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Research

Knockdown of *Methoprene-Tolerant* Arrests Ovarian Development in the *Sogatella furcifera* (Hemiptera: Delphacidae)

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Abstract

Juvenile hormone (JH) is responsible for repressing larval metamorphosis and inducing vitellogenesis and egg production in insects. Methoprene-tolerant (Met) is known to be an intracellular receptor and transducer of JH. We examined the role of Met in ovarian development in the rice pest *Sogatella furcifera* (Horváth). We first cloned and sequenced *S. furcifera Met* (*SfMet*). The SfMet protein belongs to the basic helix–loop–helix/Per-Arnt-Sim (bHLH-PAS) family with a bHLH domain and two PAS domains (PAS-A and PAS-B). *SfMet* was expressed in all developmental stages and tissues but was most highly expressed in the ovaries of adult females. Furthermore, RNA interference (RNAi) mediated silencing of *SfMet* substantially reduced the expression of *SfVg*, decreased yolk protein deposition and blocked oocyte maturation and ovarian development. These results demonstrate that *SfMet* plays a key role in female reproduction in *S. furcifera* and suggest that targeting this gene could be an effective way of controlling this pest.

Key words: *Sogatella furcifera*, methoprene-tolerant, juvenile hormone, vitellogenin

The sesquiterpenoid juvenile hormone (JH) and the steroid 20-hydroxyecdysone (20E) are the main hormones that regulate insect development, metamorphosis, and reproduction (Jindra et al. 2013, Roy et al. 2018). JHs are synthesized and secreted by the corpora allata (CA), which are a pair of endocrine glands located posterior to the brain (Toyomi et al. 2009). JH maintains the larval state by suppressing the expression of metamorphosis-initiation genes (Nijhout 1994). A drastic decrease in JH in the final instar allows a spike in 20E to induce the metamorphic molt (Kayukawa et al. 2017). The secretion of JH resumes in adults, in which it regulates the reproductive maturation of females, including vitellogenesis and oogenesis (Li et al. 2019, Santos et al. 2019).

JH acts through its receptor, methoprene-tolerant (Met) (Wilson and Ashok 1998, Jindra et al. 2013), a basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) transcription factor first identified in *Drosophila melanogaster* (Meigen) (Diptera: Drosophiladae) (Ashok et al. 1998). JH directly binds to the PAS-B domain of Met to form JH/Met (Charles et al. 2011), which enters into the nucleus by interacting with heat shock protein (He et al. 2014, He et al. 2017). In the nucleus, Met dimerizes with its partners, SRC (steroid receptor coactivator)/Taiman/FISC (Ftz-F1-interacting steroid receptor coactivator), to form a receptor complex that acts as a functional transcription factor (Miura et al. 2005, Li et al. 2011, Li et al. 2019). This JH receptor complex activates downstream gene transcription to transduce JH signals by binding to an E box-like sequence in the promoter region of JH response genes. For example, *Krüppel homolog 1* (*Kr-h1*), a zinc-finger transcription factor that is one of the early response genes directly targeted by the JH receptor complex (Kayukawa et al. 2012).

Met is, therefore, essential for JH to effectively regulate physiological activity in insects. It has been demonstrated that Met is involved in the anti-metamorphic function of JH. For example, depletion of *TcMet* in young larvae of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) induced premature pupation and precocious metamorphosis (Konopova and Jindra 2007). Similarly, in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), silencing of *HaMet* in final instar larvae resulted in premature and abnormal pupation (Ma

et al. 2018). In addition to regulating metamorphosis, Met is known to be an intracellular receptor of JH that regulates the expression of the *vitellogenin* gene (*Vg*) (Zou et al. 2013, Santos et al. 2019). Vg is the precursor of vitellin and is involved in providing nutrition for oogenesis and embryonic development (Shang et al. 2018). JH induces the synthesis of Vg in the fat body and intercellular spaces in the follicular epithelium, after which it is absorbed by developing oocytes from the hemolymph via receptor-mediated endocytosis (Li et al. 2019, Santos et al. 2019). Knockdown of *Met* in female adults dramatically reduces the transcription of *Vg*, reduces Vg yolk protein deposition and Vg uptake in oocytes, ultimately impairing fecundity (Konopova et al. 2011, Lin et al. 2015, Ma et al. 2018, Yue et al. 2018).

