RNAi Reveals Role of Insulin-Like Androgenic Gland Hormone 2 (IAG2) in Sexual Differentiation and Growth in Hermaphrodite Shrimp

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Insulin-like androgenic gland hormone (IAG) is the most widely known hormone that regulates sexual differentiation in crustaceans. Previously, a transcriptome study described two transcripts of IAGs (Lvit-IAG1 and Lvit-IAG2) in the peppermint shrimp Lysmata vittata, a species characterized by a rare reproductive system of protandric simultaneous hermaphroditism (PSH). Herein, we explored the function of Lvit-IAG2 via RNA interference (RNAi) experiments, and then compared the functional differences between the two IAGs. We demonstrated that Lvit-IAG2 positively regulated the expression of crustacean hyperglycemic hormone (Lvit-CHH) in eyestalk ganglion but exhibited no significant effect on the expression of gonad-inhibiting hormone (Lvit-GIHs) and crustacean female sex hormone (Lvit-CFSHs). Besides, Lvit-IAG2 gene knockdown caused degeneration in appendices masculinae (AM) and suppressed germ cells at the secondary spermatocyte stage. Moreover, silencing the Lvit-IAG2 gene impeded ovarian development, including smaller previtellogenic oocytes, and lower expression of vitellogenin (Lvit-Vg) gene in hepatopancreas and vitellogenin receptor (Lvit-VgR) gene in the ovarian region. Notably, silencing the Lvit-IAG2 gene impeded individual growth of the species. Collectively, findings from this study demonstrate that Lvit-IAG2 and Lvit-IAG1 coordinatively function to modulate sexual differentiation in L. vittata; meanwhile, Lvit-IAG2 stimulates the growth of the PSH species.

Keywords: sexual differentiation, IAG, PSH, reproductive endocrine, crustacean

INTRODUCTION

Gonochorism is the most common reproductive strategy in decapod crustacean species (Juchault, 1999). Males and females have significantly different reproductive systems, as well as external features (Nagaraju, 2011). For instance, the reproductive system of male American lobster Homarus americanus consists of the paired testes, vas deferens, spermiducts, and gonopores located on the fifth pair of walking legs. Besides, the specialized first pair of pleopods serves as a gonopod during mating (Comeau and Benhalima, 2018b). However, the female reproductive system of H. americanus consists of the paired ovaries, oviducts, and gonopores located on the third pair of walking legs. The specialized organ, seminal receptacle, arises on the thoracic ventral side between the fourth and the fifth pairs of walking legs (Comeau and Benhalima, 2018a).
Interestingly, a review of the literature shows that Caridean shrimps exhibit several other protandry sexual systems apart from gonochorism (Bauer, 2000). Among them, strictly sequential protandric hermaphroditism (SPH) and protandric simultaneous hermaphroditism (PSH) are well-studied. For example, Northern spot shrimp *Pandalus platyceros*, a SPH species, first mature as functional males with hermaphroditic gonads, and then go to a transitional phase followed by a functional female phase (Levy et al., 2020b). Meanwhile, all the *Lysmata* species with the PSH sexual system, eventually acquire reproductive functions for both males and females following the male phase and a short transitional phase (Bauer, 2000; Chen et al., 2019).

As with insect, sexual differentiation is controlled by key reproductive hormones in decapods. It is generally believed that insulin-like androgenic gland hormone (IAG) regulates male sexual differentiation in dioecious decapods (Ventura et al., 2009). In decapod species, IAG was first identified in the androgenic gland (AG) of the red-claw crayfish *Cherax quadrispinosus* in 2007 (Manor et al., 2007). Since then, numerous studies have found IAG in dioecious (Ventura et al., 2009; Rosen et al., 2016; Ventura et al., 2012; Chung, 2014; Huang et al., 2014), parthenogenetic (Levy et al., 2017), and hermaphroditic (Zhang et al., 2017; Levy et al., 2020a; Liu et al., 2020) species. Furthermore, its critical role in sexual differentiation has been properly studied via loss-of-function experiments (Ventura et al., 2012; Zmora and Chung, 2014; Priyadarshi et al., 2017; Fu et al., 2020). For instance, in the giant freshwater prawn, *Macrobrachium rosenbergii*, IAG gene silencing induced degeneration of male primary and secondary characteristics (Ventura et al., 2009), and feminization in young males (Ventura et al., 2012). Silencing IAG gene could also feminize male-related phenotypes in the intersex red-claw crayfish *C. quadrispinosus* (Rosen et al., 2010) and the male Chinese mitten crab *Eriocheir sinensis* (Fu et al., 2020). Moreover, augmentation of IAG hormone transcripts in adult *M. rosenbergii* males resulted in significantly higher (orange claw) BC transformations and vice versa (Priyadarshi et al., 2017). Considering its universal and pivotal role as a master regulator of crustacean sexual development, IAG is regarded as the sexual “IAG-switch” in decapod crustacean (Levy and Sagi, 2020).

