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IGFBP-rP1, a strongly conserved member of the androgenic hormone signalling pathway in Isopoda

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ABSTRACT

The first protein which has been described to interact with the malacostracan Androgenic Gland Hormone (AGH) is a binding protein called IGFBP-rP1. It has been identified and studied in several species of decapods, in which its interaction with the masculinizing hormone and its expression patterns have been established in several ways. However, this protein remains uncharacterised to date in the other malacostracan orders, like Amphipoda and Isopoda, although they were historically the first ones in which the androgenic gland and the corresponding hormone were respectively described. In this article, we identified the IGFBP-rP1 of isopods and established its implication in the pathway of the AGH with a silencing approach in the model species *Armadillidium vulgare*. We also showed that this gene is expressed in all the tissues of males and females, with a similar pattern in animals infected with *Wolbachia*, a feminizing endosymbiont of several isopod species. The expression pattern did not differ during the development of uninfected and infected animals either. We finally studied the evolution of the IGFBP-rP1 in 68 isopod species, looking for conserved motifs and evidence of natural selection. Altogether, our results showed that this gene is constitutively expressed and strongly conserved in isopods, in which it likely constitutes a key element of the insulin/IGF signalling pathway. However, we also illustrated that IGFBP-rP1 is not sufficient on its own to explain the different developmental paths taken by the males and the females or feminized genetic males.

1. Introduction

In malacostracan crustaceans (e.g. amphipods, decapods and isopods), sexual differentiation relies on a unique proteinaceous hormone, called the Androgenic Gland Hormone (AGH). The latter, produced by the so-called androgenic glands, is necessary and sufficient to induce male differentiation of the malacostracan juveniles. This hormone has been studied for several decades, revealing it belongs to the Insulin-Like Peptide (ILP) Signalling Pathway (ISP), which includes Insulin-like Growth Factors (IGFs) and insulin-related ILPs like the AGH, also called IAG for Insulin-like Androgenic Gland hormone (Ventura et al., 2011). Yet, the binding peptides of AGH remained unknown until a first discovery in a decapod cDNA library in 2013 (Rosen et al., 2013). This first AGH partner was called Cq-IGFBP (Insulin-like Growth Factor Binding Protein) due to its belonging to the IGFBP superfamily. In the canonical ISP, these proteins regulate the distribution and bioavailability of their ligand by sequestering it (Baxter, 2000; Kelley et al., 2002; Bach et al., 2005). This way, they might either inhibit the action of the ligand or promote it by carrying and releasing it to its target receptors (as a result of various post-translational modifications or upon interactions with cell surface or matrix) (Forbes et al., 2012). Members of the IGFBP are found widely in metazoans and are particularly well known in vertebrates, in which they bind IGFs and not insulin (Hwa et al., 1999). However, using a recombinant Cq-IGFBP, Rosen et al. (2013) proved the specific binding of the AGH (i.e. Cq-IAG) by Cq-IGFBP, confirming the unusual interaction between this IGFBP and an insulin-related ILP. Moreover, this first description enabled the subsequent identification of IGFBPs in a variety of other decapod species (Chandler et al., 2015; Huang et al., 2015, 2016; Li et al., 2015; Ventura-López et al., 2017; Song et al., 2018). Interestingly, Li et al. (2015) and Huang et al. (2016)

Abbreviations: AGH, Androgenic Gland Hormone; ILP, Insulin-Like Peptide; IAG, Insulin-like Androgenic Gland hormone; IGFBP, Insulin-Like Growth Factor Binding Protein; IGFBP-rP1, IGFBP-related Protein 1; ISP, Insulin/IGF Signalling Pathway; SIBD, Single Insulin-Binding Domain

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chose to call this protein IAGBP and ILPBP, respectively, referring to its specific function as a binding protein of the IAG hormone (belonging to the ILP family, and not IGF). Furthermore, Song et al. (2018) referred to this gene as IGFBP7 whereas Huang et al. (2015) preferred to call this protein an IGFBP-rP (IGFBP-related protein). Indeed, structural and phylogenetic studies have brought out the existence of two distinct families within the IGFBP superfamily (Hwa et al., 1999; Rodgers et al., 2008). The *sensu stricto* IGFBP (IGFBP1-6) have a high affinity for IGF peptides only (and a low one for insulin) and are found only in vertebrates. They all display two conserved domains: an insulin binding domain (IB), followed by a thyroglobulin domain. On the other hand, a variety of proteins (including IGFBP7) have been described in both vertebrates and invertebrates and belong to a family of proteins with a low affinity for IGF peptides (Oh et al., 1996; Radulović et al., 2015) and a high affinity for insulin (Yamanaka et al., 1997; Radulović et al., 2015). Contrary to IGFBP1-6, their modular architecture can display various structural domains (Hwa et al., 1999). Considering the structural, functional and phylogenetic evidence, members of this IGFBP family have been consensually renamed IGFBP-rP since 1998 (Baxter et al., 1998). Thus, as pointed out by Huang et al. (2015), given its structure (Rosen et al., 2013) and phylogenetic position (Chandler et al., 2015), Cq-IGFBP is actually a homologue of IGFBP-rP1 (also previously known as IGFBP7), just like all the crustaceans IGFBPs identified so far. The IGFBP-rP1 is characterized by an IB domain, followed by a Kazal domain (protease inhibitor) and an immunoglobulin domain (Ig), all domains also found in the described decapod IGFBPs. Its belonging to the IGFBP-rP family explains why Cq-IGFBP displays such an affinity for an insulin-related ILP (Cq-IAG). More recently, the molecular interaction between the AGH and IGFBP-rP1 was confirmed using a yeast-two hybrid assay (Song et al., 2018). In addition to the evidence at the molecular level, the interaction between decapod IGFBP-rP1 and the AGH was also demonstrated at the transcriptional level, using RNAi (Li et al., 2015) and modelled *in silico* (Chandler et al., 2017). Interestingly, Chandler et al. (2017) predicted that Sv-IGFBP could bind not only the AGH but also two other ILPs (ILP1 and ILP2), which remain undiscovered to date in other malacostracan orders. This characteristic is consistent with the fact that IGFBP-rPs seem to be involved in varied biological processes in invertebrate species with no AGH (metabolism, immunity, growth…) (Li et al., 2012a,b; Mulenga and Khumthong, 2010; Wang et al., 2015, 2016). The crustacean IGFBP-rPs, due to their additional interaction with AGH, are however the first ones potentially involved in sexual differentiation. Yet, the exact function of IGFBP-rP1 is still difficult to assess.

In crustacean isopods, using mostly the common woodlouse *Armadillidium vulgare* as a model species, the sexual development has been extensively studied since the late 1950s, revealing the precise anatomical sequence of male gonad differentiation that begins after the third moult following the release of the *larvae* from the female ventral pouch (stage 4) (Suzuki and Yamasaki, 1995). Moreover, male differentiation seems to be especially sensitive and is often altered in isopods, leading to numerous pathological phenotypes such as various intersex individuals (Legrand and Juchault, 1986; Rigaud and Juchault, 1998). Notably, some strains of the well-known bacteria *Wolbachia*, a common endosymbiont of woodlice, can alter the sexual differentiation of their hosts (Bouchon et al., 1998). These strains, known as feminizing *Wolbachia*, have drawn attention as they induce the differentiation of genetic male *larvae* into functional females and occasionally into partially feminized males, called male intersexes. In the functional females induced by the presence of *Wolbachia*, the androgenic glands do not differentiate so that the AGH is not expressed at all. Besides, these infected females are refractory to the action of the AGH, as androgenic gland grafts do not masculinize them. In contrast, the male intersexes become refractory in the course of male differentiation, which results in mixed sexual characters, in particular the coexistence of hypertrophied androgenic glands and female genital apertures (Legrand and Juchault, 1986; Rigaud and Juchault, 1998). In this context, all the molecular partners of the AGH are putative targets of the feminizing *Wolbachia* (Juchault and Legrand, 1985). This includes IGFBP-rP1, as well as the transmembrane tyrosine kinase receptors recently described in decapods and now identified in *A. vulgare* (Herran et al., 2018). Interestingly, some interaction between *Wolbachia* and the ISP has been described in arthropods (Ikeya et al., 2009; Grönke et al., 2010). To date, the molecular investigations of the isopod sexual differentiation are however limited to the AGH, so that subsequent potential alterations in this pathway are not known on the molecular scale either. In this context, the discovery of IGFBP-rP1 in decapods thus raises several questions relating to its existence and function in isopods.

