Molecular Features and Expression Patterns of Vitellogenin Receptor in *Calliptamus italicus* (Orthoptera: Acrididae)

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**Abstract**

Vitellogenin receptor (VgR) mediates the intake of vitellin via oocytes, thus exerting an important role in vitellogenesis. In this study, reverse transcription-polymerase chain reaction (RT-PCR) and rapid-amplification of cDNA ends techniques were adopted to clone the CiVgR gene, namely the VgR gene of *Calliptamus italicus*, i.e., Orthoptera. The full length of CiVgR was 5,589 bp, and the open reading frame was estimated to be 5,265 bp, which encoded 1,754 amino acids (aa). Sequence alignment analysis showed that CiVgR belonged to the superfamily of low-density lipoprotein receptor genes, which contained several conserved domains, including ligand-binding domains, epidermal growth factor precursor homology domains, transmembrane domains, and cytoplasmic domains. However, no O-linked sugar domain was identified. Phylogenetic analysis showed that CiVgR had the closest genetic relationship to Blattaria. RT-PCR showed that CiVgR was only specifically expressed in the ovarian tissue of females. Quantitative real time polymerase chain reaction showed that the transcription of CiVgR already appeared in the fourth-instar nymph of *C. italicus*, which gradually increased after adult emergence, peaked at the previtellogenesis stage, and then started to decrease. The expression pattern of CiVgR was closely associated with vitellogenesis. The findings of this study further our understanding of the molecular mechanisms involved in the reproduction of *C. italicus*, and provide new ideas to control this insect.

**Key words:** *Calliptamus italicus*, vitellogenin receptor, vitellin, gene clone, sequence analysis

Considering serious issues caused by the application of chemical pesticides, such as unsustainability and environmental pollution, it is of utmost importance to identify new strategies for prevention of injurious insects. Reproduction is one of the most important physiological behaviors of insects, which is also the basis for the population growth. Further investigations on the processes of reproduction and underlying mechanisms of the injurious insects could reveal potential molecular targets for the prevention of the insects, which are current research hotspots across the world (Roy et al. 2018; Shang et al. 2018; Yao et al. 2018; Zhao et al. 2018; Liu et al. 2019).

Vitellogenesis, the key regulator in the reproduction of insects, includes two major processes, i.e., the synthesis and uptake of vitellogenin (Vg). Vitellogenin is mainly synthesized in the fat body of insects, which then is transferred to ovary through hemolymph. Consequently, vitellogenin is uptaken by developing oocytes through endocytosis mediated by vitellogenin receptor (VgR). Then, following the deposition of vitellogenin in oocytes vitellin, which provides sufficient nutrients to support oogenesis and embryo development, is formed. Therefore, the deposition of vitellin in oocytes is essential for the development and maturation of oocytes (Gong and Zhai 1979; Raikhel and Dhadialla 1992; Tufail et al. 2014). Vitellogenin receptor is the exclusive receptor for endocytosis of vitellogenesis, which has a critical role in the uptake of vitellogenesis (Tufail and Takeda 2009). Previous studies have shown that when the expression of VgR gene is abnormal, the deposition of vitellin in oocytes is disrupted, which in turn delays the development of ovary and can even lead to female sterility (Lu et al. 2015; Upadhyay et al. 2016; Shang et al. 2018; Yao et al. 2018).