Crustacean female sex hormone (CFSH) is a neurohormone that is closely related to female sexual differentiation in decapods. It was first isolated from the eyestalk ganglion in the Atlantic blue crab *Callinectes sapidus*, and was found to be pivotal in the development of the female’s mating and egg brooding systems, including the gonopores and ovigerous setae (Zmora and Chung, 2014). Similar functions have also been established in the mud crab *Scylla paramamosain* (Jiang et al., 2020). Besides, CFSH was also reported as an inhibitor of IAG, as recombinant Sp-CFSH protein significantly suppressed Sp-IAG expression in the AG explants of the mud crab *S. paramamosain in vitro* (Liu et al., 2017).

In crustacean males, previous studies have revealed that the X-organ-sinus gland complex (XO-SG) in the eyestalk ganglion, a major source of neuropeptides, modulates reproductive functions via the eyestalk-AG-testicular axis (Khalaila et al., 2002; Nagaraju, 2011). Besides, its inhibitory effects on AG development and IAG expression have been intensively explored through eyestalk ablation. The active components suppressing AG development have been identified in XO-SG. Among them, researchers have comprehensively characterized the crustacean hyperglycemic hormone (CHH) superfamily neuropeptides as well as CFSH mentioned above. Most members of the CHH superfamily exerted inhibitory effects on either AG development or IAG expression (Das et al., 2015; Li et al., 2015; Guo et al., 2019). Following the silencing of gonad-inhibiting hormone (GIH), molt-inhibiting hormone (MIH), and CHH gene, IAG transcription level increased significantly in the black tiger prawn *Panaeus monodon* (Das et al., 2015), the oriental river prawn *Macrobrachium nipponense* (Li et al., 2015), and the Pacific white shrimp *Panaeus (Litopenaeus) vannamei* (Guo et al., 2019).

To date, however, there are rare reports elaborating sexual differentiation mechanism mediated by IAG in protandric crustaceans. In the SPH shrimp *P. platyceros*, *Pnp-IAG* knockdown elevated vitellogenin gene expression in the hepatopancreas and transformation of the gonad from ovotestis to ovary (Levy et al., 2020a). Zhang et al. (2017) identified an IAG transcript (*Lw-IAG*) in a PSH shrimp *Lysmata wurdemanni*, and examined its expression profile during gonadal development. The very low but detectable expression of *Lw-IAG* during the euhermaphroditic phases suggested that *Lw-IAG* was possibly responsible for the maintenance of the male reproductive activity in euhermaphroditic phase. Recently, a transcriptomic study identified two insulin-like peptide (ILP) transcripts (*Lv-it-IAG1* and *Lv-it-IAG2*) in the peppermint shrimp *Lysmata wurdemanni* (Bao et al., 2020), and biological functions of *Lv-it-IAG1* have been explored in detail (Liu et al., 2020). In *L. wurdemanni*, silencing *Lv-it-IAG1* impeded development of male-related phenotypes (appendices masculinae (AM) and male gonopores) while suppressing the germ cells at the primary spermatocyte stage, demonstrating that *Lv-it-IAG1* was indeed a functional IAG. Meanwhile, *Lv-it-IAG1* was also suggested to regulate the ovarian development by inhibiting *Lv-it-GIHs* and *Lv-it-CFSHs* expression in the eyestalk ganglion (Liu et al., 2020). In decapods, four types of insulin and related peptides were identified: insulin-like androgenic gland hormone (IAG), insulin, relaxins, and gonadulins (Veenstra, 2020). Besides, there is usually only one IAG gene identified in a species (Li et al., 2012; Huang et al., 2017b). Therefore, further in-depth studies are warranted to explore whether *Lv-it-IAG2* is another functional IAG or other insulin and related peptides.

The peppermint shrimp *L. vittata*, a relatively small caridean species (maximum carapace length < 1 cm), like other species, displays a unique sexual system, PSH, whereby individuals first mature as males; however, with increasing age and size, they acquire reproductive functions for both males and females (Bauer, 2000; Alves et al., 2019). Given its impressive reproductive fecundity, short generation time, and relatively clear ontogenetic gonad development staging system, *L. vittata* is a good model organism for exploring the molecular mechanisms for endocrine regulation of the unique PSH sexual system in crustaceans (Chen et al., 2019).
In this study, we continuously explored the putative function of Lnvt-IAG2 in *L. vittata*. Following its similar spatial and temporal expression profiles with Lnvt-IAG1, we hypothesized that Lnvt-IAG2 and Lnvt-IAG1 exert similar biological functions and they coordinatively regulate sexual differentiation of the species. To validate this hypothesis, we performed both short-term and long-term gene knockdown via RNA interference (RNAi). In addition to morphological characteristics, expression levels of gonadal development-associated genes [GIH, vitellogenin (Vg), and vitellogenin Receptor (VgR)], carbohydrate metabolism (CHH), and sexual differentiation (CFSH) were assessed via qRT-PCR.

