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Expression analysis and molecular characterization of aquaporins in *Rhodnius prolixus*

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ABSTRACT

Aquaporins (AQPs) are water channels responsible for transport of water and, in some cases, transport of small solutes such as urea and glycerol across lipid bilayer membranes. Hematophagous insects, such as *Rhodnius prolixus*, ingest large volumes of fluid and must rapidly eliminate the excess of water and salts from the blood meal within the gut. In order to deal with this increase in body fluid volume, a hormone-controlled diuresis is activated, during which a high rate of water and salt absorption occurs across the anterior midgut, followed by secretion of water and salts by the Malpighian tubules (MTs). Previously, one member of the MIP family (major intrinsic protein that includes the AQP family) was identified in the MTs of *R. prolixus*, and named RpMIP. We have described here that the RpMIP gene has different variants, and is present in tissues other than MTs. In addition, we have characterized a new AQP (RhoprAQP1) found in different tissues of *R. prolixus*. The expression of these transcripts in unfed insects as well as blood fed insects was evaluated using real-time quantitative PCR. Molecular models of the predicted proteins were constructed and the characteristics of their pores evaluated. A yeast complementation assay was used to validate that the products of these transcripts were bona fide AQPs. Both RhoprAQP1 and RhoprMIP-A were capable of transporting water whereas RhoprMIP-A was also capable of transporting H_2O_2. Taken together, these analyses suggest that RhoprMIP is probably an aquaglyceroporin, while RhoprAQP1 appears to be a strict aquaporin that transports only water.

1. Introduction

Aquaporins (AQPs) are water channels that allow rapid osmotic water flow across cell membranes. These proteins are present among all animal taxa, from the simple *Amoeba proteus* (Nishihara et al., 2008) through to mammals (Verkman, 2008), and also in plants (Maurel, 2007). Currently there are 13 known human AQPs (AQP 0–12), each of which is expressed in specific tissues and differ in both transport specificity and regulation (Spring et al., 2009).

Although overall primary sequences are not well conserved (around 30% identity), all AQPs share a relatively conserved molecular structure, containing six membrane-spanning segments (TM1–TM6) with five connecting loops (LA–LE) (Campbell et al., 2008). Each AQP half contains a conserved asparagine–proline–alanine (NPA) motif, located at LB and LE, that form short hydrophobic helices and dip halfway into the membrane from opposite sides, facing each other and participating in substrate selectivity (Gomes et al., 2009). A cysteine residue at position 189 in LE of human AQP1 and 181 of AQP2 is responsible for conferring mercury sensitivity (Preston et al., 1993). In the biological membrane, AQPs are grouped as homotetramers embedded in the lipid bilayer and each monomer functions independently as a single pore channel (Gomes et al., 2009).

Based on the permeability properties of each pore, the AQP family is divided into two subfamilies: the strict AQPs, which only allow water to permeate the pore, and the aquaglyceroporins, which are also permeable to glycerol and other small neutral solutes (Gonen and Walz, 2006). The molecular basis for AQP substrate specificity consists of two constriction points within the pore: the NPA constriction and the aromatic/arginine (ar/R) selective filter. The selection mechanism is based on size and hydrophobicity exclusions (Hub and de Groot, 2008).

Despite the obvious importance of AQPs to all animals, there is surprisingly little information reported on invertebrate AQPs relative to non-invertebrate AQPs (Campbell et al., 2008). In insects,

