Identification and Characterization of the Vitellogenin Receptor Gene and Its Role in Reproduction in the Alligatorweed Flea Beetle, Agasicles hygrophila

Hong Zhang, Yiran Liu, Jisu Jin, Zhongshi Zhou and Jianying Guo*

State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

Agasicles hygrophila is an effective biological control agent for the invasive weed Alternanthera philoxeroides, and because of this it has been introduced to many parts of the world where A. philoxeroides is a problem. Despite this, there are no reports at present about the reproduction of this important insect. Vitellogenin receptors (VgRs) belong to the superfamily of low-density lipoprotein receptors (LDLRs). One of the roles of VgRs is to regulate the absorption of yolk protein in insects. In this study, the full length vitellogenin receptor gene (AhVgR) from A. hygrophila was sequenced and found to encode a predicted protein of 1,642 amino acids. Sequence analysis of AhVgR revealed that it contains conserved structural motifs common to LDLR family members, and a phylogenetic analysis placed AhVgR as a separate group among the order Coleoptera. AhVgR was found to be specifically expressed in ovarian tissues, and it is first transcribed in the newly-emerged females. The expression patterns are consistent with VgR genes in other insects. RNA interference (RNAi)-mediated suppression of AhVgR gene expression in adult A. hygrophila females inhibited yolk protein deposition in the ovaries, shortened the ovariole, drastically reduced egg production, and ultimately led to a decrease in fecundity. In summary, our work shows that AhVgR is critical for transporting Vg into the oocytes and plays an important role in A. hygrophila reproduction.

Keywords: Agasicles hygrophila, vitellogenin receptor, RNAi, ovarian development, fecundity

INTRODUCTION

Alternanthera philoxeroides, (Mart.) Griseb (Amaranthaceae), commonly known as alligatorweed, is a perennial aquatic plant native to South America that has invaded many regions of the world, including North America, Asia, and Australia (Julien et al., 1995; Xu et al., 2003). In China, A. philoxeroides was introduced as a forage crop in the late 1930s, and subsequently spread to the country’s eastern and southern regions (Ye et al., 2010). A. philoxeroides is highly competitive, and causes economic and ecological problems almost everywhere it is found (Maddox and Rhyme, 1975). Agasicles hygrophila (Selman & Vogt) (Coleoptera: Chrysomelidae), the alligatorweed flea beetle, has proven to be an efficient control agent for A. philoxeroides (Buckingham, 1996). The beetle was introduced into China from Florida in 1987, and was first released in Chongqing,
Agasicles hygrophila became established in the release sites and is now distributed mostly in southern China, where it has controlled A. philoxeroides for over 30 years (Guo et al., 2011). As the precursor of the major yolk protein vitellin (Vn), vitellogenin (Vg) provides nutrition necessary for egg development, and plays an important role in insect reproduction (Tufail and Takeda, 2009). During reproduction, Vg is primarily synthesized in the fatbody and then secreted into the hemolymph, where it is absorbed by the developing oocytes via the vitellogenin receptor (VgR) (Lu et al., 2015). Thus, VgR plays an essential role in the process of oocyte maturation (Sappington and Raikhel, 1998). In insects, the VgR gene encodes a large ovary-specific protein of 180–214 kDa, which is twice the size of the vertebrate VgR proteins (Sappington and Raikhel, 1998). The amino acid sequences of insect VgRs show that they are members of the low-density lipoprotein receptor (LDLR) family, with two ligand binding domains (LBDs) containing several LDLR-domain class A repeats. The function of VgR is different from that of Vg, but silencing either of the genes can lead to malformed ovaries and decreased fecundity, as has been shown in Helicoverpa armigera (Zhang et al., 2016), Nilaparvata lugens (Lu et al., 2015), Spodoptera litura (Shu et al., 2011), and Aphis citricidus (Shang et al., 2018).

Although VgRs have been described in other insects, there are no studies that describe the VgR of A. hygrophila. In our study, we characterized the AhVgR gene and explore its expression patterns in A. hygrophila. We cloned the complete sequence of AhVgR, and showed its molecular characteristics, phylogenetic relationships and expression patterns. Furthermore, we verified the function of AhVgR in ovary development and reproduction through RNA interference experiments.