Sogatella furcifera (Horváth) is a migratory pest of rice crops in Asia. This species causes major economic damage to rice crops by sucking phloem sap, oviposition and transmitting the southern rice black-streaked dwarf virus to rice plants (Zhou et al. 2008, Zhou et al. 2019). Insect reproduction has been a focus of pest control, and clarification of the genes related to reproduction should provide ideal targets for pest management. Although the role of Met in the reproduction of many insects have been systematically studied, relevant information is limited in *S. furcifera*. In this study, we cloned and characterized the open reading frame (ORF) sequence of *SfMet* from *S. furcifera* and analyzed its spatial and temporal expression profile. We then used RNA interference to knock down *SfMet* in female adults, and found that this reduced *SfVg* expression and severely impeded oocyte maturation and ovarian development. These results provide further evidence that SfMet is essential for female reproduction, and that targeting it could be a potential way of controlling this pest.

Materials and Methods

Insects

The *S. furcifera* used in this study were collected from paddy fields in Hunan Agricultural University, Changsha, China. The colony was reared on 'Fengyou No. 9' rice seedlings in a climatic chamber at 26 ± 1°C with 80 ± 5% relative humidity (RH) under a 16:8 (L:D) h photoperiod.

Sample Preparation

To determine the expression of *SfMet* in different developmental stages we measured its expression in randomly chosen samples of individuals from the 1st, 2nd, 3rd, 4th, and 5th instars (n = 30, 30, 15, 10, and 10 individuals contained in one replicate, respectively), and also in 96-h-old female and male adults (five insects in one sample). We also measured the expression of *SfMet* in female adults in the first 132 h after eclosion (0, 24, 48, 72, 96, and 132 h after eclosion, five insects contained in each sample). In addition, the expression level of *SfMet* in different tissues had also been measured, namely the head, midgut, ovary, fat body and thorax which were dissected from 96-h-old female adults in phosphate-buffered saline (PBS, pH = 7.4) (Each tissue sample contained 20 dissected organs). All samples were frozen immediately in liquid nitrogen and stored at –80°C until required for RNA extraction, three replicates were performed per sample.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from *S. furcifera* whole bodies or tissues using a MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). First-stand cDNA was synthesized by reverse transcribing 0.5 µg of total RNA in a 20 µl reaction mixture with a PrimeScript RT Reagent Kit and gDNA Eraser (TaKaRa). cDNA products were stored at –20°C until required.

Cloning and Bioinformatic Analysis

The cDNA sequence of *SfMet* was obtained from *S. furcifera* transcriptome databases. The ORF sequence was amplified with special primers (Table 1) and the resultant polymerase chain reaction (PCR) product inserted into the pMD-18T vector (Takara) for sequencing. The putative molecular weight (Mw) and isoelectric point (pI) were calculated using the compute pI/Mw tool of online EXPASy proteomics server (https://web.expasy.org/compute_pi/). The conserved domains of *SfMet* were identified using the InterPro program (https://www.ebi.ac.uk/interpro/beta/). The amino acid sequence of *SfMet* was aligned with those of other hemipteran insects using the DNAMAN 8.0 software package (Lynnon Corporation, Quebec, Canada). Finally, a neighbor-joining (NJ) phylogenetic tree was constructed using on a p-distance model and MEGA 5.0 software. One thouand bootstrap replications were performed to test topology.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to determine the spatio-temporal distribution of *SfMet*. qPCR was performed using TB Green Premix Ex Taq II (TaKaRa) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, California, United States). The reaction was performed in a 10 µl volume containing 1 µl of cDNA, 5 µl of TB Green Premix Ex Taq II, 0.4 µl of each primer (10 mM) and 3.2 µl of ddH_2O. The two-step qPCR program protocol was as follows: one cycle of 95°C for 30 s, followed by 40 cycles of 5 s at 94°C, and 30 s at 60°C. Three technical replicates were performed for each sample. The qPCR primers were designed using information on the NCBI (National Center for Biotechnology Information) profile sever (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The efficiency of each primer pair was determined by generating standard curves from a fivefold dilution series of cDNA templates. Relative expression levels of *SfMet* were analyzed using the $2^{-\Delta\Delta Ct}$ method, normalized to two reference genes, *SfTub* (*a-1 tubulin*, GenBank accession No. KP735521) and *SfEF1α* (*elongation factor 1α*, GenBank accession No. KP735517). The primer sequences and their amplification efficiencies are shown in Table 1.