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AQPs have been characterized in *Cicadella viridis* (Le Cahérec et al., 1996), *Drosophila melanogaster* (Kaufmann et al., 2005; Yanochko and Yool, 2002), *Aedes aegypti* (Pietrantonio et al., 2000; Duchesne et al., 2003; Drake et al., 2010; Marusalin et al., 2012), *Chilo supressalis* (Izume et al., 2006), *Acheta domesticus* (Spring et al., 2007), *Polypedilum vanderplanki* (Kikawada et al., 2008), *Bombyx mori* (Kataoka et al., 2009; Azuma et al., 2012), *Acyrthosiphon pisum* (Shakesby et al., 2009; Wallace et al., 2012), *Coptotermes formosus* (Kambara et al., 2009), *Bemisia tabaci* (Mathew et al., 2011), *Blatella germanica* (Herraiz et al., 2011), *Belgica antarctica* (Goto et al., 2011; Yi et al., 2011), *Anopheles gambiae* (Liu et al., 2011), *Eurosta solidaginis* (Philip et al., 2011), *Phormia regina* (Ishida et al., 2012), *Anomala cuprea* (Nagae et al., 2013) and *Rhodnius prolixus* (Echevarría et al., 2001). Insect AQPs appear to be ubiquitous and affect cellular function in every tissue (Spring et al., 2009). In liquid feeders, AQPs are responsible for osmoregulation in diuresis and excretion after their intermittent or periodical feeding or in respiratory system function. In phytophagous caterpillars, they appear to be important for the larvae to conserve water from dietary plant, since they produce very dry faeces (Kataoka et al., 2009). In *A. gambiae*, AQPs are suggested to play a role in water homeostasis during blood feeding and humidity adaptation (Liu et al., 2011). Also, AQPs have roles in desiccation and cold hardiness in insects (Cohen, 2012).

R. prolixus, a hematophagous insect found in Central and South America, is a vector for the Chagas' disease parasite *Trypanosoma cruzi* (Schofield, 1988). During each instar, *R. prolixus* consumes one blood meal that can be up to 10 times their unfed body weight. This severely restricts the mobility of the insect and it is essential that much of the fluid load (particularly water and salt) be voided as rapidly as possible. Thus, *R. prolixus* begins to urinate even before it has finished the blood meal (see Orchard, 2006). Echevarría et al. (2001) isolated a water-transporting protein from the Malpighian tubules (MTs) of *R. prolixus*, called RpMIP. When expressed in *Xenopus oocytes*, RpMIP exhibits relatively low water permeability and it is not inhibited by $HgCl_2$. Martini et al. (2004) showed that mRNA expression levels are increased 6 h after feeding, and that the same effect is observed in tissues treated with serotonin and cAMP *in vitro*. Spring et al. (2009) suggested that the time course for RpMIP mRNA expression is far too slow to affect post-prandial diuresis and that RpMIP is more of a housekeeping protein, associated with the slow absorption of the blood meal, rather than one involved in the extremely rapid diuresis that is initiated by feeding.

Here, we isolate a new AQP gene in *R. prolixus*, RhoprAQP1. Also, we identified a unique transcript variant of the RpMIP gene that was described previously. The expression of the genes was evaluated at different times in several tissues. The molecular structures of the predicted proteins were modelled, and their properties evaluated using bioinformatic tools.

2. Material and methods

2.1. Insects

R. prolixus, Stål 1859, were reared at high relative humidity in incubators at 25 °C and routinely fed on defibrinated rabbit's blood. Tissues were dissected from insects under physiological saline prepared in diethylpyrocarbonate-treated double distilled-water to remove contaminating nucleases, as described previously (Paluzzi et al., 2008).

2.2. Cloning of R. prolixus AQP genes

Plasmid DNA was collected from a *R. prolixus* upper MTs cDNA library (Paluzzi and Orchard, 2010) prepared by standard maxiprep procedure and was used as template for 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR. Gene-specific primers (gsp) were designed based on the analysis of the sequences from the *R. prolixus* Trace Archives EST database available at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), using other insect AQP sequences as queries. For 3′ RACE, gsp was used successively combined with the cDNA library plasmid reverse primer (pDNR-LIBREV1, with the sequence GCCAAACGAATGGTCTAGAAAG) in a semi-nested PCR approach to increase the specificity of the amplified 3′ RACE products. Similarly, 5′ RACE gsp was used successively combined with the cDNA library plasmid forward primer (pDNR-LIBFOR1, with the sequence GTGGATAACCGTATTACCGCC) in a semi-nested PCR approach to increase the specificity of the amplified 5′ RACE products. Conditions for both 5′ and 3′ RACE PCR were 3 min initial denaturation at 95 °C, 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 61 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. Amplified fragments were visualized on an agarose gel stained with ethidium bromide, extracted, and cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). Sequencing was carried out at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, Ontario, Canada), and sequences were confirmed from at least three independent clones to ensure base accuracy.