**MATERIALS AND METHODS**

**Animals**

The experimental animals (*L. vittata*) were captive-bred at the Fisheries Research Institute of Fujian Province in Xiamen city, China. They were then acclimated in seawater aquaria for 2 days under these conditions: temperature of 25.5 ± 0.5°C and salinity of 32 ± 1 PSU. During this period, the animals were fed on a commercially formulated shrimp diet daily. A recent study from this laboratory had described two developmental phases covering four gonadal development stages for *L. vittata*, which we followed to define the gonadal stages in the present experiment. Three gonadal development stages (Stage I: ovarian region is smaller than testicular region and both regions are transparent; Stage II: both regions are cloudy white and bigger than the testicular region while testicular region is still cloudy white) are included in the male phase, during which the testicular part of the gonad becomes sequentially mature while the ovarian part is still immature. Both testicular region and ovarian region are fully developed and filled with mature germ cells in the euhermaphroditic phase (Stage IV), during which individuals acquire reproductive functions for both males and females (Chen et al., 2019). Animal handling and experimental procedures were performed with strict adherence to the guidelines approved by the Xiamen University Animal Care and Use Committee.

**Fragment Cloning of Lnvt-IAG2**

The total RNA was extracted from the androgenic gland at gonadal development stage I using HiPure Universal RNA Kit (Magen) following protocol stipulated by the manufacturer. The first-strand cDNA was generated from 1 µg total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas). To verify the accuracy of the predicted open reading frame (ORF), we performed both short-term and long-term gene knockdown via RNA interference (RNAi). The primers used for fragment cloning and dsRNA preparation were designed based on the JTT matrix-based model in MEGA7. The Maximum Likelihood method with 1,000 bootstrap replicates based on the PHYML program was applied to generate a phylogenetic tree entailing the deduced amino acid sequence alignments and excluding signal peptide of insulin-related peptides. IAG sequences were shown in Table 2.

1 https://www.ncbi.nlm.nih.gov/orffinder/
2 http://www.cbs.dtu.dk/services/SignalP/
3 http://clavius.bc.edu/~clotelab/DiANNA/
4 https://www.ebi.ac.uk/OsloTools/msa/clustalo/
5 http://www.cbs.dtu.dk/services/NetNGlyc/

| Primer | Sequence (5′–3′) | Application |
|--------|------------------|-------------|
| IAG2F  | TAACCGAAGAACACTCAGCTGAAATTG3 | Fragment validation |
| IAG2R  | ACGCTGTAGCCAGAAGCGATCGACC | RT-PCR/qRT-PCR |
| CFSh1QF | ATCCACACCCTCAGAATCTCATC | |
| CFSh1QR | GCAAGGCTACGGTTATCT | |
| CFSh2QF | CAAGGACCGCGATGATGA | |
| CFSh2QR | GCGGAAGATCTCGAGTGTGA | |
| IAG10QF | CTAATCCTCTGCGTCTTACTCAC | |
| IAG1QR | GCGCTGCTTCGTTCTAATACGTG | |
| IAG2QF | TGAACGACCACCTCCTATGT | |
| CHHOF  | CATCTATGACGCGTATCTTTT | |
| CHHQF  | TACTGGCGAOCATCTGA | |
| GhH1QF | GACTTCTGCTGTCGCTGTTA | |
| GhH1QR | GCTCGAATGTCTGCTATGQA | |
| GhH2QF | ATAGGCGCTGCTGTTTCTG | |
| GhH2QR | GAACTGACCGAGACTACATT | |
| VgQF  | GCCGAGGGTGCAGCGAGAAG | |
| VgQR  | ATCCACCGTAGAGGAGTAAGGG | |
| VgRQF | CTCGGTCTGCGAACCTCA | |
| VgRQR | GTCTGTGCTGGAAGATQGA | |
| actinF | GTCAAGCTCACTGATTACC | |
| actinR | GCTTACCCGATATGCTTACTAC | |
| IAG2dsF | CTCGCTTAACACTGCTGCTCAGGACTCTA | dsRNA synthesis |
| IAG2dsR | ATACCCGCTCTGAGAATTTCCA | |
| GFPdsF | TATGCGTGCTGATAGGCGTGTG | |
| GFPdsR | GGTCCGGAGTGGCGGTGAG | |

**TABLE 1** Summary of primers used in this study.
TABLE 2 | Summary of IAG sequences used in multiple sequence alignment and phylogenetic analysis.