To tackle these issues, we performed the first characterisation of an isopod IGFBP-rP1 (Av-IGFBP-rP1). Its affiliation to the IGFBP-rP family was first established by structural analyses. We then investigated the expression pattern of this gene among tissues of adult males and females and looked for differential expression during development, in both *Wolbachia*-infected and uninfected lineages of *A. vulgare*. Av-IGFBP-rP1 displayed a broad and constitutive expression, suggesting a major functional importance. Silencing experiments of the AGH and of the IGFBP-rP1 confirmed their implication in the physiology of the androgenic glands, as some got hypertrophied following the inhibition of these genes. The evolutionary conservation of IGFBP-rP1 was thus investigated in the Isopoda order. Using a transcriptomic approach, as well as RT-PCR and direct Sanger sequencing, we identified 68 IGFBP-rP1 sequences, one for each of the studied species. Homology to IGFBP-rP1 was confirmed by phylogenetic reconstructions. Negative selection was further demonstrated with dN/dS analyses. Altogether, our results showed that this gene is a key element of the ISP in Isopoda, although not responsible on its own for the different developmental process between male and female isopods and probably not the target of *Wolbachia*.

2. Material and methods

2.1. Animals

Tissue and developmental expression studies were performed on two lineages of the common woodlouse (*A. vulgare*) coming from our laboratory rearing: the lineage harvested in Celles/Belle (France) in 1991, which is infected by the *w*VulC *Wolbachia* strain and the *Wolbachia*-free lineage, harvested in Nice (France) in 1967. Tissues from intersexes were collected from individuals of the *Wolbachia*-infected lineage or intersexes generated from males of the uninfected lineage that were previously experimentally infected with *Wolbachia* (Juchault and Legrand, 1985; Rigaud et al., 1991). The silencing experiment was performed with the uninfected lineage only. For the phylogenetic analysis, we included isopod species for which RNAseq data were already available and species either sampled from our laboratory rearing or collected from various field sites in 2015 and 2016. These species represent a wide diversity of ecology (soil-dwelling, terrestrial, coastal, freshwater and marine species) and mostly a diversity in taxonomic ranks (Table S1).

2.2. RNA extractions

To study the spatial expression of Av-IGFBP-rP1, six to eight *A. vulgare* adults for each sex (male, female, intersex) and each lineage were dissected in Ringer's solution (394 mM NaCl, 2 mM KCl, 2 mM $CaCl_2/2H_2O$, 2 mM $NaHCO_3$). The following tissues were gradually pooled and frozen in liquid nitrogen: hemolymph (HE), brain (BR), nerve cord (NC), emptied digestive tract (DT), digestive *caeca* (CK), muscle/fat (MF), legs (LE) and the different parts of the gonads (ovaries (OV) in females; androgenic glands (AG), utricles (UC), seminal vesicles (SV), *vasa deferens* (VD) and sperm (SP) in males). All the dissections were repeated three times independently (four times for males of the uninfected lineage), requiring around a hundred animals in total. For

all the other analyses (RT-PCR as well as RNAseq and RNAi experiments), whole animals were used. To study Av-IGFBP-rP1 expression during development, we sampled pools of *larvae* just after birth (i.e. when they were released from the ventral pouch, week 0) and one, two, three weeks later. From the fourth week on, juveniles were sampled individually and measured to infer their developmental stage (4–8) according to Suzuki and Yamasaki (1995). Both dissected tissues or whole animals were homogenized using a Vibra Cell 75,185 sonicator (amplitude of 35%). Total RNA was then extracted using the RNeasy kit (Qiagen) or TRIzol® reagent and treated with RNase-free DNase I (Qiagen), according to the manufacturer's protocols. After quantification with NanoDrop™ technology, the RNAs were stored at −80 °C.

2.3. RT-PCR and RT-qPCR

Reverse transcriptions were achieved on 500 ng of the previously extracted total RNA using the SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) following the supplier's instructions. Degenerated primers (Sigma-Aldrich) were manually designed using the three sequences of the IGFBP-rP1 of *A. vulgare*, *A. nasatum* and *Porcellio dilatatus* (Table S2), identified by TBLASTX in the corresponding public cDNA libraries, using Cq-IGFBP as query. RT-PCR were carried out using Phusion® High-Fidelity DNA Polymerase and annealing temperatures varying between 45 °C and 60 °C according to the characteristics of the primers (Table S2), with an elongation time of 60 s. The amplification products were separated on 1.5% agarose gels and visualised with ethidium bromide and UV.

RT-qPCR were performed using Applied Biosystems™ SYBR™ Green master mixes (5 µL Sybergreen 5X, 0.5 µL of each primer, 2.5 µL cDNA and sterile water to 10 µL). Primer sequences are given in Table S2. The reactions were performed on a LightCycler® 480 System (Roche), using the following program: 95 °C for 10 min followed by 45 cycles of (95 °C for 10 s, 60 °C for 10 s, 72 °C for 20 s). Melting curves were established (65 °C–97 °C) to check the specificity of the PCR products. The Av-IGFBP-rP1 expression levels were analysed relatively to the ribosomal protein RbL8 expression (Chevalier et al., 2012), using LightCycler® 480 Software. Statistics on the tissue and development RT-qPCR data were calculated using the Kruskal-Wallis test, complemented by Dunn's *post-hoc* tests implemented in the R package PMCMR (Pohlert, 2014). The resulting p-values were adjusted using Holm's correction in order to take multiple comparisons into account. The Wilcoxon-Mann-Whitney test was used to assess the efficiency of the gene silencing in the RNAi experiment.

2.4. Sequencing

The 26 IGFBP-rP1 sequences amplified by RT-PCR were treated with 2 µL of EXOAnP solution (0.05 µL of exonuclease I (1 u), 0.1 µL of AnP (0.5 u) and 1.85 µL of sterile water) at 37 °C for 1 h. Enzymes deactivation was achieved at 80 °C for 20 min. New PCR using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) were then performed (3 µL of PCR product, 0.5 µL of BDT, 0.32 µL of primer, 3 µL of 5X buffer and sterile water to 15 µL). The products of the PCR were precipitated with a mix of 50 µL ethanol (100%), 2.2 µL NaOAc (3 M, pH 5.2) and 10 µL water. Pellets were washed with 70% ethanol and solubilized in deionized formamide. Finally, DNAs were sequenced on both strands using Sanger method. The accession numbers of the published IGFBP-rP1 sequences and of the public cDNA libraries on GenBank are available in Table S1.

2.5. RNA silencing

Av-AGH and Av-IGFBP-rP1 cDNAs were first generated by RT-PCR as described above, with specific primers (Table S2). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and then cloned using the pGEM®-T Easy Vector system, according to the supplier's instructions. Inserts were checked with Sanger sequencing as previously described. Single-stranded RNAs were generated in both directions, overnight, at 37 °C the using MEGAscript T7 Kit (Ambion), treated with TURBO DNase (15 min at 37 °C), precipitated with LiCl, washed with 70% ethanol and dissolved in nuclease-free water, according to the manufacturer's instructions. Hybridisation of forward and reverse RNAs was performed first for 15 min at 70 °C followed by 10 min at room temperature. Double-stranded RNAs (dsRNAs) were quantified and diluted to 1 µg/µL. Adult males were injected with 1 µL of dsRNA (Av-AGH or Av-IGFBP-rP1) or 1 µL of nuclease free water (vehicle group), to account for the trauma response, aging and environmental rearing conditions. These injections were performed using a Hamilton syringe through a hole drilled on the lateral side of the fifth segment of the pereion. Another control group was not injected at all. At one, four, 12 and 28 weeks post-injection, five animals of each group were frozen individually in liquid nitrogen for RNA extraction and another two were dissected to investigate the morphology of the androgenic glands (six per males) and the presence of female genital apertures.