RNAi Experiment

Products for a dsRNA template were obtained by amplifying a 552 bp fragment of *SfMet* and a 401 bp fragment of *EGFP* (*enhanced green fluorescent protein*, GenBank accession No. U55762) with special primers containing a T7 promoter sequence. dsRNAs of *SfMet* and the negative control *EGFP* were then prepared using the T7 RiboMAX Express RNAi System (Promega, Wisconsin, United States) following the manufacturer's protocol.

To investigate the function of *SfMet* in ovarian development, 100 ng (2,000 ng/µl) dsRNA targeting *SfMet* was injected into newly emerged female adults as described previously (Hu et al. 2019). A control group were given the same dose of dsEGFP. RNAi efficiency, and the expression levels of *SfKr-h1* and *SfVg*, were determined 48, 72, and 96 h, after injection. After 132 h, the ovaries of females in the treatment and control groups were observed, compared and photographed with an SMZ-161 microscope equipped with a D3400 digital camera (Nikon, Tokyo, Japan).

Table 1. The special primers used in this study

Purpose	Primer name	Primer sequence (5′→3′)	E (%)[a]	R^2
	Met-ORF-F	TCGCCGTTCCGACGACAATG	n.a.	n.a.
	Met-ORF-R	TATCAGCTCATCACAACGGGG		
qPCR	qMet-F	TGTGGACTATGGTCGCCTTG	103.8	0.994
	qMet-R	GCTCAATGTAGCCGTGTGTC		
	qKr-h1-F	GGCATTTGGCTACAACCACG	99.6	0.992
	qKr-h1-R	CCACCCACACTAGCATCAGG		
	qVg-F	CACAAGGTTGCTTCTGGCATC	93.8	0.999
	qVg-R	TTGGCCAAAGCTAGAGTAGCC		
	qTub-F	GAGGACACTACACCATCGGC	93.6	0.995
	qTub-R	TCAACAGCGAGGTGAATCCG		
	qEf1α-F	AAGATCGGTTACAACCCGGC	103.8	0.989
	qEf1α-R	TCCTTGCGCTCAATGTTCCA		
RNAi	Met-F	GGATCC*TAATACGACTCACTATAGG*ACATCGACGGCAGGATTATCTACA[b]	n.a.	n.a.
	Met-R	GGATCC*TAATACGACTCACTATAGG*AGTTCTCAATGTCCTTGCAGTCGT		
	EGFP-F	GGATCC*TAATACGACTCACTATAGG*GAGGACGACGGCAACTACAAG	n.a.	n.a.
	EGFP-R	GGATCC*TAATACGACTCACTATAGG*GGTCCATGCCGAGAGTGATCC		

[a]PCR efficiency.
[b]T7 RNA polymerase promoter is indicated with italics; n.a. = not applicable.

Statistical Analysis

All analyses were performed using the GraphPad Prism 8 software package (GraphPad Software Inc., San Diego, CA). The statistical significance of differences in the relative expression of *SfMet* among different developmental stages or tissues was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. The statistical significance of differences in expression levels between adults treated with dsMet and those treated with dsEGFP was assessed with Student's *t*-test. All data are expressed as mean ± SE.

Results

Sequence Data

The ORF sequence of *SfMet* (GenBank accession No. MN229742) is 2,877 bp long, encodes a 958 amino acid protein with a calculated molecular weight (Mw) of 106.96 kDa and a theoretical isoelectric point (pI) of 6.20. *SfMet* is a typical bHLH-PAS transcription factor, containing the bHLH, PAS-A, and PAS-B motifs (Fig. 1). Sequence alignment and identity analysis revealed that *SfMet* has highest amino acid identity with *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) NlMet (73.78%). The neighbor-joining phylogenetic tree indicates that the amino acid sequence of *SfMet* is highly homologous to that of other hemipteran insects (Fig. 2).

Expression of *SfMet*

We profiled the expression of *SfMet* in 1st to 5th instar nymphs and 96-h-old female and male adults. The *SfMet* transcript was detected in all of the above, and there was no significant difference in expression between developmental stages (Fig. 3A). Expression levels, however, increased in female adults in the first 132 h after eclosion (Fig. 3B). Expression of *SfMet* in 96-h-old females was highest in the ovary, followed by the fat body and midgut, and lowest in the thorax (Fig. 3C).

RNAi Knockdown

Newly emerged female adults were injected with dsMet to investigate the function of SfMet in Vg synthesis. Injecting dsMet reduced the expression of *SfMet* 48, 72, and 96 h after injection by 46.6, 51.9, and 45.4%, respectively (Fig. 4A). Injection of dsMet also reduced the expression of *SfKr-h1* 48, 72, and 96 h after injection by 29.1, 34.3, and 52.5%, respectively (Fig. 4B), and *SfVg* mRNA levels by 89.3, 45.5, and 61.1%, respectively, relative to their expression in the dsEGFP control group (Fig. 4C).