Over recent years, VgRs have been extensively investigated in insects including Blattaria, such as *Leucophaea maderae* (Tufail and Takeda 2007), *Blatella germanica* (Ciudad et al. 2006), and *Periplaneta americana* (Tufail and Takeda 2005); Coleoptera such as *Colaphellus bouvungi* (Liu et al. 2019); Diptera such as *Bactrocera dorsalis* (Cong et al. 2015; Lin et al. 2015) and *Drosophila melanogaster* (Schonbaum et al. 1995); Hemiptera such as *Aphis* (Toxoptera) *citricidus* (Shang et al. 2018), *Bemisia tabaci* (Upadhyay et al. 2016), *Nephotettix cincticeps* (Chen et al. 2016), and *Nilaparvata lugens* (Lu et al. 2015); Hymenoptera such as *Apis mellifera* (Guidugli-Lazzarini et al. 2008) and *Solenopsis invicta* (Chen et al. 2004); and Lepidoptera such as *Thitarodes pui* (Wu et al. 2018), *Conopomorpha sinensis* (Yao et al. 2018), *Spodoptera exigua* (Zhao et al. 2018), *Bombyx mori* (Han et al. 2017), *Helicoverpa armigera* (Zhang et al. 2016), *Bombyx mandarina* (Qian et al. 2015), *Antheraea pernyi* (Liu et al. 2011),
and Spodoptera litura (Shu et al. 2011). Previous studies have shown that vitellogenin receptor belongs to the superfamily of low-density lipoprotein receptors (LDLR) (Schneider 1996), and contains the typical conserved domains including ligand-binding domains (LBD), epidermal growth factor precursor homology domains (EGFPD), O-linked sugar domain (OLSD), transmembrane domain (TMD), and cytoplasmic domain (CPD) (Sappington and Raikhel 1998; Tufail and Takeda 2009).

Although VgRs have already been investigated in some species of insects, VgRs in insects of Orthopteran are rarely studied. Calliptamus italicus is the dominant species of injurious insect most commonly found in the deserts and semi-deserts of Xinjiang, China, where it severely jeopardizes the steppe ecology (Li and Xia 2006). The role of VgR in the reproduction of C. italicus is still unclear. In this study, the full length of VgR of C. italicus was cloned, and the sequence information and expression patterns were investigated. The findings from this study helped unravel the reproductive regulation mechanisms, and have important theoretical significances for screening of the new preventive strategies.

Materials and Methods

Insect Raising and Tissue Collection

C. italicus was collected in the Changji Manas county, China (43° 54’ N, 86° 21’ E; altitude: 1,310 m) between June 2017 and August 2017. The insects were kept in the cages 1 m x 1 m x 1 m in size. Fresh Medicago sativa and Artemisia frigida were provided every day for mixed feeding. After adult emergence, the ovaries, head, feet, wings, and other tissues (pooled samples of epidermis, fat body, midgut, and muscle) of the 7-d-old female adults, as well as the whole body of the 7-d-old male adults, were collected for the analysis of tissue specificity of the gene expression. The ovarian tissues from the fourth- and fifth-instar nymph of C. italicus, as well as 1- to 25-d-old female C. italicus after adult emergence were collected to explore the differences in the gene expression at different developmental stages. RNAstore reagent (Tiangen, Beijing, China) was immediately added after the collection of the tissue samples. The samples were stored at −80°C until use.

Total RNA Extraction and First Strand cDNA Synthesis

Trizol reagent (Invitrogen) was used to extract the total RNA from the tissues of C. italicus. Nano Drop spectrophotometer (Thermo Fisher Scientific, DE) was used to measure the purity and concentration of the total RNA, and 1% agarose gel electrophoresis was conducted to measure the integrity of total RNA. The RNA samples were stored at −80°C until use. PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan) was used to remove the possible gDNA contamination, and then the first strand cDNA was synthesized. Next, 1 µg total RNA was added in the 20 µl reaction system.

Full-Length VgR Gene Cloning

The fragment sequences of VgR gene from the previously reported transcriptome data of C. italicus (Xiang et al. 2017) were obtained; based on which the specific primers were designed for the amplification. PCR amplification was conducted using 2x Taq PCR Master Mix (Tiangen, Beijing, China) for the validation of CiVgR fragment. The conditions for the PCR reactions were as follows: pre-denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The products were then extended at 72°C for 5 min. Universal DNA Purification Kit (Tiangen, Beijing, China) was used for the purification and recovery of PCR products, which were then cloned to pGM-T vector (Tiangen, Beijing, China). Afterwards, the transformation of TOP10 competent cells was conducted according to the manufacturer instructions (Tiangen, Beijing, China). The positive clones were screened, verified by PCR, and then sequencing analysis was conducted. The cDNA fragments 1, 2, and 3 were obtained.