| Sequence | Species | GenBank accession number |
|----------|---------|-------------------------|
| Poh-IAG1 | Penaeus chinensis | AFU60548.1 |
| Poh-IAG2 | Penaeus chinensis | AFU60549.1 |
| Pm-IAG  | Penaeus monodon   | ADA67878.1 |
| Pv-IAG  | Penaeus vannamei  | ARO09497.1 |
| Py-IAG  | Penaeus japonicus | BAK20460.1 |
| Jv-IAG  | Jasus edwardsii   | AIMS5892.1  |
| Sv-IAG  | Sagmariaus verreauxi | AHY99679.1 |
| Cq-IAG  | Cherax destructor | ACD91988.1  |
| Cqua-IAG | Cherax quadricarinatus | ABH07705.1 |
| Pc-IAG  | Procambarus clarkii | ALX72789.1 |
| Pn-IAG  | Procambarus fallax | AKW43106.1 |
| Lvit-IAG1 | Lysmata vittata  | MT114196    |
| Lvit-IAG2 | Lysmata vittata  | MT114197    |
| Es-IAG  | Eriocheir sinensis | AVK43106.1  |
| Cqui-IAG | Chaceon quinquedens | ASA45642.1 |
| Sp-IAG  | Scylla paramamosain | AFY09905.1 |
| Cs-IAG  | Callinectes sapidus | AEI72263.1 |
| Pp-IAG  | Pandalus platyceros | ASM4212.1 |
| Ppau-IAG | Palaemon pacificus | BAJ84109.1 |
| Mi-IAG  | Macrobrachium lar  | BAJ78349.1  |
| Mv-IAG  | Macrobrachium vollenhovenii | AHZ49275.1 |
| Lw-IAG  | Lysmata wurdemanni | 10.1371/journal.pone.0172782 (Zhang et al., 2017) |
| Mn-IAG  | Macrobrachium nipponense | AHA33398.1 |
| Mh-IAG  | Macrobrachium rosenbergii | AWU67706.1 |

TABLE 3 | Summary of qPCR efficiency of the primer pairs.

| Gene | Primer pairs | Annealing temperature (°C) | Amplification efficiency (%) |
|------|--------------|----------------------------|-----------------------------|
| Lvit-IAG1 | IAG1QF/IAG1QR | 58.5 | 98.9 |
| Lvit-IAG2 | IAG2QF/IAG2QR | 58.5 | 104.5 |
| Lvit-CFSH1 | CFSH1QF/CFSH1QR | 58.5 | 99.1 |
| Lvit-CFSH2 | CFSH2QF/CFSH2QR | 58.5 | 103.2 |
| Lvit-GIHI | GIHIQF/GIHIQR | 58.5 | 98.8 |
| Lvit-GIHI2 | GIHI2QF/GIHI2QR | 58.5 | 98.5 |
| Lvit-CHH | CHHQR/CHHQR | 58.5 | 101.7 |
| Lvit-Vg | VgQR/VgQR | 58.5 | 98.3 |
| Lvit-VgA | VgAQR/VgAQR | 58.5 | 101.8 |
| Lvit-β-actin | actinQF/actinQR | 58.5 | 101.2 |

Insulin, relaxins, and gonadulins sequences were borrowed from previous works by Veenstra (2020).

The qRT-PCR Assays

Primers used for quantitative real-time PCR (qRT-PCR) were designed by the Beacon Designer 8 software. RT-PCR products were sequenced as described in section “Fragment cloning of Lvit-IAG2” for accuracy. The melting curves were subjected to intensive analysis to ensure primer specificity. A standard curve was used to calculate the amplification efficiency of each primer pair. Notably, each selected primer pair exhibited a suitable PCR amplification efficiency (96.3–104.5%, see Table 3). The first-strand cDNA was generated from 300 ng total RNA using TransScript® II One-Step gDNA Removal and cDNA short SuperMix Kit (TransGen). The cDNA was diluted to four folds using RNase-free water before it was utilized in qRT-PCR detection. Components including, 10 μl TB Green Premix Ex Taq II (2X) (TaKaRa), 2 μl diluted cDNA, 0.5 μl forward/reverse primer (1 mM), and 7 μl RNase-free water, were used for a 20 μl qRT-PCR reaction system. The reaction was performed using 7500 Real-Time PCR (Applied Biosystems) with the following steps: 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, 58.5°C for 15 s, and 72°C for 30 s. The result was calculated using the 2^−ΔΔCt method, Lvit-β-actin (GenBank accession number: MT114194) as the reference gene.

Tissue Expression Profile of Lvit-IAG2 in L. vittata

As described in section “Fragment cloning of Lvit-IAG2,” total RNA was extracted from various tissues (eyestalk ganglion, brain, thoracic ganglion, abdominal ganglion, ovary, testis, AG, hepatopancreas, stomach, intestine, heart, gill, and muscle). The first-strand cDNA synthesis was performed as described in section “The qRT-PCR assays.” RT-PCR tissue expression profile detection was conducted under the following conditions: 95°C for 3 min; 35 cycles of 95°C for 30 s, 58.5°C for 30 s and 72°C for 30 s, followed by 72°C for 5 min final extension. Meanwhile, Lvit-β-actin was amplified as a positive control, with similar PCR conditions as described above. RT-PCR products were examined using 1.5% agarose gel, then images taken by a UV detector (Geldoc, Thermo Fisher Scientific).