2.6. Bioinformatics

The assembly of both newly generated and already published reads was achieved on our servers (Table S1). The raw reads were first filtered with Trimmomatic (leading and trailing quality of 5, minimum length of 36 bp, Illumina adapters removed) (Bolger et al., 2014). The trimmed data were then assembled with IDBA-tran assembler (maximum k-mer size of 100 bp) (Peng et al., 2013). The IGFBP-rP1 sequences were searched locally with TBLASTX, using first Cq-IGFBP and then Av-IGFBP-rP1 as queries.

The post-translational modifications were predicted using CBS prediction servers (SignalP 4.1 (Petersen et al., 2011), NetPhos 3.1 (Blom et al., 1999), ProP 1.0 (Duckert et al., 2004)). The conserved domains of the IGFBP-rP1 sequences were obtained using the Conserved Domain Search service (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2015). Coding sequences were retrieved with ORF Finder from the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/orf_find.html). Sequences logos were drawn using the WebLogo toolsite (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al., 2004).

Phylogeny reconstructions were carried out using the programs implemented in Seaview (Gouy et al., 2010). Nucleotidic coding sequences were aligned using Muscle (Edgar, 2004). The final set of sites was determined with Gblocks (Castresana, 2000; Talavera and Castresana, 2007). The reconstructions themselves were performed using PhyML (maximum likelihood method (Guindon and Gascuel, 2003)), using GTR + I + G model of evolution as determined using jModelTest 2.1.9 (Darriba et al., 2012; Guindon and Gascuel, 2003). Branch support was estimated with both the non parametric Shimodaira-Hasegawa-like approximate Likelihood-Ratio Test (SH-like aLRT) (Anisimova and Gascuel, 2006; Guindon et al., 2010) and the bootstrap proportions (1000 repetitions) (Felsenstein, 1985). Branch supports were further checked with a bayesian method using BEAST 1.8.4 (Drummond et al., 2012), using the same model of evolution, an uncorrelated relaxed clock (log normal) and a run of 50,000,000 generations (log sampling every 1000 generations). Posterior probabilities (PP) were retrieved using TreeAnnotator.

Evidence for natural selection in our IGFBP-rP1 dataset was investigated using PAML (Yang, 1997, 2007). An overall dN/dS value was calculated with codeml (seqtype = 1, model = 0). A dN/dS value was calculated for each site with the tools available on the Datamonkey server (SLAC, FEL, REL (Kosakovsky Pond, 2005; Murrell et al., 2012)) (http://datamonkey.org) (Pond et al., 2005; Pond and Frost, 2005).

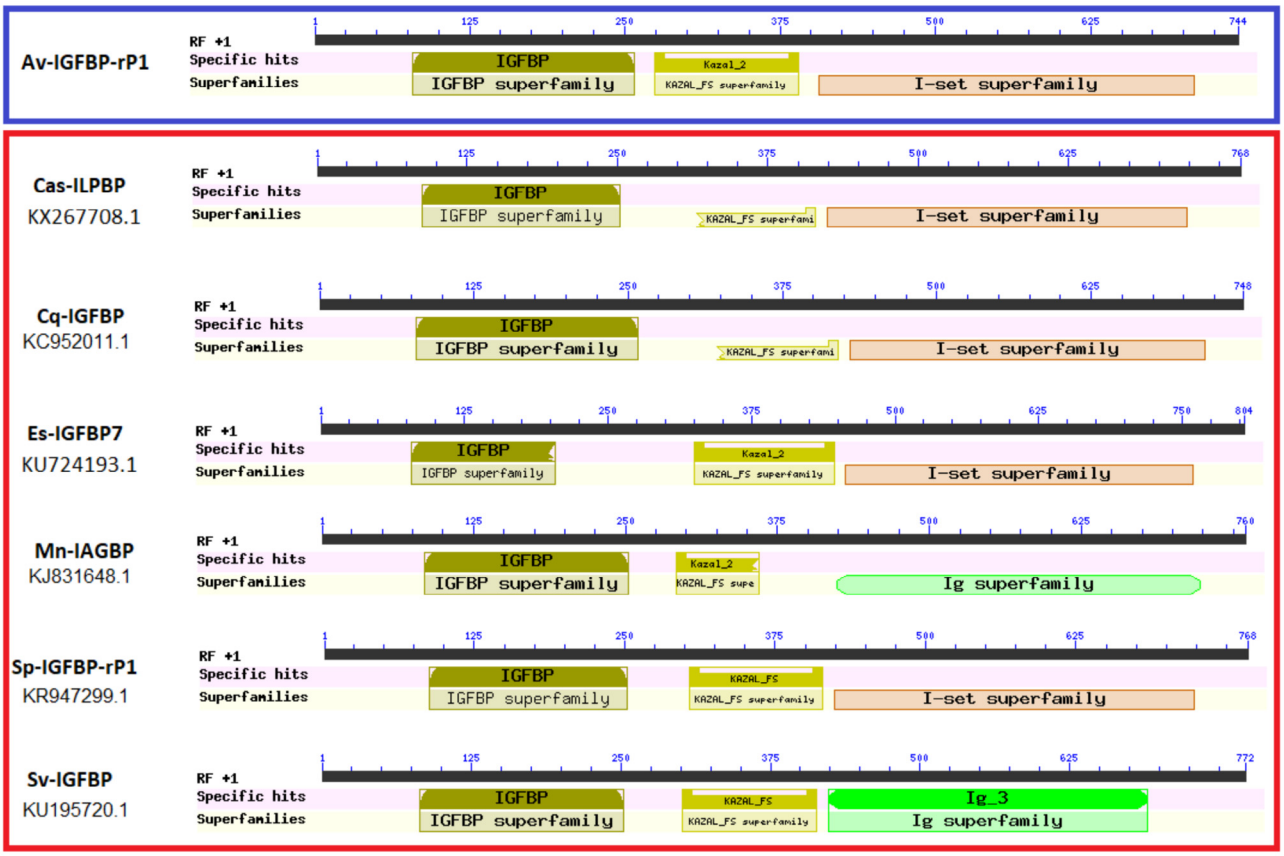

Fig. 1. Comparison of the conserved domains in the published decapod IGFBP-rP1s and the newly characterized isopod IGFBP-rP1, the sequence of *A. vulgare* being shown for illustration. The conserved domains were mapped using CDsearch (Marchler-Bauer et al., 2015).

3. Results

3.1. Av-IGFBP-rP1 sequence and structure

With a transcriptomic approach using publicly available reads, we identified in *A. vulgare* a unique transcript coding for the decapod homologue of IGFBP-rP1, named Av-IGFBP-rP1. Its sequence was confirmed using the Sanger method, revealing a coding sequence of 744 bp (247 amino acids). A signal peptide was predicted, with a cleavage site between positions 22 and 23. Two sites of phosphorylation (Ser145, Ser232) but no R/K cleavage site were predicted.

In keeping with descriptions in Decapoda, three conserved domains were predicted in Av-IGFBP-rP1. It included the IB domain itself, to which the ligand binds, followed by the Kazal domain (a serine protease inhibitor) and finally an Immunoglobulin I-set domain (Fig. 1).

3.2. Expression analysis of Av-IGFBP-rP1

The spatial expression of the Av-IGFBP-rP1 mRNA was investigated by RT-PCR (Fig. S1) and RT-qPCR (Fig. 2) in adult *A. vulgare*. Av-IGFBP-rP1 was expressed in all the individuals, regardless of their sex (male, female or male intersex) or their *Wolbachia*-infection status. In all cases, we found a broad expression of Av-IGFBP-rP1 in the analysed tissues. The RT-qPCR analyses in the uninfected lineage showed that this expression was especially strong in the nerve cord (NC), the muscles and fat (MF), the digestive tract (DT) and the ovaries (OV) (Fig. 2A). In contrast, this expression was especially weaker in the digestive *caeca* (CK). The same expression pattern was observed in the *Wolbachia*-infected lineage (Fig. 2B) and in the intersexes generated experimentally by injecting *Wolbachia* in uninfected males (Fig. 2A).