To obtain the sequences of the full-length cDNA of CiVgR, SMARTer rapid-amplification of cDNA ends (RACE) cDNA Amplification Kit (Clontech) was used for the synthesis of the first strand cDNA and nested PCR amplification. Specific primers were designed according to the sequences of the cDNA (Table 1). The conditions for the RACE reactions were as follows: 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. Then, the products were extended at 72°C for 5 min. After the products from RACE amplification were recovered and purified, In-Fusion HD Cloning Kit (Clontech) was used for cloning and sequencing. The cDNA fragments were assembled after sequencing analysis to obtain the full-length CiVgR sequences.

Sequence Analysis

Expy translate tool (http://web.expasy.org/translate) and Expasy MW/pI tool (http://web.expasy.org/compute_pi/) were used to obtain the sequences of the encoded protein, as well as the theoretical molecular weight and isoelectric point. National Center for

### Table 1 Primers used for VgR sequence of Calliptamus italicus

| Primer name | Primer sequence | PCR product length (bp) |
|-------------|-----------------|------------------------|
| Fragment 1  | VgR1-F          | 5'-AGCCAGCATTGAAGGTAACAGC-3' | 1,497 bp |
| Fragment 1  | VgR1-R          | 5'-AGTCCATATGGATGCTGCTGCTG-3' | 1,540 bp |
| Fragment 2  | VgR2-F          | 5'-GCCAAGCTGATGCTAATAGATC-3' | 1,33 bp |
| Fragment 2  | VgR2-R          | 5'-CAGACAGCTTCTTCTATCTGACC-3' | 1,466 bp |
| Fragment 3  | VgR3-F          | 5'-TCTCAGGTTGAGCAGTGGNCACTG-3' | 1,540 bp |
| Fragment 3  | VgR3-R          | 5'-AAACATCCCAGAGCGGATACNAC-3' | 1,466 bp |
| RACE        | 5'-RACE         | 5'-GATTGCTAGTGTGCTTTCAGCAG-3' | 1,466 bp |
| RACE        | 5'-RACE-net     | 5'-GGCGGTCTTCTTCTTTCTAG-3' | 1,466 bp |
| RACE        | 3'-RACE         | 5'-GGCGGGTCTCTTCTCTAGGCT-3' | 1,466 bp |
| RT-PCR and qRT-PCR | VgR-F | 5'-GAAAGAGAAGCTGGATTGATG-3' | 114 bp |
| RT-PCR and qRT-PCR | VgR-R | 5'-GCGCTATCTCTCTGTCCTCC-3' | 114 bp |
| RT-PCR and qRT-PCR | β-actin-F | 5'-ACAGCCGTTCTTCTATGAAGG-3' | 133 bp |
| RT-PCR and qRT-PCR | β-actin-R | 5'-TCAGCAGGCTAGTCG-3' | 133 bp |
Biotechnology Information open reading frame (ORF) finder (https://www.ncbi.nlm.nih.gov/orfinder/) was used to predict the ORF. SignalIP 4.1 Server (http://www.cbs.dtu.dk/services/SignalIP/) was used to identify the signal peptides, TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to analyze the potential TMD, NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict the phosphorylation sites, and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) were used to predict the N- and O-linked glycosylation sites. BLASTP search (https://blast.ncbi.nlm.nih.gov/) was used for the homology comparisons of VgR sequences of the other insects. SMART (http://smart.embl-heidelberg.de/) was used to identify the conserved domains of the protein. The MEGA6.0 software was adopted to construct the phylogenetic tree, using the Neighbor-Joining method. Bootstrap test was used for the statistical analysis of each branch, with the repetition times of 1,000.

Expression Pattern of CiVgR

The total RNA was extracted from the ovaries, head, feet, wings, and other tissues (pooled samples of epidermis, fat body, midgut, and muscle) of the 7-d-old female adults, as well as from the whole body of the 7-d-old male adults, which was reversely transcribed to cDNA. According to our previous findings (data not shown), β-actin was selected as the internal reference for the reverse transcription-polymerase chain reaction (RT-PCR) analysis, to investigate the tissue specificity of CiVgR expression. The conditions for the PCR reactions were as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Finally, extension at 72°C for 5 min was conducted to complete the reactions. The PCR products were analyzed by 1% agarose gel electrophoresis.

