full-length of RyRs from lepidopterans *P. xylostella*, *Cnaphalocrocis medinalis*, *Bombyx mori*, *Ostrinia furnacalis* and *Pieris rapae* have been cloned and characterized [16–19].

* Corresponding author. Institute of Plant Protection, Chinese Academy of Agricultural Sciences, No. 2 Yuanmingyuan West Road, Haidian District, Beijing, 100193, China. Fax: +86 010 62815941.
E-mail address: hzhyuan@ippcaas.cn (H. Yuan).

http://dx.doi.org/10.1016/j.pestbp.2015.03.002
0048-3575/

The beet armyworm, *Spodoptera exigua* (*S. exigua*) (Lepidoptera: Noctuidae), is a polyphagous pest that targets numerous cultivated crops, including vegetables, cotton, fruit tree seedlings and ornamentals [20,21]. *S. exigua* has emerged as a significant pest in the last 20 years in China that causes enormous economic losses. *S. exigua* has developed resistance to diverse chemical classes due to a long history of treatment with various insecticides, including chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids and benzoylphenylureas [22,23]. Since 2009, chlorantraniliprole has been used to control *S. exigua* in China. The risk for developing resistance to chlorantraniliprole in populations of *S. exigua* was assessed. The LC_{50} increased 12-fold after 22 generations of selection, which is considered a moderate increase [24]. Li et al. reported that chlorantraniliprole inhibited high-voltage activated L-type calcium channels in *S. exigua* larvae central neurons. The peak current of the calcium channel significantly negatively correlated with the concentration of chlorantraniliprole (>100 pmol L^{-1}). In addition, chlorantraniliprole inhibits the recorded calcium currents in a concentration-dependent manner, and this inhibition cannot be reversed by washout [25].

RyR is widely expressed in the insect body, such as in the head, gut, hemolymph, and bodywall muscle [18]. However, the expression of RyR in the fat body has been unclear. The insect fat body has historically been considered a single tissue with multiple and diverse metabolic and detoxification functions [26]. This tissue consists of cells of mesodermal origin and sometimes also contains ectodermic cells, which are distributed in the spaces between insect organs. Detoxification enzymes associated with insecticide resistance are highly expressed in the insect fat body [27,28].A fat body cell lines from *S. exigua* was cultured in our laboratory, and the complete developmental period of the cell line is significantly shorter than that of the *S. exigua* insect. So the cDNAs encoding the RyR gene from the fat body cells of *S. exigua* (named *Se*RyR) were cloned and sequenced, and the effects of chlorantraniliprole on the expression of *Se*RyR mRNA were determined in this study in order to investigate the relationship between the fat body and the development of resistance to diamides in *S. exigua*.

2. Material and methods

2.1. Culture of insect cells

The cell line IOZCAS-Spex-II, which was derived from the *S. exigua* fat body, was provided by the Institute of Zoology of Chinese Academy of Science (Beijing, China) and maintained in Grace's insect medium containing 10% fetal bovine serum (FBS, Heat-inactivated, Invitrogen, CA, USA) in T-25 cm^2 tissue culture flasks (Corning, NY, 14831) incubated at 27 °C under sterile conditions.

2.2. Primer design

The primers for *Se*RyR are listed in Fig. 1 and Table 1 and were designed according to previously reported methods [16].

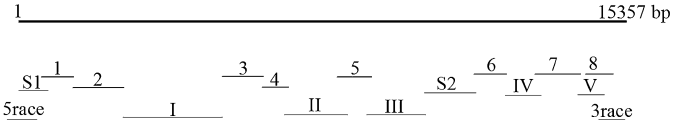

Fig. 1. Cloning strategy of the *Spodoptera exigua* ryanodine receptor (*Se*RyR) cDNA. Degenerate primers were used to generate eight PCR fragments (1–8). Based on sequences 1 and 6, S1 and S2 were amplified by using a special primer and degenerate primer, respectively. Seven gaps (I–V) were amplified based on sequences 1–8. The 5race and 3race fragments were obtained by RACE. The full-length of *Se*RyR cDNA sequence was obtained by overlapping all amplified fragment sequences.

Table 1
Primers used for cloning *Se*RyR cDNA.