The expression of the Av-IGFBP-rP1 mRNA was also investigated in whole individuals at each developmental stage by RT-qPCR (Fig. 3). In the uninfected lineage, Av-IGFBP-rP1 gene expression was elevated after birth (week 0), decreased after the first and second weeks and remained stable until adulthood (stages 4–9) (Fig. 3A). A similar expression pattern was observed during the development in the *Wolbachia*-infected lineage (Fig. 3B). Overall, this gene was found continuously expressed throughout development.

3.3. Silencing of Av-IGFBP-rP1 and Av-AGH

To confirm the implication of Av-IGFBP-rP1 in the ISP, its cross-talk with Av-AGH was investigated using a RNA silencing approach (Fig. 4). One week post-injection (wpi) of the Av-AGH dsRNA, the expression of Av-AGH was successfully inhibited by 98% compared to the vehicle group ($p = 0.001$). The silencing efficiency was still of 95% ($p = 0.001$), 89% ($p = 0.002$) and 64% ($p = 0.032$) at four, 12 and 28 wpi, respectively (Fig. 4A). In the same way, the expression of Av-IGFBP-rP1 in the IGFBP-rP1 silencing experiment was reduced by 88% ($p = 0.001$), 86% ($p = 0.001$), 94% ($p = 0.002$) and 92% ($p = 0.008$) at one, four, 12 and 28 wpi, respectively (Fig. 4B). Whereas the inhibition of the Av-AGH gene did not impact the expression of the Av-IGFBP-rP1 gene (Fig. 4C), the silencing of Av-IGFBP-rP1 gene induced an increase in the Av-AGH mRNA level, which significantly reached a two-fold increase at four ($p = 0.019$) and 12 wpi ($p = 0.002$), and up to a seven-fold increase at 28 wpi ($p = 0.016$) (Fig. 4D).

The impact of these two silencing experiments on the gonad phenotypes was also investigated (Fig. 5). No altered phenotype could be observed one week after the silencing of both genes. Four weeks after AGH dsRNA injection, half of the investigated androgenic glands appeared hypertrophied (bigger and more nodular) (5/10) (Fig. 5B). Four weeks after IGFBP-rP1 dsRNA injection, hypertrophied androgenic

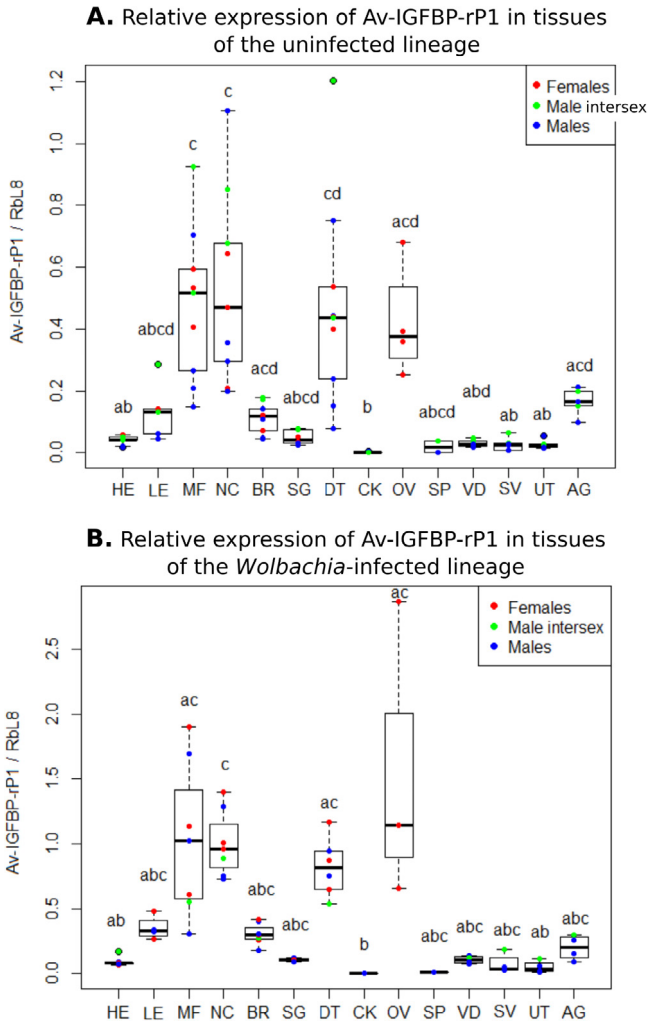

Fig. 2. Tissue expression of the Av-IGFBP-rP1 mRNA by RT-qPCR. The following tissues were dissected from *A. vulgare* adults of uninfected (A) and *Wolbachia*-infected lineages (B): HE: hemocytes, LE: legs, MF: muscle and fat, NC: nerve cord, BR: brain, SG: salivary glands, DT: digestive tract, CK: digestive *caeca*, OV: ovaries, SP: sperm cells, VD: *vasa deferens*, SV: seminal vesicle, UT: utricles, AG: androgenic glands.

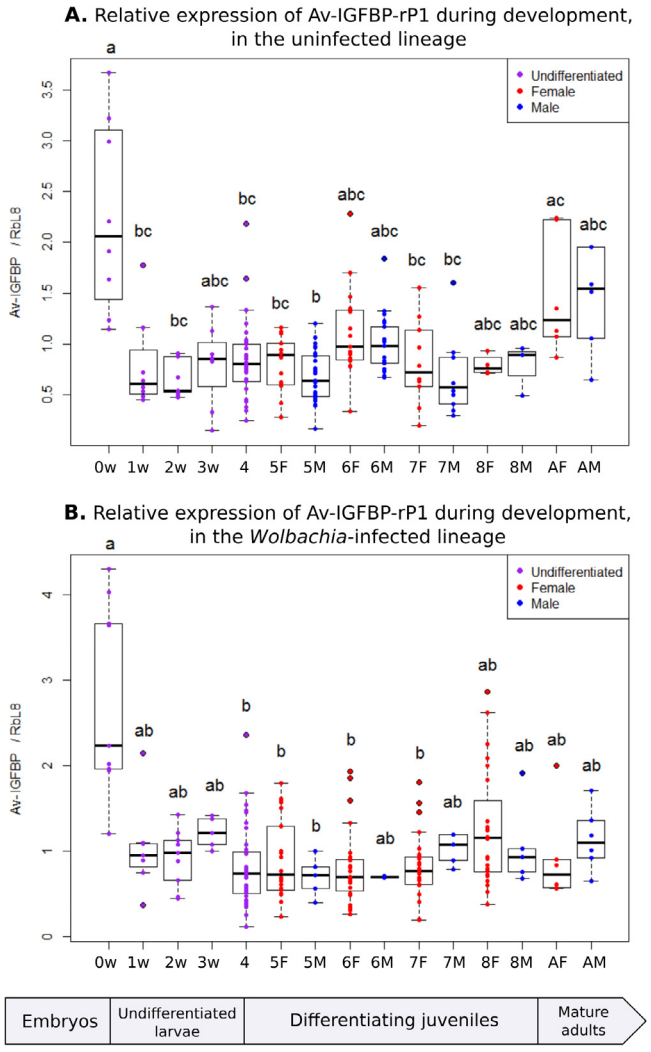

Fig. 3. RT-qPCR expression profiles of the Av-IGFBP-rP1 mRNA during development in uninfected (A) and *Wolbachia*-infected lineages (B). *Larvae* were sampled after birth (0w), one (1w), two (2w) and three weeks (3w) after birth. Then, each step represents a developmental stage (4–8), characterized by a new moult, until adulthood (AM for adult males, AF for adult females). External sex characters start to appear at stage 5, allowing gender-specific sampling.

glands also began to appear (2/10) (Fig. 5E). At 12 and 28 wpi, we observed hypertrophy for all of the androgenic glands in the AGH silencing experiment (12/12 and 12/12) (Fig. 5C and D), as well as the appearance of female genital apertures in half cases (Fig. 5H). In contrast, at 12 and 28 wpi in the IGFBP-rP1 silencing experiment, the androgenic glands seemed normal (hypertrophy: 0/12 and 0/9) in the dissected individuals (Fig. 5F and G), which did not display female genital apertures either (Fig. 5I).