The total RNA was extracted from the ovarian tissues of the fourth- and fifth-instar nymph of C. italicus, as well as 1- to 25-d-old female C. italicus after adult emergence, which was reversely transcribed to cDNA. Quantitative real time polymerase chain reaction (qRT-PCR) was conducted using LightCycler 96 fluorogenic quantitative PCR instrument (Roche Applied Science, Switzerland) and SYBR Premix Ex Taq II (TaKaRa, Tokyo, Japan) to investigate the expression pattern of CiVgR at different developmental stages, using β-actin as the internal reference. The primers for the qRT-PCR are shown in Table 1. The PCR amplification system contained 1 µl cDNA, 10 µl 2x SYBR mix, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), and then ddH2O was added until the total volume was 20 µl. The conditions for the qRT-PCR reactions were as follows: pre-denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Next, 1 cycle of 95°C for 1 min, 65°C for 30 s, and 95°C for 1 s was conducted to obtain the melting curve, which was used to verify the specificity of the qPCR primers. The reactions were repeated for 3 times for each sample, using sterilized ddH2O as the blank control. The Cq values were obtained after the reactions were completed. 2^ΔΔCt method was used to calculate the relative expression of CiVgR in different developmental stages (Livak and Schmittgen 2001).

Results

CiVgR Sequences and Structures

According to the previously published transcriptome data, the full-length cDNA of CiVgR, which was 5,589 bp was obtained (GenBank accession number: GenBank MK358118). The cDNA contained an 86 bp 5'-untranslated region (UTR), 5,265 bp of ORF, and 238 bp of 3'-UTR, which encoded 1,754 amino acids (aa) (Fig. 1). The theoretical molecular weight of the predicted protein encoded by the gene was 191.19 kDa, and πw was 5.15. The N-terminal of CiVgR contained a signal peptide composed of 27 amino acids, whose sequence was MSCGPGGSMKILTAVLLLLLGVAEVC. CiVgR also contained 7 N-linked glycosylation sites and 35 O-linked glycosylation sites with 155 phosphorylation sites including 76 serine sites, 51 threonine sites, and 28 tyrosine sites. BLASTP analysis showed that the similarity of the amino acid sequences of CiVgR was 46% with the VgR of P. americana, 45% with the VgR of Cryptotermes secundus and Zootermopsis nevadensis, and 44% with the VgR of Franklinkiella occidentalis and B. germanica. The multiple sequence alignment between the predicted amino acid sequences of CiVgR and other insects showed that the amino acid sequences of VgRs were highly conserved, and CiVgR had consistent conserved domains and conserved motifs with other insects (Fig. 2).

CiVgR belonged to the superfamily of LDLR, and contained the typical domains of this superfamily (Fig. 3). CiVgR contained two LBD, located at 28–238aa and 931–1,256aa. The LBD1 contained 5 LDLR Class A repeat (LDRLa), while LBD2 contained 8 LDLR. Each LBD was located at an EGF motif, located at 1,257–1,643aa. The EGF motif contained 7 LDLR Class B repeat (LDLRb) and 6 YWXD motifs. EGF motif was followed by the TMD, located at 1,673–1,695aa. The TMD formed a transmembrane α-helix, which anchored the CiVgR to the membrane. The CPD was located at 1,696–1,754aa. No OLS was found in CiVgR.

Phylogenetic Analysis of CiVgR

MEGA6.0 software was used for the sequence alignment of the 36 amino acids of VgR, after which the phylogenetic tree was constructed (Fig. 4). The results showed that the VgRs of the insects of Hymenoptera, Siphonaptera, and Coleoptera were in the same branch, the VgRs of the insects of Thysanoptera and Blattaria were in the same branch, and the VgRs of the insects of Anoplura and Hemiptera were in the same branch. In addition, the VgRs from different orders were categorized into smaller classes. The genetic relationship between CiVgR and Blattaria insects, including B. germanica, Rhyparobia maderae, P. americana, C. secundus, and Z. nevadensis, were closer compared to other insects.