Expression Profiles of Lvit-IAG2 and Lvit-IAG1 During Gonadal Development

The AGs of L. vittata at different gonadal development stages (I–IV) (n = 5) were obtained. This was followed by RNA extraction, the first-strand cDNA synthesis, and qRT-PCR analysis as described in sections “Fragment cloning of Lvit-IAG2” and “The qRT-PCR assays.”

dsRNA Preparation

Lvit-IAG2 fragment encoding C peptide and A chain and green fluorescent protein gene (GFP) (exogenous gene control) fragment were cloned into pGEM-T Easy Vector (Promega). dsRNA synthesis was performed using T7 RNA Polymerase (Takara) and SP6 RNA Polymerase (TaKaRa) following the standard protocols. Finally, dsRNA was diluted with 10 mM phosphate-buffered saline (PBS, pH 7.4).

Short-Term Silencing Experiment in vivo

The efficacy of gene knockdown via RNAi was evaluated by a short-term silencing experiment carried out with L. vittata at gonadal development stage I. A total of 15 shrimp (carapace
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RESULTS

Sequence Analysis of *Lvit-IAG2*

A schematic diagram of preproprotein of *Lvit-IAG2* was depicted in Figure 1A. The *Lvit-IAG2* (GenBank accession number: MT114197) coding region was 441-bp in length and encoded a 146-aa polypeptide, including a 27-aa signal peptide, 36-aa B chain, 45-aa C peptide, and 38-aa A chain. We predicted 6 conserved cysteine residues in the B chain (C$_{B12}$ and C$_{B23}$) and A chain (C$_{A13}$, C$_{A14}$, C$_{A19}$, and C$_{A27}$). The predicted mature *Lvit-IAG2* peptide comprised the B and A chains with two interchain disulfide bonds (between C$_{B12}$ and C$_{A14}$, C$_{B23}$, and C$_{A27}$, respectively) and an intrachain disulfide bond (between C$_{A13}$ and C$_{A19}$).

Homology and Phylogenetic Analysis

Multiple sequence alignment of the putative B chain and A chain of IAGs from decapod species was highlighted in Figure 1B. Notably, 6 cysteine residues forming two interchain disulfide bonds (between C$_{B15}$ and C$_{A18}$, C$_{B26}$ and C$_{A37}$, respectively) and an intrachain disulfide bond (between C$_{A18}$ and C$_{A28}$) were fully conserved among decapod species. Besides, *Lvit-IAG2* shared the highest identity with *Lvit-IAG1* (58.82% for B chain and 44.12% for A chain, respectively). Based on phylogenetic analysis, insulin and related peptides formed four major clades: IAG, insulin, gonadulin, and relaxin. Meanwhile, the IAGs in decapods formed three subclades: (i) The family Caridea; (ii) sequences from the family Brachyura; (iii) the families Astacidea, Achatelata, and Penaeoidae (Figure 2). We classified *Lvit-IAG2* into the subclade containing the family Caridea.

Spatial and Temporal Expression Profiles of *Lvit-IAG2*

To explore the spatial distribution profiles of *Lvit-IAG2*, RT-PCR was performed on *L. vittata* at gonadal development stage I. Results demonstrated exclusive expression of *Lvit-IAG2* in the AG (Figure 3A). The relative expression of *Lvit-IAG2* in the AG during gonadal development was also assessed through qRT-PCR (Figure 3B). Notably, the expression levels of *Lvit-IAG2*...
reached a peak at stage I, decreased sharply at stage II, and were continuously maintained at low levels at stages III and IV \( [F_{(3, 16)} = 19.797, p < 0.05] \). Similar trend was observed in \textit{Lvit-IAG1} expression profiles except that the sharp decrease presented at stage III \( [F_{(3, 16)} = 161.408, p < 0.05] \), which was consistent with results of the former study (Liu et al., 2020).

**FIGURE 1** | Sequence analysis of \textit{Lvit-IAG2}. (A) Schematic diagram of preproprotein of \textit{Lvit-IAG2} containing a signal peptide, B chain, C peptide, and A chain. The six cysteine residues of B chain and A chain were predicted to form three putative disulfide bridges connected with lines. (B) Multiple sequence alignment of putative B chain and A chain of decapod IAGs. The sequence information was given in Table 2. Six conserved cysteine residues were boxed in red, and three putative disulfide bridges were connected with lines. Predicted N-glycosylation sites were marked by gray shadow. \textit{Lvit-IAG1} was marked with solid arrows.
Short-Term Silencing Experiment in vivo

Results demonstrated that the transcript level of Lvit-IAG2 was explicitly inhibited up to 77% \( F(2, 12) = 22.851, p < 0.05 \). Meanwhile, Lvit-IAG2 knockdown significantly suppressed the expression of Lvit-CHH \( F(2, 12) = 113.857, p < 0.05 \). On the contrary, no significant difference in Lvit-IAG1 \( F(2, 12) = 0.173, p > 0.05 \), Lvit-CFSH1 \( F(2, 12) = 0.876, p > 0.05 \), Lvit-CFSH2 \( F(2, 12) = 2.270, p > 0.05 \), Lvit-GIH1 \( F(2, 12) = 0.687, p > 0.05 \), and Lvit-GIH2 \( F(2, 12) = 1.378, p > 0.05 \) expression was found (Figure 4).