2.3. RNA extraction and cDNA synthesis

Total RNA extraction was performed with TRIzol Reagent (Invitrogen), according to the manufacturer's protocol. RNA was quantified using a NanoDrop System (ND-1000, Thermo Scientific). The same amount of RNA from each tissue was used for the synthesis of first-strand cDNA with the cDNA iScript kit (Bio-Rad) and supplied Oligo(dT) primer, following manufacturer recommendations. An aliquot of this first strand synthesis reaction was used as a template for the PCR in order to confirm the amplification of a single product using the specific primers designed for the Real-Time PCR (see below). Conditions for PCR were: initial denaturation for 3 min at 95 °C, 40 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

2.4. Tissue-specific expression profile monitored with real time PCR

Unfed and fed fifth-instar *R. prolixus* were used and the following tissues were isolated: oesophagus, salivary glands, anterior midgut, posterior midgut, MTs and hindgut. Tissues from blood-fed insects were dissected at 5 min, 30 min, and 1, 3, 6, 24, and 48 h after feeding. The tissues were stored in RNA later solution (Ambion, Austin, TX) until use in RNA isolation and cDNA synthesis as described above. As a control, RNA was extracted from the rabbit's blood used in the feeding assays and PCR was performed to exclude amplification of any product due to contamination that could have occurred from the ingested blood, during the dissection of tissues. Real time PCR analyses were carried out on a Mx4000® Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) using the Maxima SYBR Green qPCR Master Mix (Fermentas, Burlington, ON). Primers were optimized to amplify target fragments of similar size across all experimental and reference genes (rp49 and β-actin) (Paluzzi and Orchard, 2006; Paluzzi et al., 2010). rp49 is a functional constituent of the 60S ribosomal subunit and, like beta-actin, is a commonly utilized reference gene for target gene normalization in various organisms. These and other reference genes were validated previously for studies examining expression across various tissues of fifth-instar *R. prolixus* (Paluzzi and O'Donnell, 2012). In addition, each primer set consisted of at least one primer designed over an exon–exon splice boundary to ensure target

amplification was solely cDNA synthesized from the RNA isolation. The reaction parameters were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 59 °C, and extension for 1 min at 72 °C, with data acquisition during the extension step. To validate the specificity of the SYBR Green detected products, a melting curve analysis along with gel electrophoresis was performed. The expression levels were quantified using the $\Delta\Delta C_T$ method (Pfaffl, 2001) and quantities were normalized to the reference genes rp49 and β-actin. Results are representative of at least two biological replicates with each individual assay measurement quantified in duplicate. In every assay, a no template control was included to assess reagent contamination or primer dimer generation. *t*-tests were performed on the mean at each time point to assess statistical significance in the differences between fed and unfed insects.

2.5. Computational tools

Homology models were generated with the Swiss-Model Workspace (Arnold et al., 2006), using crystal structures of the human AQP1 (PDB ID: 1J4N), human AQP4 (PDB ID: 1FQY) and bovine AQP1 (PDB ID: 3GD8) as templates. The models were energy minimized in order to remove any steric strain introduced during modeling, and the predicted structures were validated using the PROCHECK (Laskowski et al., 1993). The best generated structures were used for pore analysis. The pore inner surface and distances were calculated using Mole software (Petrek et al., 2007) as a Plugin for PyMOL Molecular Graphics System, v0.99 (Schrödinger, LLC).

Phosphorylation sites were predicted using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 1999).