Ovaries from both dsMet and dsEGFP treated females were observed 132 h after injection to assess the effect of silencing *SfMet* on ovarian development. Depletion of *SfMet* caused markedly less yolk protein deposition and prevented ovarian development (Fig. 4D).

Discussion

Preventing the premature metamorphosis of larvae and stimulating vitellogenesis in adult females are the two major functions of JH (Riddiford 2008, Li et al. 2019, Santos et al. 2019). The molecular action of JH relies on the intracellular receptor Met, which acts as a JH-activated regulator inducing vitellogenesis in adult females (Smykal et al. 2014, Roy et al. 2018). We successfully cloned the ORF sequence of *SfMet* from *S. furcifera*. Its sequence alignment and predicted structure indicate that it is a homologue of other insect JH intracellular receptors, and that it contains a conserved HLH domain and two variably spaced PAS (PAS-A and PAS-B) domains (Li et al. 2010, Li et al. 2011, Zhang et al. 2019). There is evidence to suggest that the bHLH domain is required for JH III induction of the *Kr-h1* (Cui et al. 2014). PAS-A, another Met domain, is important for dimerization with FISC in *Aedes aegypti* (L.) (Diptera: Culicidae) (Li et al. 2011), and the PAS-B domain of Met is involved in binding to JH, regulating the protein-protein interaction with SRC (Tai/FISC) to form the JH receptor complex and recognize JHRE (Charles et al. 2011, Kayukawa and Shinoda 2015). These features of Met highlight its importance to the action of JH.

We found that *SfMet* is expressed in both nymphs and adults of both sexes in *S. furcifera*. This is not unexpected given that JH is involved in processes that occur throughout an insect's life-span, including mating, foraging, aging, polymorphism, and caste differentiation in social insects (Marchal et al. 2014). In addition, increased transcription of *SfMet* was observed within 132 h of the emergence of adult females. Ovarian development in *S. furcifera* began after eclosion, and oviposition began about 96 h after emergence. We, speculate, therefore, that JH may be involved in ovarian maturation in *S. furcifera*; similar results had been reported in