**MATERIALS AND METHODS**

**Insect Rearing and Sample Collection**

Alternanthera philoxeroides plants were collected at the Institute of Plant Protection, Hunan Academy of Agricultural Sciences, and grown in the greenhouse at Langfang Experimental Station, Chinese Academy of Agricultural Sciences. Plants were grown in sterilized soil in plastic boxes (40 cm × 18 cm × 15 cm) and watered every other day. Agasicles hygrophila adults were initially collected from the field in Changsha. The population was reared on A. philoxeroides plants in the laboratory at the Chinese Academy of Agricultural Sciences (Beijing, China) under controlled conditions (28 ± 1°C, 12 h light:dark photoperiod, relative humidity [RH] = 75 ± 5%) (Guo et al., 2011).

To clone the AhVgR gene sequence, the ovaries of A. hygrophila females were dissected in 1X PBS (phosphate-buffered saline, pH 7.4) using an Olympus stereo microscope (SZX16, Olympus, Tokyo, Japan). For tissue-specific expression of AhVgR, the head, thorax, ovary, fat body, midgut, and wing tissues of 6-day-old beetles were also dissected in PBS using the Olympus SZX16 stereomicroscope. To analyze the expression of the different developmental stages, freshly pupated pupa were collected daily, and female adults were collected every 2 day after emergence. All samples were immediately frozen in liquid nitrogen and stored at −80°C until they were used for RNA isolation.

**RNA Isolation and Gene Cloning**

Total RNA was extracted from A. hygrophila tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, United States) according to the manufacturer's protocol. The concentrations of the RNA samples were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, United States), while degradation and genomic DNA contamination were monitored by electrophoresis through 1% agarose gels. First-strand cDNA was synthesized using the Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China) following the manufacturer's protocol. Our lab mined the A. hygrophila transcriptome data to obtain expressed sequence tags (ESTs) that showed similarities to other insect vitellogenin receptor genes. The PCR primers were designed based on the A. hygrophila vitellogenin receptor gene cDNA fragment from the transcriptome (Supplementary Table 1). Positive clones were confirmed by PCR amplification and then sequenced. To obtain full-length cDNA sequence of the AhVgR gene, we used a SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, United States) to amplify the 5′- and 3′-ends. Gene-specific primers were designed (Supplementary Table 1), and the RACE cycling conditions were performed as described in Zhang et al. (2016). The RACE products were then gel-purified and sequenced.

**Gene Sequence Analysis and Phylogenetic Relationships**

Sequence similarities were analyzed using the BLAST website. The open reading frames (ORFs) of the AhVgR clones were predicted using the NCBI ORF finder. The molecular weights and isoelectric points (pIs) of the inferred AhVgR protein sequences were predicted with the ExPASy proteomics server. The conserved domains and signal peptide were analyzed by the online SMART server and the SignalP 4.1 Server. The VgR amino acid sequences from other insects were downloaded from the GenBank database and used to in the phylogenetic analyses. The VgR sequence alignments were carried out using Clustal X software. A phylogenetic tree was constructed with molecular evolution analysis software (MEGA version 5.0), using the neighbour-joining (NJ) method with a bootstrap test of 1,000 replicates (Tamura et al., 2011).

**Synthesis of Double-Stranded RNA (dsRNA) and Injection of dsRNA**

dsRNA was synthesized from the AhVgR cDNA, and EGFP (Enhanced Green Fluorescent Protein, GenBank Accession No. 1http://www.ncbi.nlm.nih.gov/BLAST/ 2https://www.ncbi.nlm.nih.gov/orffinder/ 3http://www.expasy.org 4http://smart.embl-heidelberg.de/ 5http://www.cbs.dtu.dk/services/SignalP
To ensure the interference effect, we synthesized two dsRNA fragments from AhVgR (dsVgR-A and dsVgR-B) and EGFP (ds EGFP- and, ds EGFP-B). The target gene dsRNAs were synthesized and purified using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England BioLabs, Ipswich, MA, United States) as directed by the manufacturer. The purity of the dsRNA was checked by electrophoresis on a 1.0% agarose gel and the concentration of the dsRNA was measured on a NanoDrop ND-2000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States). To ensure the injection volume between the control group and treatment group was consistent, we adjusted the concentration of all synthesized dsRNA to 10,000 ng/µl. Newly-emerged adult females were collected, and the dsRNA was injected into the conjunctivum using a PLI-100 Pico-Injector (Harvard Apparatus, Holliston, MA, United States) under an Olympus stereomicroscope. Each experiment was repeated three times. Each repeat consisted of 60 female individuals for later experimental observation after injection. The injection dose was 0.1 µl of each dsRNA (dsVgR group: dsVgR-A 0.1 µl, dsVgR-B 0.1 µl; dsEGFP group: dsEGFP-A 0.1 µl, dsEGFP-B 0.1 µl). After injection, female adults were kept in separate plastic bottles (8 cm x 10 cm) and paired with freshly emerged wild-male adults. Each bottle was kept moist with a piece of moistened filter paper at the bottom, and fresh A. philoxeroides stems were added.