Fragment name	Primers	Position	Primer sequence (5′–3′)	Length of fragment sequence (bp)
1	1F	571	TTYCAYGTRACNCAYTGGTC	824
	1R	1394	TGYTTYTCYTCGTGYTCCAT	
2	2F	1366	GMWGANGAYATGGARCACGA	1307
	2R	2672	ATYTTRTTYATWGCCCACAT	
3	3F	5170	MRDCCRCAYCARTGGGCTAG	1046
	3R	6215	TCHCKVGGHGGACANCGGAA	
4	4F	6187	ACNARRGARTACCGNTGTCC	674
	4R	6860	GTRTTCATCAYNACNGCCAT	
5	5F	8089	CGHGARGCKGTBTCMGACTT	848
	5R	8936	CKYTCVGCCATRTTYTGCAT	
6	6F	11680	ATMCAYGARCAAGARATGGA	839
	6R	12518	CCRTTDACRACRTTYCCTTC	
7	7F	13121	ARGAYGCHATHTTTGARATG	1168
	7R	14288	ATYTCYTTYTCNCGYTTGAA	
8	8F	14404	TGGGACAARTTYGYRAAGAA	716
	8R	15119	ATRAARCARTTGGAYTCCATGT	
S1	SF1	1	ATGGCBGANRSNGAGGGNRGNKC	753
	SR1	753	GCAAGTATCTCTCAGTAGCGAC	
S2	SF2	10318	GADARYGCBCCDTGGATGAC	1431
	SR2	11748	CACTCCCCTGTTCGCTAAA	
I	IF	2646	TCACGAAATGTGGGCAAT	2550
	IR	5195	GCGTATGCGAATGGAAAC	
II	IIF	6760	AGGCTTTGGAAACTCGTC	1612
	IIR	8371	GTTCATAGTCCATCTTGCTG	
III	IIIF	8849	ACTCAGCGACTCCGTTTA	1516
	IIIR	10364	ATTTGCTGCGTGTAGGTCG	
VI	VIF	12482	TGATGCTGTCTATGCTGGA	825
	VIR	13306	AGAATGCTGCGTAGATGC	
V	VF	14215	GTGTCCCTTGCCATACTTA	688
	VR	14902	AAAGGTTGAATACGAAGCAC	
3race	GSP1	14730	CGTCACGCATAACGGAAAAC	670
	GSP2	14752	CTGGTCCTGACAGTGATGCTATTG	
5race	GSP3	529	GCAAGTATCTCTCAGTAGCGAC	768
	GSP4	459	TCTCTGTTTGCTGGCAGG ATGTAGGGTC	

Note: R = A/G, Y = C/T, M = A/C, K = G/T, S = C/G, W = A/T, H = A/C/T, B = C/G/T, V = A/C/G, D = A/G/T, N = A/C/G/T. 3OP was the primer of 3RACE outer PCR, and 3IP was the primer of 3RACE inner PCR. 5OP was the primer of 5RACE outer PCR, and 5IP was the primer of 5RACE inner PCR. These primers were synthesized by BGI (Beijing, China).

2.3. Isolation of RNA from IOZCAS-Spex-II cells, the first strand cDNA synthesis and PCR amplification

The total RNA was extracted and isolated from IOZCAS-Spex-II cells with Trizol reagent (Invitrogen, San Diego, CA) following the manufacturer's instructions. The first strand cDNA was synthesized using the D6110A kit (Takara™, Dalian, China).

Each fragment was amplified by PCR in three steps using the *Ex* and *LA* Taq kits (Takara, Dalian, China), and the annealing temperature was defined based on the melting temperatures (Tm) of the paired primers. To obtain the full-length of *Se*RyR cDNA sequence, the 3′- and 5′-RACE ends were assayed using the 3′-full RACE core set (Takara, Dalian, China) and SMARTer™ RACE (Clontech Laboratories, Mountain View, USA), respectively. Nested PCR was utilized for both RACE reactions. The gene-specific primers GSP1, GSP2, GSP3 and GSP4 utilized in this experiment are listed in Table 1. The amplified products were cloned, and the positive recombinant clones were sequenced by BGI (Beijing, China).

2.4. Real-time quantitative RT-PCR (qRT-PCR) for measuring the mRNA expression abundance of the ryanodine receptor

Chlorantraniliprole (98%) was obtained from DuPont Co. and dissolved in dimethyl sulfoxide. IOZCAS-Spex-II cells were maintained in medium at final chlorantraniliprole concentrations