Long-Term Silencing Experiment in vivo

Effects of Lvit-IAG2 Silencing on Gene Expression

At the end of the 30 day long-term trial, the efficacy of gene knockdown was evaluated. Compared to the PBS treatment, Lvit-IAG2 transcript was 95% inhibited \( F(2, 13) = 172.195, p < 0.05 \). Besides, Lvit-IAG2 knockdown not only significantly inhibited the expression of Lvit-CHH in the eyestalk ganglion \( F(2, 13) = 38.197, p < 0.05 \) but also repressed Lvit-Vg expression in the hepatopancreas \( F(2, 13) = 6.851, p < 0.05 \) and Lvit-VgR expression in the ovarian region \( F(2, 13) = 9.605, p < 0.05 \). However, we found no significant difference in the expression levels of Lvit-CFSH1 \( F(2, 13) = 0.953, p > 0.05 \), Lvit-CFSH2 \( F(2, 13) = 1.129, p > 0.05 \), Lvit-GIH1 \( F(2, 13) = 1.333, p > 0.05 \), and Lvit-GIH2 \( F(2, 13) = 1.249, p > 0.05 \); similar results were reported with short-term silencing experiment (Figures 4, 5).

Effects of Lvit-IAG2 Silencing on Growth and Development of Sexual Characteristics

After a 30 day long-term experiment, we recorded the average carapace length and bodyweight of the shrimps. Shrimps from the Lvit-IAG2 silencing treatment group (4.27 ± 0.05 mm, 90.05 ± 3.08 mg) were much smaller compared to those of the PBS (4.83 ± 0.05 mm, 144.78 ± 3.04 mg) or dsRNA GFP (4.96 ± 0.05 mm, 147.87 ± 4.33 mg) treatment groups [carapace length: \( F(2, 13) = 7.619, p < 0.05 \); body weight:
F(2, 13) = 11.082, p < 0.05 (Figures 6A,B). Moreover, changes in male and female sexual characteristics were documented at the end of the experiment through photography (Figure 7). Notably, Lvit-IAG2 gene knockdown retarded appendices masculinae (AM) development [F(2, 13) = 24.824, p < 0.05] (Figures 6C, 7). No significant difference in female characteristics (female gonophores) and other male sexual characteristics (male gonophores and cincinnuli) was observed (Figure 7).

Effects of Lvit-IAG2 Silencing on Gonadal Development

We examined the morphological and histological characteristics of gonads following Lvit-IAG2 knockdown; notably, gene silencing resulted in abnormal development of gonad (Figure 8). In the testicular regions, different compositions of germ cell types were reported across the three treatments. For both GFP-injected and PBS-injected control, active spermatogenesis occurred (evident from the abundant spermatogonia, spermatid, and spermatozoa found in the testicular region) (Figures 8D,E). However, with Lvit-IAG2 silencing, a large number of secondary spermatocytes and little spermatid were found, demonstrating arrested spermatogenesis (Figure 8F). Besides, the ovarian region was much smaller (Figure 8C), and in terms of the arrangement, germ cells were tighter and more disordered compared to those of the controls (Figure 8I). Additionally, the oocyte size significantly decreased following Lvit-IAG2 knockdown [F(2, 13) = 67.576, p < 0.05] (Figures 6D, 8I).

**DISCUSSION**

Following a previous transcriptome study, two ILP transcripts, Lvit-IAG1 and Lvit-IAG2, exist in the PSH shrimp, L. vittata
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FIGURE 5 | Effects of long-term Lvit-IAG2 silencing on gene expression of L. vittata. The effectiveness of gene knockdown in the long-term Lvit-IAG2 silencing experiment was evaluated by qRT-PCR. The expression levels of Lvit-CFSH1, Lvit-CFSH2, Lvit-GIH1, Lvit-GIH2, Lvit-CHH, Lvit-Vg, and Lvit-VgR were detected following in vivo injection with PBS, dsRNA GFP or dsRNA Lvit-IAG2. The gene expression levels were standardized by Lvit-β-actin expression levels and represented as mean ± SEM ("a, b, and c," p < 0.05; one-way ANOVA followed by Tukey’s multiple range tests; n = 4–6).

FIGURE 6 | Effects of Lvit-IAG2 silencing on carapace length, body weight, AM length and oocyte diameter of L. vittata. (A) Carapace length; (B) body weight; (C) the normalized length of AM (AM/Al); (D) the oocyte diameter. Data were represented as mean ± SEM ("a and b," p < 0.05; one-way ANOVA followed by Tukey’s multiple range tests; n = 4–6).