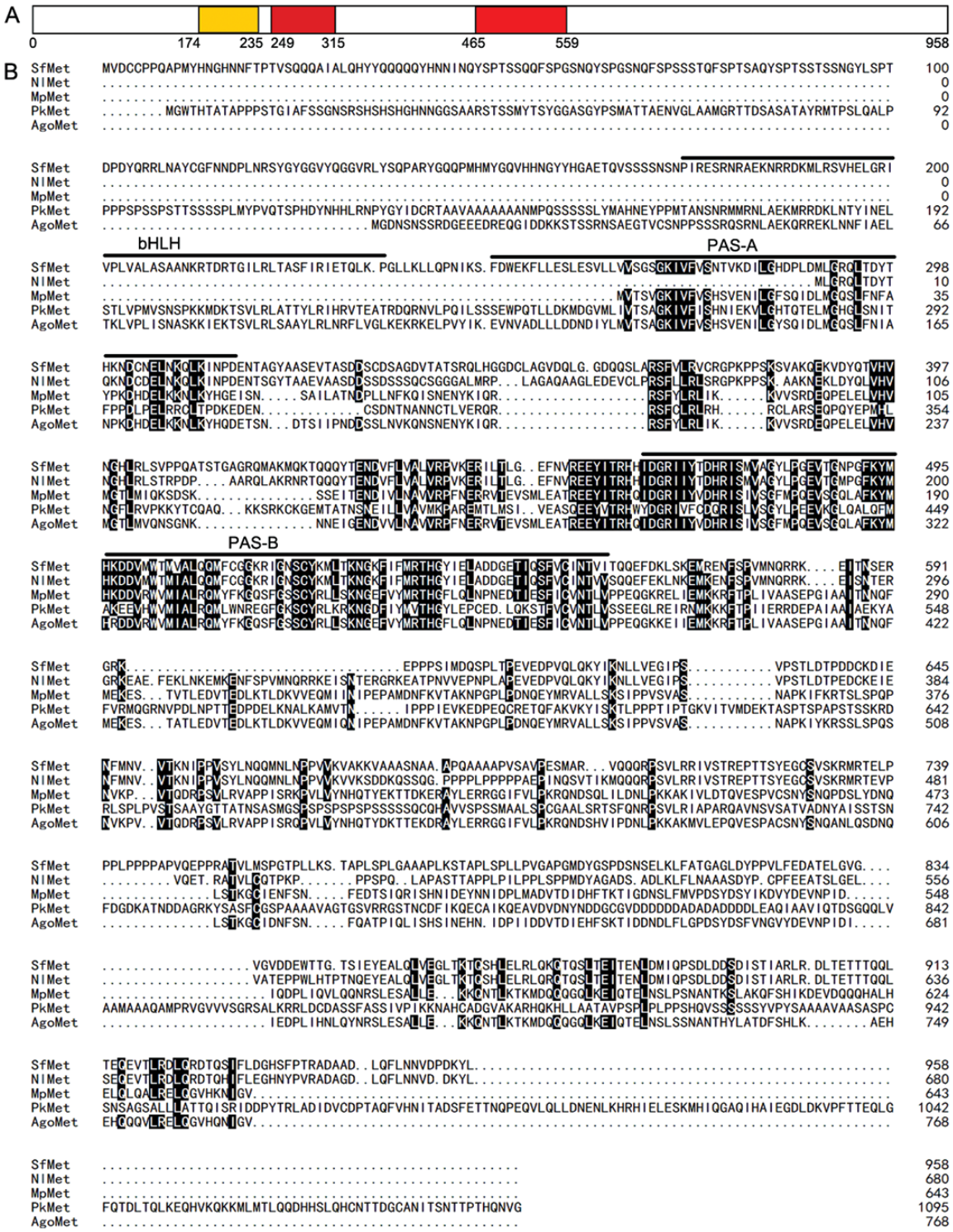

Fig. 1. Identification and sequence analysis of *Sogatella furcifera Methoprene-tolerant* (*SfMet*). (A) Gene structure of SfMet predicted by InterPro tool. (B) Alignment of amino acid sequences of SfMet with those of other hemipteran species; *Nilaparvata lugens* (NlMet, ALT45968.1), *Myzus persicae* (MpMet, AYI50057.1), *Planococcus kraunhiae* (PkMet, BAU79435.1), and *Aphis gossypii* (AgoMet, ANZ54966.1). The bHLH, PAS-A and PAS-B domains are indicated by the black line.

N. lugens (Lin et al. 2015). *SfMet* was also, however, quite highly expressed in the midgut. JH has been found to be synthesized by the gut of adult *Drosophila* (Diptera: Drosophiladae); this gut specific JH regulates the survival and cellular growth of intestinal stem cells and enteroblast populations through the JH receptors Gce/Met and Tai-dependent manner. This local JH plays an important role in damage responses and is necessary for intestinal tumor growth driven by activating mutations in the Wnt and EGFR/Ras pathways (Rahman et al. 2017). However, further research is required to determine whether *S. furcifera* also synthesizes JH in the gut, and whether