3.4. IGFBP-rP1 in Isopoda: sequences, structure and evolution

Considering the constitutive expression of the IGFBP-rP1 gene in the different tissues and during the development of *A. vulgare*, as well as the consequence of the silencing experiment, we suspected an essential role of Av-IGFBP-rP1 in the ISP and thus investigated its global conservation in isopods. IGFBP-rP1 sequences were obtained using two complementary approaches: by RT-PCR with degenerated primers and by using both public and newly generated cDNA libraries. After the sequencing of the PCR fragments and assembly of the RNAseq data, a total of 68 IGFBP-rP1 sequences was obtained from isopod species belonging to 18 different families: five consisting of aquatic species and 13 comprising terrestrial species (Table S1).

All of the sequences shared the canonical domains of this protein: the IB domain, the Kazal domain and an Immunoglobulin domain (Ig, Ig_3) or Immunoglobulin I-set domain (Fig. 6). The open reading frame length varied between 720 bp (239 amino acids) for *Bragasellus peltatus* and *Proasellus ibericus* and 756 bp (251 amino acids) for *Armadillo officinalis*. About 68% (26/38) of the terrestrial isopods (Oniscidea) displayed an IGFBP-rP1 coding sequence of 744 bp (247 amino acids) (Fig. 6). Decapod IGFBP-rP1 sequences have a length within the same range, from 747 bp (248 amino acids) for Cq-IGFBP but up to 771 bp (256 amino acids) for *Sagmariasus verreauxi*. In isopods, 74 amino acids were found completely conserved in all the sequences (Fig. 6). They constituted ~30% of the total sequence and were especially represented in the Ig domain (47/101). Among them, ~25% were cysteines (18 residues), mainly distributed in the IB domain (10/60) and the Kazal domain (4/39) but rare in the Ig domain (2/101) or out of the conserved domains (2). Several conserved motifs are described as being implicated in ligand binding: (R/L)xLxxLL in humans (Imai et al., 2000; Hong et al., 2002), RxLxxL in decapods (Rosen et al., 2013) and CGCCxxC in vertebrates (Hwa et al., 1999; Daza et al., 2011). We found in isopod IGFBP-rP1 the same CGCCxxC determinant as the one identified in vertebrates (Fig. 6, framed in green). In contrast, the

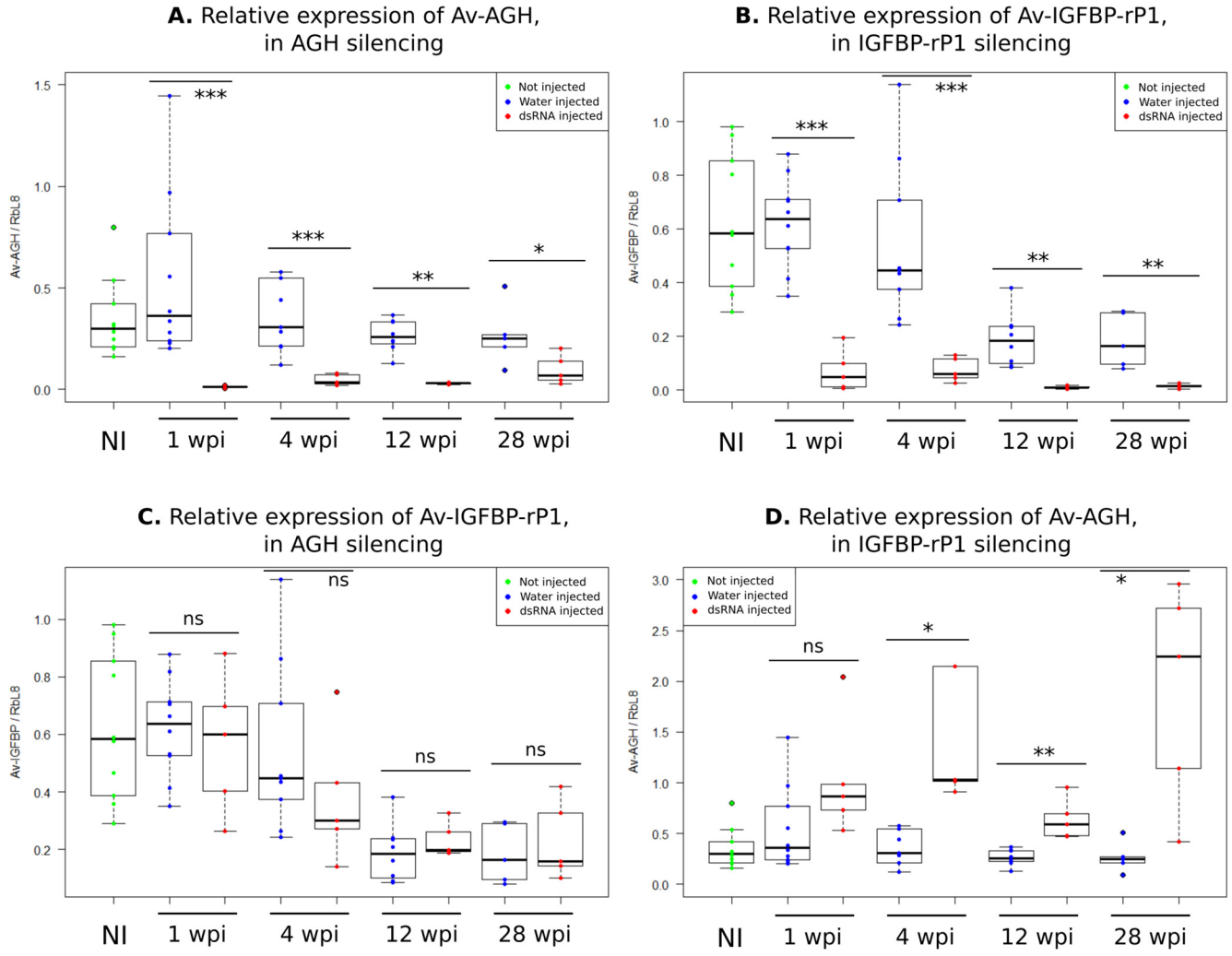

Fig. 4. RT-qPCR expression profiles of the Av-AGH mRNA in the Av-AGH silencing experiment (A), the Av-IGFBP-rP1 mRNA in the Av-IGFBP-rP1 silencing experiment (B), the Av-IGFBP-rP1 mRNA in the Av-AGH silencing experiment (C) and the Av-AGH mRNA in the Av-IGFBP-rP1 silencing experiment (D). Two kinds of controls were included: animals which were not injected (NI, in green) and animals injected with water (in blue), which correspond to the vehicle group. The dsRNA-injected animals appear in red. Animal samplings were performed one, four, 12 and 28 weeks post-injection (wpi). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

homologue region of the decapod motif RxLxxL (Fig. 6, framed in blue) did not correspond to the same sequence. However, a close and overlapping region was found to be similar to the described motif (Fig. 6, framed in orange), even if not fully conserved: (I/V)xLxxL (or (I/V) xxLxL in *Proasellus sp.*). In this conserved determinant, the second conserved leucine in Isopoda was found homologous to the first conserved leucine of Decapoda.

