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# Molecular cloning, spatiotemporal and functional expression of GABA receptor subunits RDL1 and RDL2 of the rice stem borer *Chilo suppressalis*

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ABSTRACT

Insect γ-aminobutyric acid (GABA) receptor (GABAR) is one of the major targets of insecticides. In the present study, cDNAs (*CsRDL1A* and *CsRDL2S*) encoding the two isoforms of RDL subunits were cloned from the rice stem borer *Chilo suppressalis*. Transcripts of both genes demonstrated similar expression patterns in different tissues and developmental stages, although *CsRDL2S* was ~2-fold more abundant than *CsRDL1A* throughout all development stages. To investigate the function of channels formed by *Cs*RDL subunits, both genes were expressed in *Xenopus laevis* oocytes singly or in combination in different ratios. Electrophysiological results using a two-electrode voltage clamp demonstrated that GABA activated currents in oocytes injected with both cRNAs. The $EC_{50}$ value of GABA in activating currents was smaller in oocytes co-injected with *CsRDL1A* and *CsRDL2S* than in oocytes injected singly. The $IC_{50}$ value of the insecticide fluralaner in inhibiting GABA responses was smaller in oocytes co-injected with different cRNAs than in oocytes injected singly. Co-injection also changed the potency of the insecticide dieldrin in oocytes injected singly. These findings suggested that heteromeric GABARs were formed by the co-injections of *CsRDL1A* and *CsRDL2S* in oocytes. Although the presence of Ser at the 2′-position in the second transmembrane segment was responsible for the insensitivity of GABARs to dieldrin, this amino acid did not affect the potencies of the insecticides fipronil and fluralaner. These results lead us to hypothesize that *C. suppressalis* may adapt to insecticide pressure by regulating the expression levels of *CsRDL1A* and *CsRDL2S* and the composition of both subunits in GABARs.



## 1. Introduction

The γ-aminobutyric acid receptor (GABAR) is the principal inhibitory neurotransmitter receptor in both invertebrate and vertebrate nervous systems, and it includes ionotropic and metabotropic receptors (Buckingham and Sattelle, 2005; Miller and Aricescu, 2014; Ozoe, 2013). The insect ionotropic GABAR, as a member of the family of pentameric ligand-gated ion channels (LGICs), is one of the major targets of insecticides, parasiticides, and acaricides (Casida, 1993; Casida and Durkin, 2015). Several representative insecticides, including phenylpyrazoles (fipronil, ethiprole, *etc.*), cyclodienes (dieldrin, α-endosulfan, *etc.*), isoxazolines (fluralaner, afoxolaner, *etc.*) and *meta*-diamides (broflanilide), demonstrate high insecticidal activity by blocking the chloride channels of insect GABARs (Casida, 2015; Casida and Durkin, 2015; Nakao, 2016).

Dieldrin is one of the first-generation noncompetitive antagonist (NCA) insecticides against insect GABARs (Kadous et al., 1983). Fipronil, as the first phenylpyrazole insecticide and the second generation of GABAR NCA insecticides, exhibits excellent insecticidal activities against both piercing-sucking and chewing insects

*Abbreviations:* ANOVA, analysis of variance; cDNA, complementary DNA; CI, confidence interval; cRNA, capped RNA; *CsRDLs*, *Chilo suppressalis RDL* genes; *CsRDL1A and CsRDL2S*, the RDL subunits of *Chilo suppressalis*; DMSO, dimethyl sulfoxide; E2F, elongation factor 2; GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric acid receptor; GSP, gene-specific primer; NCA, noncompetitive antagonist; ORF, open reading frame; qRT-PCR, quantitative RT-PCR; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SE, standard error; TEVC, two-electrode voltage clamp; TM, transmembrane segment.

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(Colliot et al., 1992), including sanitary pests (the housefly *Musca domestica*, the cat flea *Ctenocephalides felis, etc.*) and agricultural pests (the rice stem borer *Chilo suppressalis*, the small brown planthopper *Laodelphax striatellus*, the diamondback moth *Plutella xylostella, etc.*) (He et al., 2007); however, it has low toxicity towards mammals at relatively high concentrations (Moffat, 1993). Recently reported insecticides, fluralaner and broflanilide, do not demonstrate cross-resistance to insects that developed resistance to conventional GABAR NCA insecticides; therefore, these new insecticides open up a new vista of the practical use of GABAR NCAs as insecticides (Nakao et al., 2013; Ozoe, 2013; Ozoe et al., 2010).

Insect GABARs as insecticidal targets have been extensively studied, and some remarkable progress has been made and well documented (Casida and Durkin, 2015; Ozoe, 2013; Zhao and Casida, 2014; Zhao et al., 2014). To date, four insect GABAR subunits with alternative splicing have been reported, namely, RDL (resistant to dieldrin), GRD ($GABA_A$ and glycine receptor-like subunit of *Drosophila*), LCCH3 (ligand-gated chloride channel homolog 3), and CG8916 subunits (ffrench-Constant and Rocheleau, 1993; Gisselmann et al., 2004; Wei et al., 2017). Of these subunits, only RDL can form a functional GABA-gated chloride channel when heterologously expressed, and existing GABA NCA insecticides exert their insecticidal activity by acting on RDL GABARs. Hence, the A2′S mutation at the second transmembrane segment (TM) of RDLs was identified as a typical marker of insecticide resistance. In addition to this mutation, the A2′G or G2′N mutation was subsequently observed to be a cyclodiene- or fipronil-resistance-associated mutation in different insects (Nakao, 2016).