**qPCR Experiments**

Total RNA was extracted from the samples with TRIzol reagent (Life Technologies, Carlsbad, CA, United States) following the manufacturer’s instructions. First-strand cDNA was synthesized by following the procedures mentioned above. Quantitative real-time PCR (qPCR) analysis was performed with the TransStart Green qPCR SuperMix Kit (Transgen, Beijing, China) on an ABI Prism 7500 system (Applied Biosystems, Carlsbad, CA, United States). 

**Domain architecture of the Agasicles hygrophila vitellogenin receptor (VgR) protein.**

**Observation of Ovary Development and Fecundity After RNAi**

Each dsRNA-treated A. hygrophila female was observed 4 days after dsRNA injection to examine ovary development. The ovaries were dissected using high-precision tweezers (IDEAL-TEK, Balerna, Switzerland) in 1X PBS under the Olympus SZX16 stereomicroscope. Dissected ovaries were washed three times with 1X PBS and photographed as described by Zhao et al. (2016). Eggs laid by each pair of A. hygrophila adults in the control and treatment groups were collected and counted once. A total of 1,000 eggs were used to calculate the egg hatch rate, and the eggs were assessed every 12 h until they started to decay (Zhao et al., 2016).

**Statistical Analysis**

Statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, United States). All the experimental data are shown as means ± SD (standard deviation). Experimental data were checked for normality and homoscedasticity, and if needed, were arcsine square-root or log-transformed before analysis. Egg hatching rates and ovariole lengths were arcsine-transformed. The number of eggs was square root-transformed. We performed the least significant difference (LSD) test after one-way analysis of variance (ANOVA) to analyze differences in AhVgR expression levels between different tissues and developmental stages. AhVgR expression levels after injection, ovariole lengths, fecundity of females, and egg hatch rate were analyzed by Student’s t-test. We considered means significantly different at $P < 0.05$. 
RESULTS

Sequence Analysis and Phylogenetic Analysis of the AhVgR Gene

The full-length AhVgR cDNA was found to be 5,386 bp in length, including a 224 bp 5′ untranslated region (UTR), a 4,929 bp ORF encoding a predicted protein of 1,642 amino acids, and a 233 bp 3′ UTR (GenBank Accession No. MH428915). The theoretical molecular weight is 183.32 kDa, and the pI is 5.08. Analysis of the AhVgR amino acid sequence showed that a 16-amino acid signal peptide (MLVLLLLGVVTPSLGF) is located at the N-terminus of AhVgR. Domain architecture analysis showed that AhVgR is a transmembrane protein with several typical domains, including a LDLR domain class A, an epidermal growth factor (EGF)-like domain, a calcium-binding EGF-like domain, a low-density lipoprotein-receptor YWTD domain, and the transmembrane region (Figure 1). The analysis indicated that AhVgR has three LBDs with five class A (LDLa) cysteine-rich repeats in the first domain and eight in the second domain (Figure 1). Each repeat contains six cysteine residues, and each LDLa is followed by an EGF-like domain. The second and the seventh EGF-like domains contain a calcium-binding domain. The second, third, and seventh EGF-like domains are followed by 4, 3, and 1 repeats of the LDLR YWTD motif, respectively (Figure 1).
A phylogenetic analysis of the AhVgR protein showed that VgR sequences from insects are clearly separated from mammalian VgR sequences (Figure 2). In the neighbor-joining tree, VgRs from Coleoptera clustered in one clade, well-separated from VgR proteins from other insect orders, showing that VgRs from Coleoptera all had high amino acid sequence identity and are derived from a common ancestor (Figure 2). The dendrogram also showed that the VgR proteins from A. hygrophila and Leptinotarsa decemlineata are close, suggesting that the VgRs from these two insects share a close evolutionary relationship. The phylogenetic tree also showed that VgRs from the same insect orders clustered into individual clades, suggesting the conservation of insect VgRs and showing the close evolutionary relationships within the same taxonomic groups. However, the VgRs of Coleoptera clustered most closely with the VgRs of Blattaria with high bootstrap support, showing that the VgRs of these two insect orders share a closer evolutionary relationship than they do with VgRs from other insect orders.