CiVgR Expression Analysis

Tissue-specific analysis of CiVgR expression showed that CiVgR was only expressed in the ovarian tissues of female C. italicus (Fig. 5). The analysis of different developmental stages showed that CiVgR was slightly expressed in the fourth- and fifth-instar nymph of C. italicus, but the expression levels were relatively low. However, the expression of CiVgR gradually increased after adult emergence, peaking at 6 d after adult emergence, and then starting to decrease and consolidating at relatively low level (Fig. 6).

Discussion

In this study, we cloned the full-length cDNA of the CiVgR of C. italicus, and investigated the molecular features of this gene. To our knowledge, this is the first study that investigated the full-length cDNA of VgR in Orthopteran insects. The full length of the CiVgR gene was 5,589 bp, which encoded a 193.19 kDa protein, with the
The predicted amino acid sequence of CiVgR. Signal peptide have been underlined; N-glycosylation sites are marked by the boxes.

**Fig. 1.** Predicted amino acid sequence of CiVgR. Signal peptide have been underlined; N-glycosylation sites are marked by the boxes.
The current study also investigated the expression of CiVgR in different tissues. The results showed that CiVgR was only specifically expressed in the ovarian tissues, but not in male adults and the other tissues of female adults. Previous studies have demonstrated that VgR is also specifically expressed in the ovarian tissues of most other insects, such as *S. exigua* (Zhao et al. 2018), *H. armigera* (Zhang et al. 2016), *N. lugens* (Lu et al. 2015), *S. litura* (Shu et al. 2011), *L. maderae* (Tufail and Takeda 2007), *B. germanica* (Ciudad et al. 2006), *P. americana* (Tufail and Takeda 2005), and *S. invicta* (Chen et al. 2004). These findings demonstrate that VgR is closely associated with the reproductive regulation of the insects.

However, some other studies have shown that VgRs could also be expressed in other tissues in some insects (Roy-Zokan et al. 2015). VgR expression has been detected in the ovarian tissues and fat body of *B. mandarina* (Qian et al. 2015) and *A. peryyi* (Liu et al. 2011) in the pupal stage; however, VgR expression was only found in the ovarian tissues in adult insects. Low level expression of VgR has also been found in the eggs of *N. cincticeps* (Chen et al. 2016) and *B. mori* (Lin et al. 2013). In addition, the studies in *A. mellifera* have shown that VgR is not only expressed in the ovaries of the female insects, but also in the fat body, hypopharyngeal gland, and midgut at low levels (Guidugli-Lazzarini et al. 2008). The differential expression of VgR in the tissues suggested that in addition to reproductive regulation, Vg might also participate in the other physiological processes.

The important role of VgRs is to regulate the absorption of yolk protein in insects, which has been confirmed in many species. According to the ovarian morphological characteristics, the ovarian development cycle in *C. italicus* is 24–25 d after emergence, and the vitellogenesis stage is 8–13 d after emergence (data to be published). In this study, the expression of CiVgR was analyzed by qRT-PCR. The results revealed that CiVgR gene was expressed in the complete ovarian development cycle. The expression level of CiVgR increased in previtellogenesis stage (1–7 d after adult emergence) and reached the peak value, then decreased rapidly in the vitellogenic stage (8–13 d after adult emergence), and remain low with slight fluctuation in the secondary vitellogenesis stage (14–25 d after adult emergence). The developmental expression pattern of CiVgR gene is similar to that of *N. cincticeps* (Chen et al. 2016), *N. lugens* (Lu et al. 2015), and...
L. maderae (Tufail and Takeda 2007), in which the VgR mRNA levels were high at the early yolk deposition stage and low at the yolk deposition stage, suggesting that its function is related to yolk deposition.

In summary, this study successfully cloned the full-length segment of CiVgR gene, and elucidated the molecular features and expression patterns, which provide theoretical bases for further...
researches on molecular mechanisms of vitellogenesis in *Calliptamus italicus*. The regulatory function and molecular mechanism of VgR in vitellogenesis need further study.

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**Conflict of Interest**

These authors have no conflict of interest to declare.

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