The rice stem borer, *C. suppressalis*, is an important pest of rice, occurring in all rice plant areas in China (Sheng et al., 2003). At present, *C. suppressalis* is mainly controlled by fipronil, monosultap, dimehypo, chlorantraniliprole, flubendiamide, *etc.* (Gao et al., 2013; He et al., 2007; Su et al., 2014; Wu et al., 2014). However, moderate resistance to fipronil, chlorantraniliprole, and flubendiamide has been observed in China (Jiang et al., 2005; Yao et al., 2017). Fipronil has been used widely for the management of *C. suppressalis* since 1997 in China, and moderate levels of fipronil-resistance of *C. suppressalis* were investigated in the field and in lab-reared strains (Cao et al., 2004; He et al., 2013; Jiang et al., 2005). However, the mechanism of fipronil resistance in *C. suppressalis* remains unclear. In the present study, to fill the blank of information about the GABARs of *C. suppressalis*, the RDL subunits of *C. suppressalis* were cloned and characterized to determine the expression pattern of the subunits and pharmacological characteristics, which would promote the study of insect GABARs.

## 2. Material and methods

### 2.1. Ethical statement

The experimental African clawed frogs (*Xenopus laevis*) used in the present study were strictly maintained in the laboratory following the world, China, and Nanjing Agricultural University guidelines for animal protection and welfare.

### 2.2. Insect strain and chemicals

Rice stem borers were reared in the laboratory under the same conditions as those described by Peng et al. (2017). Female *X. laevis* frogs were purchased from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). GABA, fipronil, collagenase (type I), gentamicin, penicillin-streptomycin, and sodium pyruvate were purchased from Sigma-Aldrich (Shanghai, China) or Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Dieldrin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Fluralaner (≥99%) was purified from BRAVECTO® (Merk & Co., Inc., Isando, South Africa). General reagents are analytical grade and were purchased from J & K Scientific Ltd. (Beijing, China) unless otherwise noted. The plasmid vector pGH19 used for RDL expression in *X. laevis* oocytes was kindly provided by Professor Ze-Wen Liu's lab (College of Plant Protection, Nanjing Agricultural University).

### 2.3. Molecular cloning of CsRDLs

Following the database of *C. suppressalis* (http://ento.njau.edu.cn/ChiloDB/) (Yin et al., 2014) and of National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/), the full length of *CsRDLs* was searched, and cDNAs were cloned by RT-PCR and 5′- and 3′-RACE technology using kits and total RNA from the 4th instar *C. suppressalis* larva. The complete open reading frame (ORF) was amplified with gene-specific primers (GSPs) (Table S1) for *CsRDLs*, and the PCR products (Fig. S1) were verified by sequencing in GenScript (Nanjing, China).

### 2.4. Bioinformatic analysis of CsRDL profiles

The isoelectric point and the molecular weight of *Cs*RDLs were estimated using the ExPASY Proteomic Server (http://web.expasy.org/compute_pi/) (Gasteiger et al., 2005). Signal peptides and transmembrane helices were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Putative motifs and domains were predicted using the ScanProsite tool (http://prosite.expasy.org/scanprosite/) and InterPro (http://www.ebi.ac.uk/interpro/) (Sangrador-Vegas et al., 2016). A phylogenetic tree was generated using MEGA 7.0 (Kumar et al., 2016) and ClustalW by the maximum likelihood method using bootstrap values on 1000 replicated calculations and was annotated using Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA). Amino acid sequences of *Cs*RDL subunits were aligned with their homologues from other species using DNAMAN version 6.0 software (Lynnon LLC., San Ramon, CA). GenBank accession numbers are described in Supplementary Data S1.

### 2.5. Expression analysis of CsRDL mRNAs

To investigate the relative transcription levels of *CsRDLs*, the whole body in different developmental stages (such as eggs, 1st to 6th instar larvae, pupae and adults) and various tissues (including heads, nerve cords, mid-guts, and other parts containing integument, hemolymph, Malpighian tubules and silk glands) from thirty 4th instar larvae were stored at −80 °C for RNA purification. Total RNA was isolated with a TRIzol kit (Invitrogen, CA), and first-strand cDNA was obtained from 1 μg of total RNA using a PrimeScript™ 1st strand cDNA Synthesis kit (TaKaRa, Dalian, China) after genomic DNA clearance. At least three biological samples were assayed in triplicate.

qRT-PCR was performed on an ABI 7500 system (Applied Biosystems, CA) in 20 μL reactions containing 1 μL of cDNA, 10 μL of SYBR® Premix Ex Taq (Perfect Real Time) (Takara Co., Otsu, Japan), 0.4 μL of forward GSP (q*CsRDLs*-F and E2F-F) and reverse GSP (q*CsRDLs*-R and E2F-R) (10 μM), 0.4 μL of ROX Reference dye II, and 7.8 μL of RNase-free water. The thermal cycling procedures were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60 °C for 34 s. Amplification efficiency was estimated, and the uniqueness of PCR products was verified by the melting curve analysis. The elongation factor 2 (E2F) gene was used as the reference to standardize *CsRDL* expression levels (Wei et al., 2013), and the primers used for qRT-PCR were designed by Beacon Designer 8.0 (Premier Biosoft International, CA). Data of *CsRDL* expression were statistically analyzed