**Tissue-Specific Expression and Temporal Expression Patterns of AhVgR**

qPCR analysis shows that AhVgR mRNA is most highly expressed in the ovaries (Figure 3). Analysis of AhVgR mRNA expression in the pupal and adult stages revealed that AhVgR expression was not detectable in the pupal stage, but was detected in newly-emerged females, reaching a maximum level in 11-day-old individuals (Figure 4). Subsequently, expression decreased gradually to a lower level that was maintained in the adults.

Effects of dsRNA Injection on AhVgR Expression

qPCR assays showed that AhVgR expression in dsVgR-injected females decreased significantly at all sampled time points from 3 to 25 days post-injection (Figure 5). However, AhVgR expression did not show an obvious difference between the dsEGFP-injected group and dsVgR-injected group after 25 days. This may be because of the timeliness of dsRNA. Furthermore, from 3 to 25 days after the injection of dsAhVgR, the transcription levels of AhVgR decreased by 45.6–81.2% (Figure 5) compared to the dsEGFP treatment control group.

**dsVgR Injection Inhibits Ovarian Development**

After 4 days, the ovaries of the dsVgR-injected group showed a decrease in yolk protein deposition compared to the dsEGFP-injected group (Figure 6). The length of the ovariole in the dsVgR-injected group was shorter than in the dsEGFP-injected group (Figure 7), showing that ovary development in A. hygrophila females was inhibited after injection with dsVgR RNA, and that AhVgR plays an important role in ovary development and yolk protein deposition.

**Knockdown of AhVgR Affects Fecundity in A. hygrophila Females**

After the injection of dsVgR RNA into newly-emerged adult females, they laid significantly fewer eggs than did the dsEGFP-injected group (Figure 8A). Furthermore, when the hatch rates were compared, eggs produced by the dsVgR-injected females had lower hatching success than did eggs from the dsEGFP-injected group (Figure 8B). Results of fecundity and egg hatching...
rate both confirmed that RNA interference of AhVgR gene expression significantly inhibited reproduction in A. hygrophila.

**DISCUSSION**

The VgR gene have been studied in many insects, however, this gene has not been characterized in A. hygrophila, and studying it is relevant because that this species is used as a biological control agent for the invasive weed A. philoxeroides. In our experiments, we characterized AhVgR as the LDLR homolog, and analyzed the expression patterns of this gene in different tissues and developmental stages. Our study is the first to use the RNA interference method to explore the function of AhVgR, and the results showed that silencing the AhVgR gene inhibits ovary development and significantly reduces fecundity in A. hygrophila.

VgR proteins belong to the LDLR family, and regulate the process through which Vg enters the oocyte by endocytosis. All oviparous vertebrates and invertebrates have VgR family member proteins (Tufail and Takeda, 2009; Shu et al., 2011). In our study, the full-length cDNA of the AhVgR gene was cloned, and the molecular characteristics of this gene were analyzed. The AhVgR protein shares several typical domains with other insect VgRs, such as a LDLR domain class A, an EGF-like domain, a calcium-binding EGF-like domain, and a low-density lipoprotein-receptor YWTD domain. As the structural arrangement found in most insect VgRs, the AhVgR protein contains five cysteine-rich repeats in the first binding site and eight in the second binding site (Figure 1). However, the structural characteristics in other insect VgRs are different. For example, in Spodoptera litura, SlVgR has four cysteine-rich repeats in the first binding site and seven in the second binding site. Phylogenetic analysis shows that these insect VgRs cluster into six clades (Coleoptera, Blattaria, Hymenoptera, Hemiptera, Diptera, and Lepidoptera).
As expected, AhVgR is in the Coleoptera clade, and the phylogenetic tree indicates that AhVgR shares homology with other Coleoptera VgR proteins, such as those from *Leptinotarsa decemlineata*, *Diabrotica virgifera*, *Dendroctonus ponderosae*, and *Agrilus planipennis* (Figure 2).