(Bao et al., 2020). Lvit-IAG1 has also been identified as an IAG via functional study (Liu et al., 2020). In this study, we expounded on the biological functions of another ILP, Lvit-IAG2. Lvit-IAG2 contained features that were structurally conserved in IAGs: a preproprotein comprised of a signal peptide, B chain, C peptide, and A chain; six strictly conserved cysteine residues forming three disulfide bonds in the B chain and A chain among predicted mature peptides. Besides, based on phylogenetic tree analysis, Lvit-IAG2 was classified into the IAG clade and subclade of the family Caridea. These results confirmed that Lvit-IAG2 was identified structurally as an IAG. Meanwhile, we noticed that some studies reported more than one transcript of IAG in a decapod species (Li et al., 2012; Huang et al., 2017b). However, these transcripts were generated by alternative splicing of one IAG gene, and deduced mature amino acid sequences of these encoded IAG proteins were nearly identical (Li et al., 2012; Huang et al., 2017b). Herein, mature peptides of Lvit-IAG2 and Lvit-IAG1 shared only 58.82% similarity for B chain and 44.12% similarity for A chain, respectively. These results might suggest that two different IAG genes exist in the species, which has never been reported.

Earlier experiments demonstrated that IAG genes were mainly expressed in the AG in decapod crustaceans (Manor et al., 2007). More recently, studies detected IAG mRNA expression in the hepatopancreas and ovary (Chung, 2014; Huang et al., 2014). Herein, the Lvit-IAG2 gene was exclusively expressed in the AG; its expression level was significantly higher in the male phase (Stage I-III) than that in the euhemaphrodite phase (Stage IV) during the life cycle of the PSH species. These reports implied that Lvit-IAG2 potentially can initiate male sexual differentiation in the male phase and maintain male reproductive activity in the euhemaphrodite phase. Notably, Lvit-IAG2 and Lvit-IAG1 displayed slightly different expression trends during gonadal development. The expression levels of Lvit-IAG2 decreased sharply at stage II, while significant downregulation of Lvit-IAG1 expression appeared at stage III. The distinction suggested different functions of the two IAGs.

The present results demonstrated that gene silencing via RNAi successfully induced a specific knockdown of Lvit-IAG2 transcripts levels by 77 and 95% in short-term and long-term silencing experiments, respectively. Furthermore, we assessed the influence of Lvit-IAG2 on the development of male features by comparing male external features and germ cell type composition...
FIGURE 7 | Effects of Lvit-IAG2 silencing on development of male and female sexual characteristics. Both male (cincinnuli, AM, male gonopore) and female characteristics (female gonopore) were photographed at the end of the 30 day experiment. The AI was marked by white dashed lines, while the AM was marked by yellow dashed lines. Male and female gonopores were noted by blue and red dotted circle, respectively. AM, appendices masculinae; AI, appendix interna.

in the testicular region. Notably, previous studies revealed that IAG silencing delayed the appearance of male sexual characteristics and induced testicular spermatogenesis arrest in the freshwater prawn, *M. rosenbergii* (Ventura et al., 2009) and the red swamp crayfish *Procambarus clarkii* (Shi et al., 2019). Similar results were observed for *Lvit-IAG1* in *L. vittata*. *Lvit-IAG1* knockdown induced abnormal development of both AM and male gonopores, and suppressed germ cells at the primary spermatocytes (Liu et al., 2020). However, we found that *Lvit-IAG2* and *Lvit-IAG1* exerted distinct functions in the male sexual differentiation process. *Lvit-IAG2* knockdown solely suppressed AM development and germ cells arrested at the secondary spermatocytes, implying its crucial contribution to secondary spermatocyte-to-spermatid transition. These results demonstrated that *Lvit-IAG2* was indeed another functional IAG of *L. vittata*. On the other hand, either primary-to-secondary spermatocyte transition or secondary spermatocyte-to-spermatid transition was essential for the normal development of testicular region. Meanwhile, both of the two *Lvit*-IAGs might be involved in developmental processes specific to AM. Thus, *Lvit-IAG2* and *Lvit-IAG1* acted in concert, and both of them were indispensable in the male sexual differentiation process of the PSH species.