A phylogeny of all the IGFBP-rP1 sequences in isopods was reconstructed using the whole coding sequence (Fig. 7). The phylogenetic tree of the gene was globally congruent with the known phylogeny of isopod species. In particular, the IGFBP-rP1 of terrestrial isopods clustered together, which is consistent with the monophyly of Oniscidea. More extensively, the monophyly of every isopod family was respected with two exceptions. Indeed, our two representatives of Platyarthridae (Fig. 7, in red) as well as the four species of Philoscidae (Fig. 7, in purple) did not cluster together. The monophyly of species classified in Philoscidae is however deemed unlikely (Schmidt, 2008). Because of the modularity of the IGFBP superfamily members, we also constructed phylogenies of the different IGFBP-rP1 conserved domains separately, to check whether they evolved similarly or not. However, considering the very high conservation of these domains (e.g. half of the amino acids in the Ig domain strictly conserved in Isopoda), the resolution of these trees was rather poor (data not shown).

To confirm the apparent good conservation of the isopod IGFBP-rP1, we calculated the dN/dS ratios for the whole sequence set and then for each site of the coding sequence. The overall dN/dS ratio of the isopod IGFBP-rP1 alignment was between 0.12 (codeml, model 0) and 0.17 (SLAC and REL), implying a strong purifying selection on this sequence. This was further confirmed by SLAC, FEL and IFEL methods that predicted 177 (73%), 191 (79%) and 173 (72%) negatively selected codons, respectively, 166 (69%) of these codons being found under purifying selection by these three methods.

4. Discussion

In this study, we identified and described the first molecular partner of the AGH in Isopoda, namely the malacostracan order in which this unique sex hormone was initially identified and characterized (Hasegawa et al., 1987; Martin et al., 1990). In the model *A. vulgare*, we found Av-IGFBP-rP1 expressed constitutively, without significant difference between males, females or intersex individuals. The lack of clear differential expression between males and females in isopods is consistent with the fact that *Wolbachia*-uninfected females are still responsive to the AGH, as shown by the grafts of androgenic glands or the

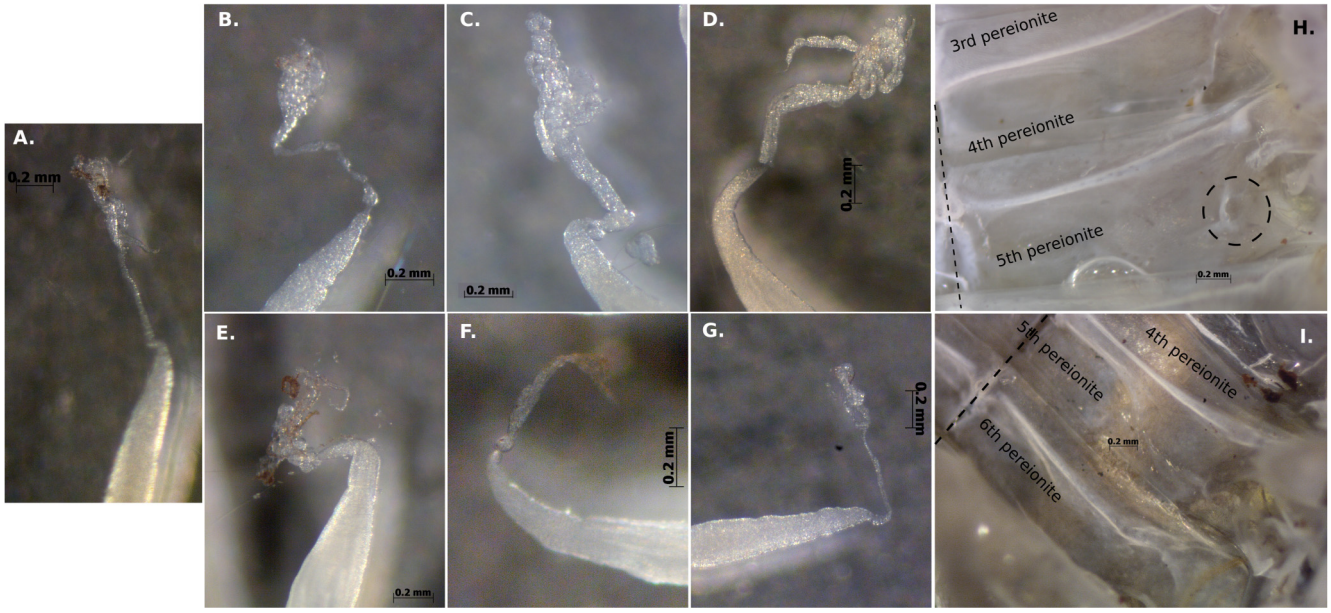

Fig. 5. Phenotypes of the AG in the silencing experiment. Normal AG in the water-injected group (A). Hypertrophied AG in the AGH silencing group at four (B), 12 (C) and 28 (D) weeks post-injection of the dsRNA (wpi). Hypertrophied AG in the IGFBP-rP1 silencing group at four wpi (E), then non-hypertrophied AG at 12 (F) and 28 (G) wpi. Female genital aperture observed during AGH silencing, 12 wpi (H) but absent during IGFBP-rP1 silencing (I). The dashed lines show the plane of bilateral symmetry whereas the dashed circle shows the female genital aperture.

AGH injections (De Lattin and Gross, 1953; Legrand, 1954; Katakura, 1959; Katakura and Hasegawa, 1983). Yet, the expression of IGFBP-rP1 in females could be surprising considering its putative role in male differentiation and that they do not express the AGH. Actually, it is likely that IGFBP-rP1 ensures several functions simultaneously within the organism, including ligand-independent functions (Hwa et al., 1999) and functions associated with additional ligands. Indeed, the AGH is not the only known member of the ILP family in Crustacea and thus, not the only candidate ligand for IGFBP-rP1. For instance, four ILPs had already been discovered in 2011 in the branchiopod crustacean *Daphnia pulex* (Colbourne et al., 2011; Dircksen et al., 2011) and two others were described in the decapod *Sagmariasus verreauxi* in 2015 (Sv-ILP1, a possible homologue of DILP7) (Chandler et al., 2015) and in 2017 (Sv-ILP2, a possible homologue of DILP8) (Chandler et al., 2017). To this date, the function of these ligands cannot be investigated in Isopoda as no homologue of ILP1 or ILP2 was described in any isopod species. Lastly, the exact interactions between the mobile IGFBP-rP1 and its target tissues are not described yet in crustaceans, so that we cannot exclude variations in its function according to the targets, such as feeding activity in the midgut (Huang et al., 2016) or ovarian development (Huang et al., 2015, 2017) for instance.

The analysis of Av-IGFBP-rP1 expression in the different tissues revealed that its spatial expression in adults was very broad, despite the variation in intensity. This is consistent with previous descriptions in decapod models in which IGFBP-rP1 is also expressed in all of the investigated tissues but hemocytes in *Scylla paramamosain* (Rosen et al., 2013; Chandler et al., 2015; Huang et al., 2015, 2016; Li et al., 2015; Song et al., 2018). More precisely, we found the highest Av-IGFBP-rP1 expression in the nerve cord, muscles and digestive tracts, and the lowest expression in the hemocytes, testis and most notably in the digestive *caeca*. A similar expression level of IGFBP-rP1 is reported in the hepatopancreas (i.e. digestive *caeca*) of *Callinectes sapidus* and *Eriocheir sinensis* (Huang et al., 2016; Song et al., 2018), whereas the other decapod IGFBP-rP1s display a high gene expression in this tissue. Contrary to Av-IGFBP-rP1, all of them also show an elevated expression in the testis. It seems however difficult to conclude on functional differences between these homologues on the basis of their gene expression patterns only. Considering both its broad expression and its role as a binding protein, circulating freely in the hemolymph, it seems plausible that IGFBP-rP1 protein is ubiquitous in the organism despite local transcriptional variations. Besides, this broad spatial expression pattern is consistent with the fact that the AGH action is not restricted to the male gonads, as this hormone indeed acts on several secondary sex characters (copulatory organs (Suzuki and Yamasaki, 1991; Takewaki and Nakamura, 1944), brushes on the pereiopods, behaviour (Barki et al., 2006, 2003; Karplus et al., 2003)) and is expressed, in some decapod species, by several organs besides the androgenic glands (ovaries, hepatopancreas, nerve cord) (Li et al., 2012a,b; Chung, 2014; Huang et al., 2014; Li et al., 2015). Likewise, the broad occurrence in the organism is in line with IGFPB-rP1 binding several ILPs with different spatial expression profiles (e.g. Sv-ILP1, Sv-ILP2, Sv-IAG; Chandler et al., 2015, 2017).