In *A. hygrophila*, expression profiles of AhVgR showed that this gene is expressed at high levels in the ovaries (Figure 3), indicating that it is an ovary-specific gene, consistent with its role in insect reproduction (Ciudad et al., 2010; Lin et al., 2013). However, VgR gene expression has also been detected in other tissues. In *Apis mellifera*, AmVgR expression was detected in the hypopharyngeal gland and other tissues (Guidugli-Lazzarini et al., 2008). The various expression patterns of VgR genes in different tissues may be related to their ligand Vg, which regulates longevity and the juvenile hormone titer in bees (Corona et al., 2007; Nelson et al., 2013). The temporal expression patterns of AhVgR were found to be closely correlated with the reproductive process, and expression levels significantly increased in the ovaries beginning 3 days after emergence (Figure 4). This pattern is consistent with VgR expression patterns in other insects, such as in *Nilaparvata lugens*, *Thitarodes pui*, and *Aphis citricidus* (Lu et al., 2015; Shang et al., 2018; Wu et al., 2018).

RNA interference is considered to be a specific tool to efficiently determine gene functions (Hannon, 2002) and also to control gene expression at the mRNA level (Fire et al., 1998). We used RNAi to explore the functions of AhVgR in vivo. The results showed that injection with dsVgR RNA displayed high efficiency and caused significant differences in fecundity and ovarian development compared to the control group (Figures 6–8). AhVgR expression decreased after the dsVgR treatment, and the effects of dsVgR RNA lasted for about 25 days after injection (Figure 5). The high gene silencing efficiency of dsRNA has also been verified in other Coleoptera species such as *Tribolium castaneum*, *Leptinotarsa decemlineata*, and *Diabrotica virgifera* (Arakane et al., 2005; Shi et al., 2016; Camargo et al., 2018). After injecting the dsVgR RNA, the ovaries exhibited a decrease in yolk protein deposition compared to the control group (Figure 6), and the lengths of the ovarioles were also reduced (Figure 7). Furthermore, we observed that *A. hygrophila* fecundity, as measured by the number of eggs laid and their hatch rate, both decreased sharply after injection with dsVgR RNA (Figure 8).

Our results are consistent with an earlier study which suggested that the capability of reproduction in insects depends on two primary steps, (1) the process of vitellogenin (Vg) formation and deposition and (2) transport of the Vg to oocytes by the vitellogenin receptor (VgR) (Sappington and Raikhel, 1998). The functions of VgR have been also explored in other insect species in which the functions of Vg have been studied, and the results are in accordance with ours (Shu et al., 2011; Lin et al., 2013; Shang et al., 2018).

CONCLUSION

In our study, the molecular characterization of AhVgR and the expression patterns of this gene in different tissues and developmental stages were analyzed. Analysis of amino acid sequences of AhVgR suggests AhVgR is a member of the LDLR family and displays high sequence similarity to VgRs from other insects. The tissue and developmental stage-specific mRNA expression patterns of AhVgR were also similar to those in other insects. We found that AhVgR was most highly expressed in the ovary of adult stage. After the basic characterization of the gene, we explored the functions of AhVgR via RNA interference, and the results indicate that the AhVgR protein is necessary to stimulate yolk uptake, as well as being critical for ovary development and egg laying in *A. hygrophila*.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.
AUTHOR CONTRIBUTIONS

HZ and JG conceived and designed the experiments, and wrote the manuscript. HZ performed the experiments. HZ, YL, JJ, and ZZ analyzed the data.

FUNDING

This work was supported by the National Natural Science Foundation of China (31572068) and the National Key Technology Research and Development Program of the Ministry of Science and Technology of China (2015BAD08B03).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00969/full#supplementary-material

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