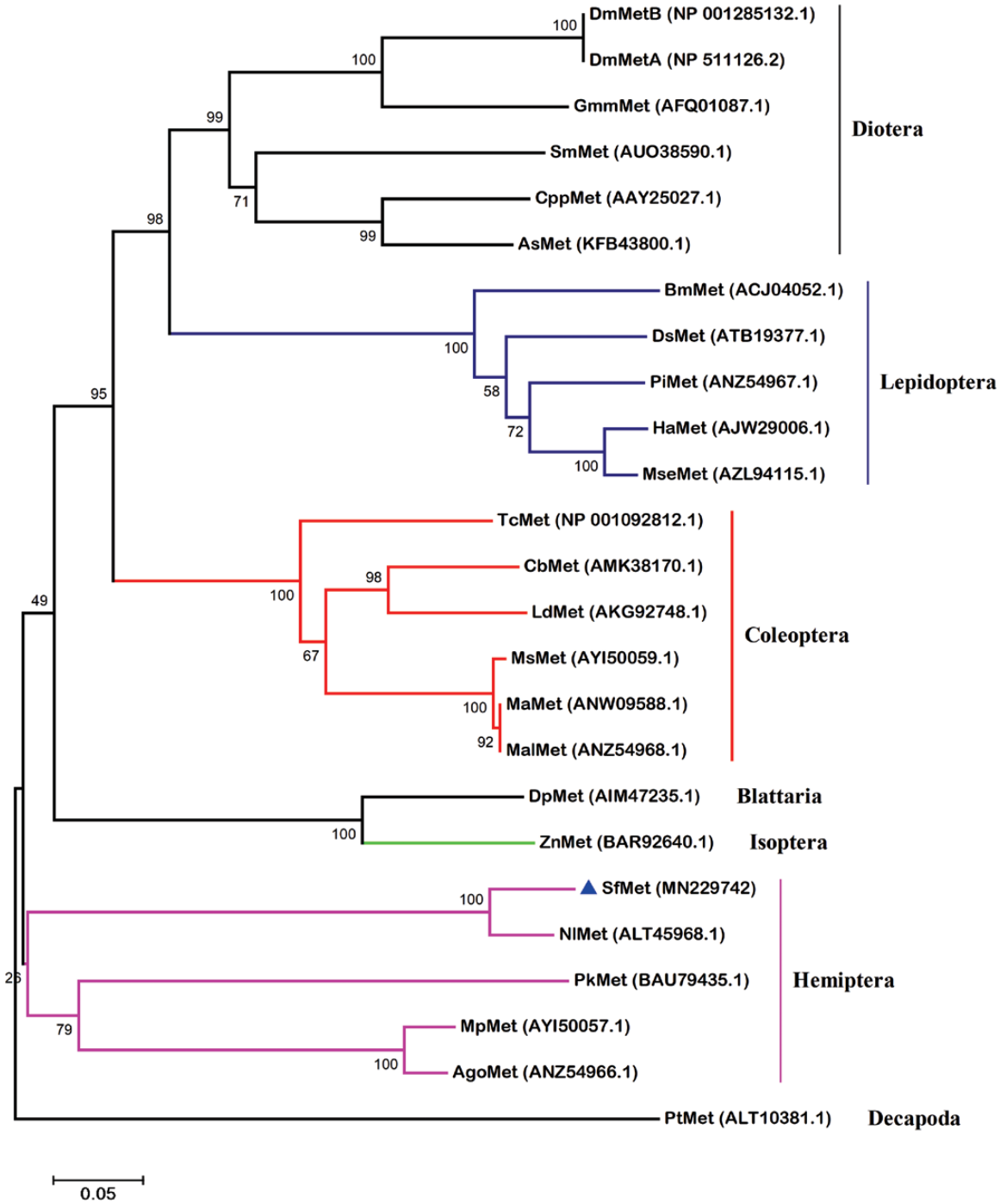

Fig. 2. Neighbor-joining phylogenetic tree of *Sogatella furcifera* SfMet and those in other insects, using *Portunus trituberculatus* (PtMet) as the outgroup. Numbers at the nodes of branches indicate bootstrap values (%) based on 1,000 replicates. The number in parenthesis are the GenBank accession numbers for each species' gene. The blue triangle indicates the protein sequence of SfMet. DmMetA and DmMetB, *Drosophila melanogaster*; GmmMet, *Glossina morsitans morsitans*; SmMet, *Sitodiplosis mosellana*; Cppmet, *Culex pipiens pipiens*; AsMet, *Anopheles sinensis*; BmMet, *Bombyx mori*; DsMet, *Dendrolimus spectabilis*; PiMet, *Plodia interpunctella*; HaMet, *Helicoverpa armigera*; MseMet, *Mythimna separate*; TcMet, *Tribolium castaneum*; CbMet, *Colaphellus bowringi*; LdMet, *Leptinotarsa decemlineata*; MsMet, *Monochamus saltuarius*; MaMet and MalMet, Monochamus alternatus; DpMet, *Diploptera punctate*; ZnMet, *Zootermopsis nevadensis*; NlMet, *Nilaparvata lugens*; PkMet, *Planococcus kraunhiae*; MpMet, *Myzus persicae*; AgoMet, *Aphis gossypii*.

the relatively high level of *SfMet* in the midgut plays the same role as it does in *Drosophila*.