Simultaneously, we evaluated the effects of *Lvit-IAG2* on female development; notably, no significant difference in *Lvit-CFSHs* gene expression among the three treatments in both short-term and long-term silencing experiments was reported. Further morphological characterization revealed similar results as *Lvit-IAG2* knockdown did not impact the development of female gonopores. Nonetheless, a significant inhibitory effect on the development of ovarian region was observed. Contrary to the controls, shrimp in the *Lvit-IAG2* silencing treatments exhibited decreased volume of the ovarian region, and germ cells were much smaller and arranged in a disorganized mass. It is interesting to note that silencing IAG gene in another hermaphrodite shrimp *P. platyceros* induced feminization of individuals, including Vg expression in the hepatopancreas and ovarian development (Levy et al., 2020a), which is consistent with studies in dioecious species. Different outcome might be
induced by the difference between SPH and PSH. For SPH shrimp *P. platyceros*, individuals first mature as male and eventually acquire reproductive function of female, indicating that functional males and females have different reproductive systems (Levy et al., 2020b). This phase-specific gender pattern is similar, to some extent, with dioecious species, and so it is with reproductive endocrine system. However, for SPH shrimp *L. vittata*, individuals finally acquire the same euhermaphrodite reproductive system with both male and female functions. It is therefore reasonable to speculate that different reproductive endocrine system presents in PSH species, and IAG is likely to possess different functions in ovarian development. Following reports from previous experiments, *Lvit*-IAG1 could regulate ovarian development by inhibiting *Lvit*-GIHs expression (Liu et al., 2020). However, in the present experiment, we reported no significant change in *Lvit*-GIHs transcript levels. Remarkably, knockdown of *Lvit*-IAG1 and *Lvit*-IAG2 suppressed ovarian development. Thus, we could not eliminate the possibility that insulin and related peptides could directly regulate Vg expression in the hepatopancreas, and promote oocyte growth and development. Following a review by Das and Arur (2017), insulin signaling played a pivotal role in the regulation of oocyte growth, development, and maturation in both vertebrates and invertebrates. For instance, in the rainbow trout *Oncorhynchus mykiss*, insulin was reported to induce the accumulation of vitellogenin in cultured oocytes (Shibata et al., 1993). Besides, ILPs could also control germline cyst growth and vitellogenesis in *Drosophila* (LaFever and Drummond-Barbosa, 2005). In the silkmoth *Bombyx mori*, knock-out of an insulin-like growth factor (IGF)-like peptide gene induced drastically lower ovary weights and number of eggs in female individuals (Fujinaga et al., 2019). In the kuruma shrimp *Marsupenaeus japonicas*, *Mj*-IAG
was proved to have insulin-type disulfide, rather than AGH-type (Katayama et al., 2014), indicating that IAG potentially possessed similar functions as IGF or insulin. Moreover, a recent in vitro experiment revealed that bovine insulin induced the Vg expression in the hepatopancreas of the mud crab *S. paramamosain*, implying that insulin-like peptide could directly modulate vitellogenesis in the hepatopancreas (Huang et al., 2017a). Thus, Lvit-IAG2 potentially possessed similar functions as IGF or insulin in the PSH *L. vittata*, which might be explained by their structural similarities (Martin et al., 1998, 1999; Manor et al., 2007). On the other hand, growth and reproduction are closely related (Michalakis et al., 2013). Herein, we noted that Lvit-IAG2 silencing induced significantly slower growth in the treated shrimp, implying that Lvit-IAG2 is potentially a growth regulator. Similar results have been reported in the freshwater prawn *M. rosenbergii* (Ventura et al., 2009) and the mud crab *S. paramamosain* (Huang et al., 2014), and with the function of insulin and related peptide in mammals (Bruning et al., 2000; Neirijnck et al., 2019), fishes (Reinecke, 2010), and insects (Wu and Brown, 2006). Moreover, transcript levels of Lvit-CHH significantly decreased following knock down of Lvit-IAG2 in both short-term and long-term experiment. CHH is defined as a metabolism associated hormone as its participation in multiple metabolic processes, including glycometabolism (Webster et al., 2012), lipid metabolism (Montiel-Arzate et al., 2020) and ammonia metabolism (Zhang et al., 2020). Of note, the low expression of Lvit-CHH implied the growth and developmental abnormalities of individuals. Hence, it could also be reasonably hypothesized that retardation in the development of the ovarian region in Lvit-IAG2 silencing shrimp potentially arose from the abnormality in the growth and development of the shrimp. Further in vitro experiments are required to test the two hypotheses.

Taken together, the present work characterized an IAG gene, Lvit-IAG2, from the AG of the peppermint shrimp *L. vittata* and explored its crucial functions in regulating primary and secondary sexual characteristics of the PSH species. Moreover, this study implicated Lvit-IAG2 and Lvit-IAG1 to act in concert, aimed at regulating male sexual differentiation in the PSH shrimp. Also, Lvit-IAG2 may directly contribute to ovarian development, or via the promotion of growth in *L. vittata*. These findings provide new insights into molecular mechanisms of sexual differentiation in PSH crustaceans, expounding our current knowledge on crustacean reproductive endocrinology.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

**AUTHOR CONTRIBUTIONS**

FL contributed to conceptualization, methodology, software, validation, formal analysis, investigation, data curation, visualization and writing—original draft preparation of the study. HY contributed to conceptualization, methodology, validation, data curation, writing—review and editing, supervision, project administration and funding acquisition. WS contributed to investigation. AL contributed to validation and writing—review and editing of the study. ZZ contributed to funding acquisition and provided resources. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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