We also investigated the differential gene expression of Av-IGFBP-rP1 during *A. vulgare* development. In crustaceans, a similar analysis was performed on *Macrobrachium nipponense* only, in which Mn-IAGBP transcription tends to the increase, particularly at the beginning of sexual differentiation (Li et al., 2015). By contrast, we observed a significant decrease of Av-IGFBP-rP1 expression after birth, followed by a relatively constant expression. Moreover, the Av-IGFBP-rP1 expression patterns between males and females and between infected and uninfected lineages were similar. This suggests that Av-IGFBP-rP1 is required throughout the life of the animal but that other factors may account for the different developmental paths taken from stage four on by the males and the females or feminized genetic males. Despite the absence of differential gene expression patterns, there are other ways to modulate IGFBP-rP1 activity besides variations of transcription intensity. First, the amount of proteins could be regulated at the translation level, which would require quantifying the IGFBP-rP1 protein. Then, the responsiveness of the different tissues, in particular the gonads, may also fluctuate: undifferentiated gonads may not interact in the same way with IGFBP-rP1 during development or according to their *Wolbachia*-infection status. Further studies should also investigate the affinity of isopod IGFBP-rP1 for its ligand in different contexts. IGFBP-rP1 pull-down combined with AGH antibodies may reveal affinity variations between sexes or between infected and uninfected lineages.

In that respect, a role of IGFBP-rP1 in the sex differentiation cannot

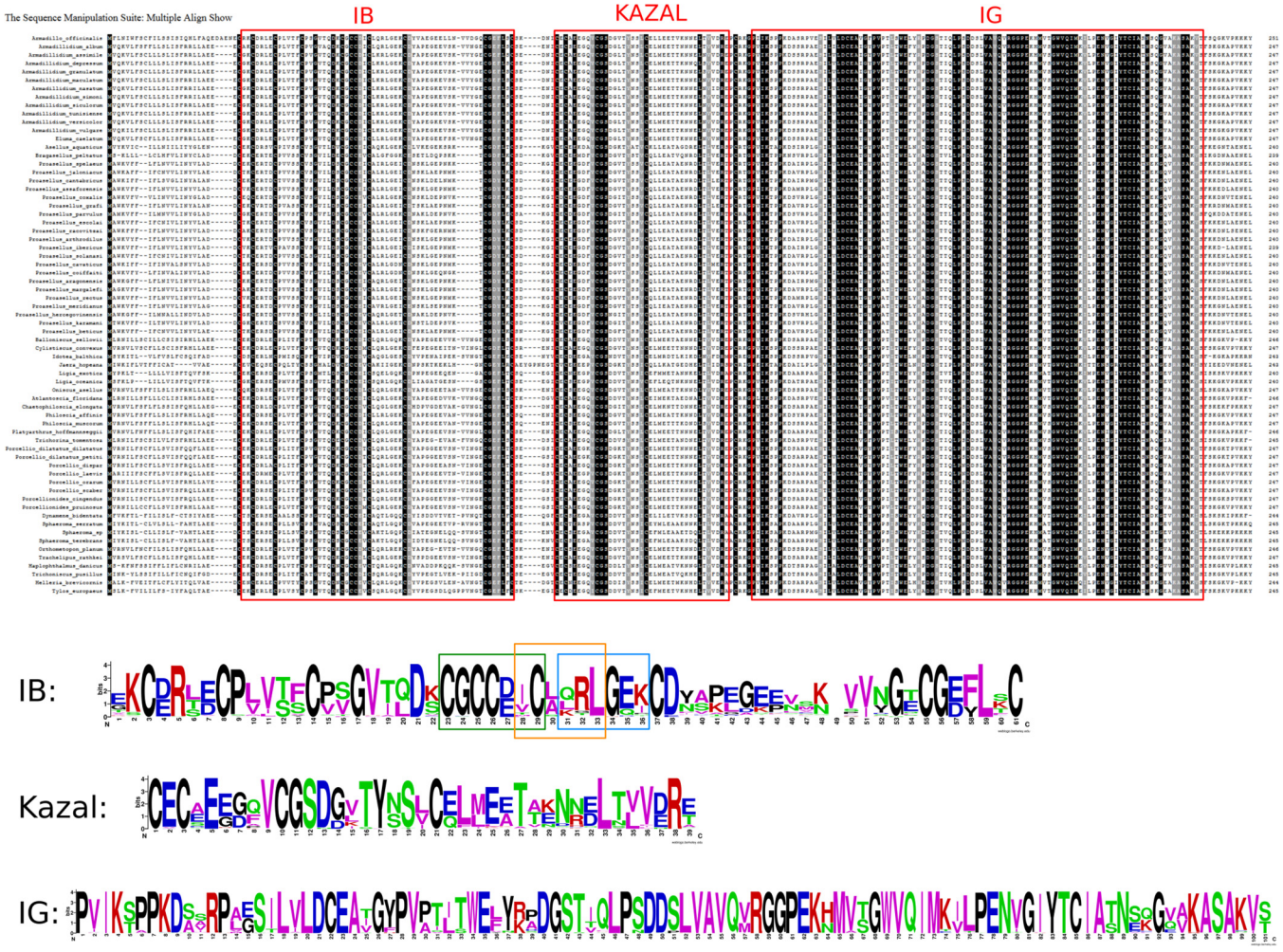

Fig. 6. Analyses of IGFBP-rP1 conservation in isopod crustaceans. An analysis of the amino acid conservation was carried out with Multiple Align Show on the alignment of all the isopod IGFBP-rP1 sequences (A). Fully conserved amino acids are highlighted in black, substitutions leading to a functional conservation of the amino acid are indicated in grey. The three conserved domains are framed in red. Sequence logos of the three conserved domains of the IGFBP-rP1 (IB, Kazal, Ig) were generated by WebLogo (B). Positively charged amino acids (KRH) are depicted in red, negatively charged amino acids (DE) in blue, polar amino acids (STNQ) in green, apolar amino acids (AVLIWFM) in purple and the others (YGPC) in black. The conserved ligand-binding motifs are framed in green (CGCCxxC) and orange ((I/V)xLxxL). The region homologous to the decapod motif is framed in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

be excluded, particularly since its implication in the AGH pathway is now well established in decapods (molecular interaction, modelling, silencing…). The isopod model also appeared particularly well-suited for the gene silencing approach, as only one injection of Av-AGH or Av-IGFBP-rP1 dsRNA could successfully inhibit the expression of the target genes for at least six months. This way, the implication of Av-IGFBP-rP1 in the AGH pathway was also confirmed in Isopoda with RNAi experiments. Indeed, both Av-AGH and Av-IGFBP-rP1 knock-down could induce the hypertrophy of the androgenic glands, which is also observed in naturally *Wolbachia*-induced intersexes. However, only the silencing of the Av-AGH induced the appearance of female genital apertures like in intersexes, leading to a more feminized phenotype. Moreover, the silencing of the Av-AGH did not affect the transcriptional level of Av-IGFBP-rP1, whereas the silencing of the latter induced the overexpression of the Av-AGH gene. Interestingly, the genetic interactions between Av-AGH and Av-IGFBP-rP1 are not consistent with the observations on *M. nipponense*, in which Mn-IAGBP silencing reduces the transcriptional level of Mn-IAG in the androgenic glands and conversely (Li et al., 2015). To better understand the divergent results, it would be interesting to know whether the authors also observed altered phenotypes of the androgenic glands during the Mn-IAGBP silencing experiment.