Vitellogenesis is the central processes of female reproduction and JH is the leading hormone inducing vitellogenesis in many insects (Parthasarathy et al. 2010, Zhu and Noriega 2016, Santos et al. 2019). The JH receptor complex formed by Met and its partner SRC (Tai/FISC/Gce) is the central part of the JH signaling pathway (Roy et al. 2018). Our results show that *SfMet* is highly expressed in the fat body and ovary of adult female *S. furcifera*. Furthermore, knockdown of *SfMet* significantly reduced *SfVg* and reduced yolk protein deposition in oocytes, preventing normal ovarian development. These results demonstrate that JH plays an important role in

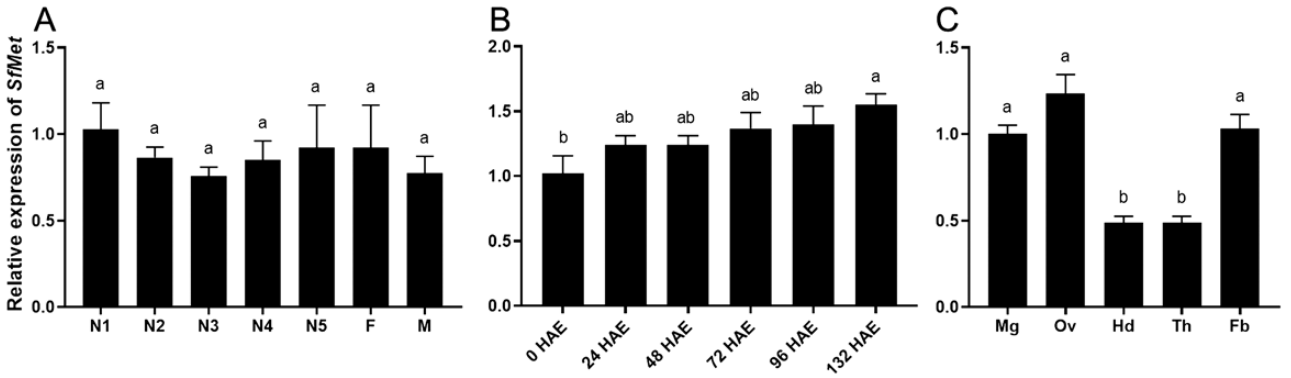

Fig. 3. Tissues and temporal expression profiles of *SfMet*. (A) Relative expression levels of *SfMet* in the first to fifth nymphal instars (N1, N2, N3, N4, N5), female (F) and male adults (M). (B) Relative expression levels of *SfMet* in females at different times after emergence. HAE, hours after emergence. (C) Relative expression levels of *SfMet* in various tissues of females. Mg, midgut; Ov, ovary; Hd, head; Th, thorax; Fb, fat body. Bars indicate the mean (±SE) of three biological replicates. Different letters above bars represent significant differences (ANOVA followed by Tukey's test, $P < 0.05$).

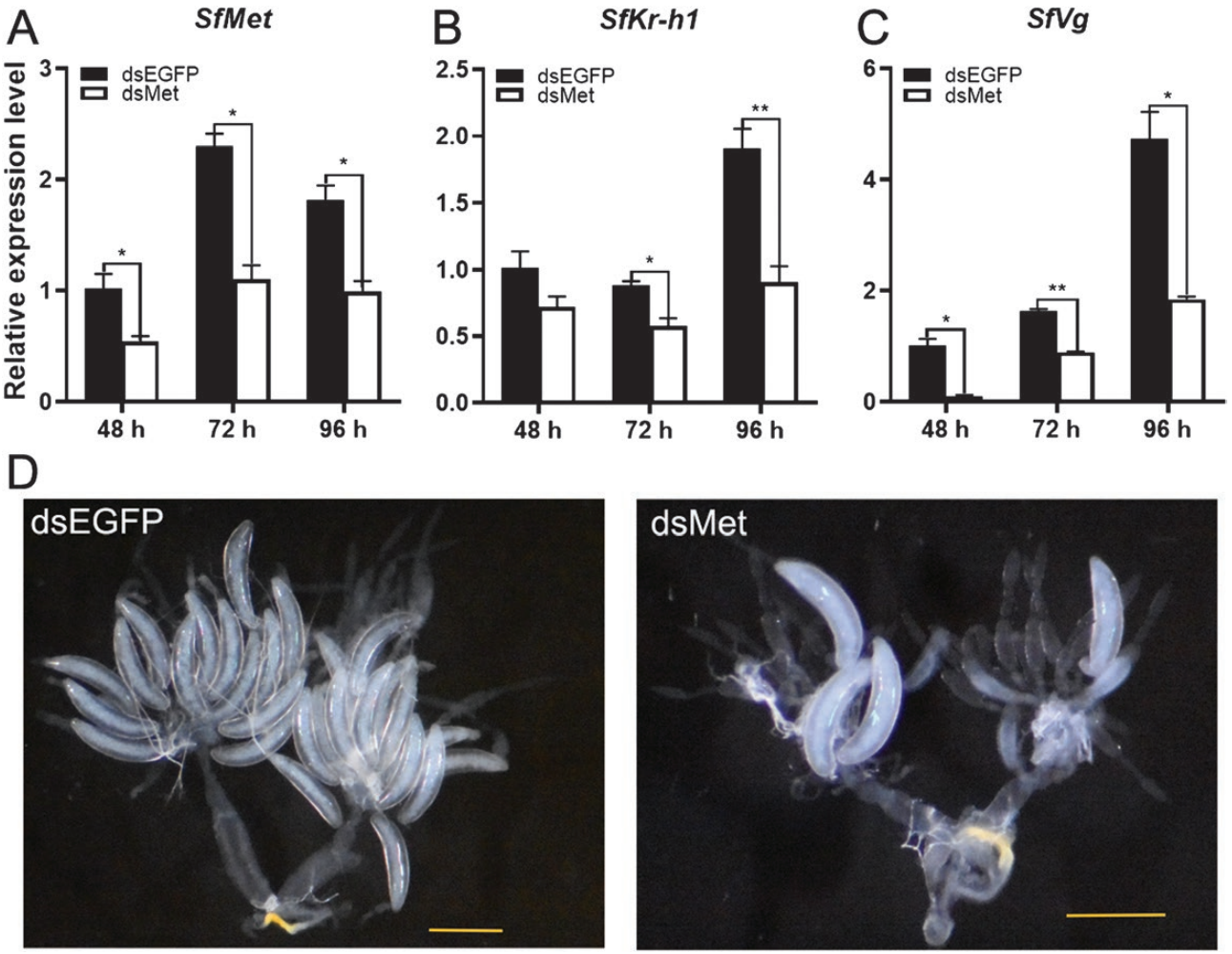

Fig. 4. Functional analysis of SfMet in the ovarian development of female *Sogatella furcifera*. Expression levels of *SfMet* (A), *SfKr-h1* (B), and *SfVg* (C) in whole bodies of females 48, 72, and 96 h after injected with dsMet and dsEGFP. (D) Effect of *SfMet* RNAi on ovarian development with dsEGFP as a control. Ovaries were dissected 132 h post-injection. Scale bar, 0.5 mm. Asterisks represent values statistically different from the EGFP dsRNA group (*t*-test: $*P < 0.05$, $**P < 0.01$).

vitellogenesis via Met in *S. furcifera*. Studies on other insects have also demonstrated that Met is involved in JH-regulated reproduction. For instance, depletion of *HaMet* reduced *Vg* transcription and prevented ovarian development in *H. armigera*, and treatment with a JH analog did not restore *Vg* expression (Ma et al. 2018). These results have been corroborated in *Locusta migratoria* (L.) (Orthoptera: Acrididae) (Song et al. 2014), *Pyrrhocoris apterus* (L.) (Heteroptera: Pyrrhocoridae) (Smykal et al. 2014) and *T. castaneum* (Parthasarathy et al. 2010).

The nucleic complex formed by Met and Tai (SRC/FISC) interacts with promoters of the downstream gene *Kr-h1* called JH response elements (JHREs) (Kayukawa et al. 2012). Kr-h1 has been found to play a crucial role in regulating vitellogenesis and oogenesis (Zhang et al. 2018, Santos et al. 2019). Our results show that knockdown of *SfMet* reduced the transcript level of *SfKr-h1*. Therefore, we speculate that SfKr-h1 transduces JH signaling during vitellogenesis and also regulates the synthesis of Vg and ovarian development in *S. furcifera*. Indeed, previous research has demonstrated that depletion of *LmMet* reduced the expression of *LmKr-h1*, and that silencing *LmMet* or *LmKr-h1* can stop oocyte maturation and arrest ovarian development in *L. migratoria*. These results indicate that the JH-Met-Kr-h1 signaling pathway is involved in reproduction in *L. migratoria* (Song et al. 2014). Other studies suggest that the JH-Met-Kr-h1 signaling pathway is conserved in insects, presumably because of its universal role in regulating female reproductive development (Marchal et al. 2014, Yue et al. 2018, Gijbels et al. 2019).

In summary, our results demonstrate that the depletion of *SfMet* suppresses *Vg* expression in the fat body and prevents oocyte development and maturation in *S. furcifera*. This JH-mediated signal conveyed by *SfMet* may be transduced by SfKr-h1 in *S. furcifera*. These findings illustrate the key role of *SfMet* in reproduction in *S. furcifera*, and suggest that targeting *SfMet* could be an effective way of controlling this pest.

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