The importance of IGFBP-rP1 in isopod biology was finally considered on the evolutionary scale. In the whole Isopoda order, each of the 68 studied species, both aquatic and terrestrial, displayed a single sequence of IGFBP-rP1. All of them featured the three same conserved domains already described in decapods (Rosen et al., 2013; Chandler et al., 2015; Huang et al., 2015, 2016; Li et al., 2015; Song et al., 2018). This description, associated with the monophyly of this gene in all Metazoa (Huang et al., 2015), definitely proves that, despite the varying nomenclature, all homologue genes identified in crustaceans derived from only one ancestral sequence. Furthermore, the various sequences quite grouped according to their taxonomic origin in the phylogenies, both on the isopod (present study) or metazoan scales (Chandler et al., 2015; Huang et al., 2015, 2016). This is inconsistent with the observation of Li et al. (2015) regarding a clade gathering vertebrate and arachnid sequences, which can be explained by the absence of tree root in their analysis. Our study, in addition with the purifying selection brought out by the dN/dS analyses, strongly suggests that the function of the IGFBP-rP1 has been well conserved during malacostracan evolution. This is also supported by the conservation in the isopod sequences of most of the negatively charged amino acids in the N-terminal region of IGFBP-rP1 identified in Decapoda to be involved in electrostatic interaction with the ligands (Chandler et al.,

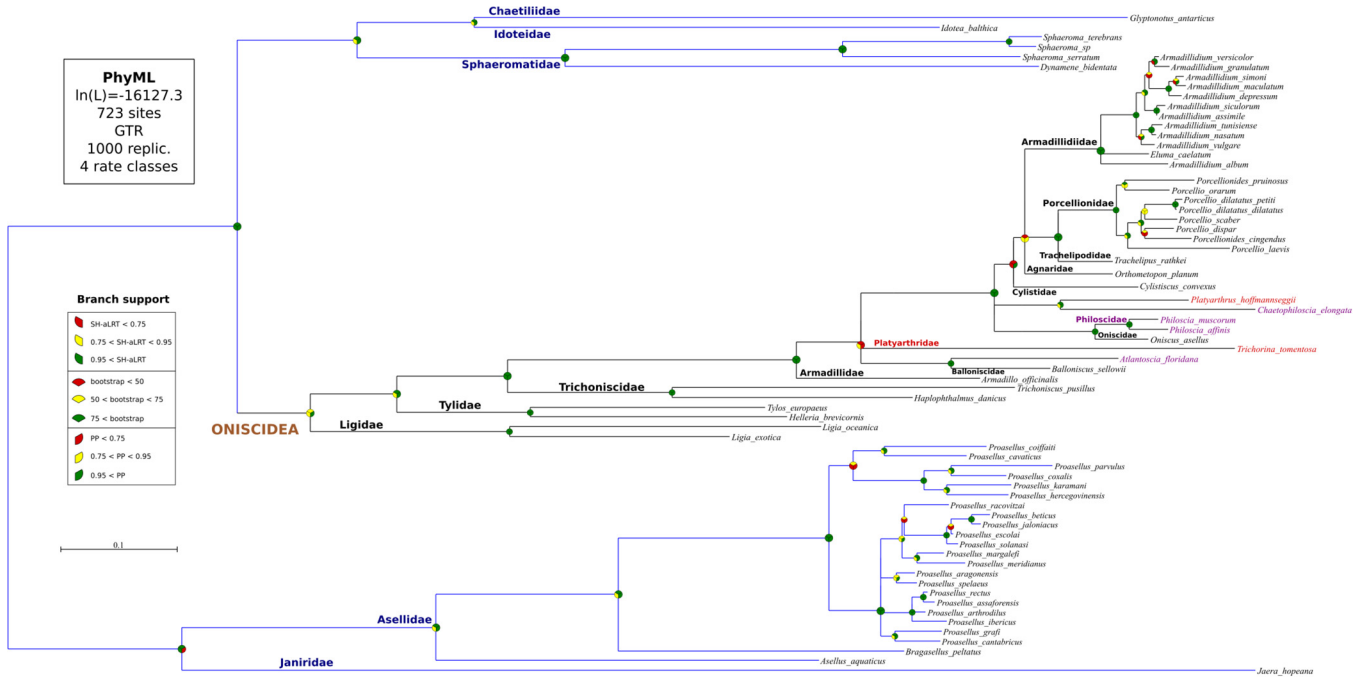

Fig. 7. Isopod IGFBP-rP1 phylogeny of the whole cDNA. Sequences aligned with Muscle were trimmed with Gblocks (723 nucleic sites left). A maximum likelihood tree was constructed using PhyML, with GTR + I + G model of evolution and 1000 bootstrap repetitions. Branch supports were confirmed with SH-aLRT and bayesian approaches. Family names are indicated in black except for families of aquatic species (in blue) and families in which the IGFBP-rP1 sequences did not cluster together (in red and purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2017).

Studies on the evolution of IGFBP1-6 in vertebrates already revealed a complicated scenario although they are all composed of the same two conserved domains and form a clear monophyletic group (Daza et al., 2011). By contrast, the evolutionary story of the IGFBP superfamily in invertebrates appears even trickier to decipher, all the more so because various proteins sharing partial similarity have now been identified in this superfamily. In Hexapoda for instance, the gene called IGFBP7 but also known as ImpL2 (Ecdysone-inducible gene L2) displays only the Ig domain, so that only this part of the sequence is homologue to the other members of the IGFBP-rP1 family (Bader et al., 2013). This receptor is however able to bind ILPs (Bader et al., 2013; Honegger et al., 2008). The Insulin-related peptide-Binding Proteins (IBPs) display only Ig domains as well, and are also able to bind several ILPs, including vertebrate ones (Gao et al., 2012; Sloth Andersen, 2000). Rosen et al. (2013) reported ImpL2 and IBPs to be, together with Cq-IGFBP, three IGFBP homologues but their structural domains clearly differ, explaining why they were excluded in broader analyses such as in Huang et al. (2015). In the same way, Single Insulin Binding Domain proteins (SIBDs), as their name implies, only display the IB domain, like neuroparsins (NP), which are also invertebrate peptides with only the IB domain (Badisco et al., 2007). All these proteins (IGFBP, IGFBP-rP, IBP, ImpL2, NP, SIBD) are visibly related, yet distantly (Rodgers et al., 2008). But considering the divergence between their sequences and given that none of the conserved domain is shared by all of them, establishing global phylogenies of this superfamily by classical means is impracticable, all the more so when exon shuffling events are likely to be involved (Rodgers et al., 2008).

The high conservation of the IGFBP-rP1 sequences in malacostracans, as reflected by the same transcript structure and the close homology, suggests that Av-IGFBP-rP1 is a key element of the ISP, which plays the same role previously described with the decapod IGFBP-rP1, that is the binding of the AGH, among other ILPs. However, the different IGFBP-rP1 expression patterns during development and the different consequences of the IGFBP-rP1 silencing experiments also suggest that its exact function is not that well conserved between the decapod and isopod models. Further studies on the sexual differentiation in Isopoda will likely focus on a more direct evidence of the interaction between the IGFBP-rP1 and the AGH, as well as a more global integration of these two interacting molecules in the ISP. Indeed, several other promising binding or receptor proteins of ILPs have now been characterized in crustaceans. Firstly, SIBDs have been described in several decapod species, in which they were thought to be involved in the immune response (Castellanos et al., 2008; Gai et al., 2010) but likely ensure several functions (Chandler et al., 2015). Lastly, new transmembrane receptors of the AGH were recently described in three decapod species: the Tyrosine Kinase Insulin-like Receptors (TK-IR), also called IAG receptors (IAGR) (Aizen et al., 2016; Sharabi et al., 2016; Guo et al., 2018). Two of these receptors have just been identified in the isopod species *P. beticus* and *A. vulgare* (Herran et al., 2018), whereas the AGH remains the only ILP identified in this order so far.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.006.

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