2.6. Yeast functional complementation assay

The *Saccharomyces cerevisae* strain YLL043W, lacking the AQP gene *fsp1*, was obtained from the EUROSCARF strain collection (Institute for Microbiology, Johann Wolfgang Goethe University, Frankfurt, Germany). This yeast strain also has a ura3 genotype. The whole open reading frame of the genes of interest were cut from the pGEMT-Easy vector and inserted into the plasmid pYES2 (Invitrogen), that contains the URA3 gene as selection marker. Thermal shock was used to transform the pYES2 plasmid constructions into competent yeast cells. Transformants were selected on uracil-deficient SC medium with glucose as the carbon source, and the presence of the gene of interest was confirmed by PCR with gene specific primers. A single colony was chosen for the growth complementation tests. The tranformants were grown overnight at 28 °C on liquid SC medium without uracil, with 2% raffinose as the carbon source. Then, the culture absorbance (600 nm) was adjusted to 0.5 with fresh SC medium. Water transport was evaluated in a hyperosmotic stress test, by supplementing the media with 1 M NaCl . Cells were spotted on the solid media without uracil, with 1% raffinose and 2% galactose as the carbon sources, in a 10× dilution series and plates were incubated for 3 days at 28 °C. To evaluate if molecules other than water were transport by the

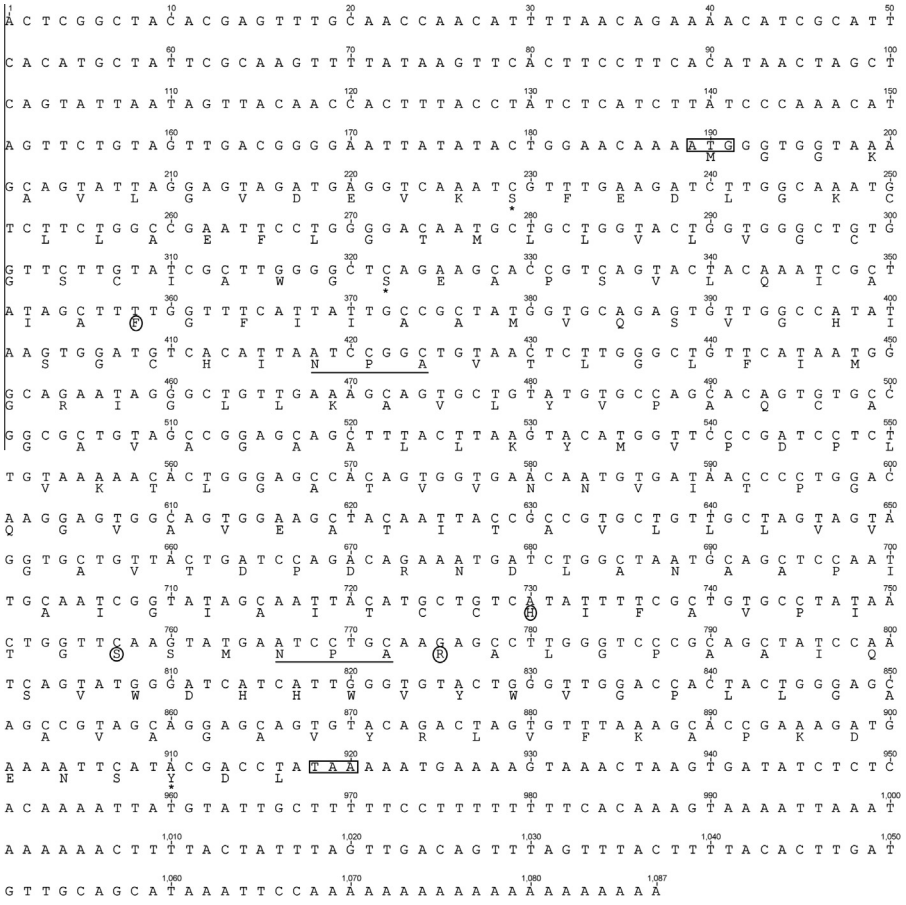

Fig. 1. RhoprAQP1 cDNA and predicted protein sequences. Start and stop codons are boxed. NPA motifs are underlined. Residues from the selective filter are circled. Predicted phosphorylation sites are denoted by "∗".

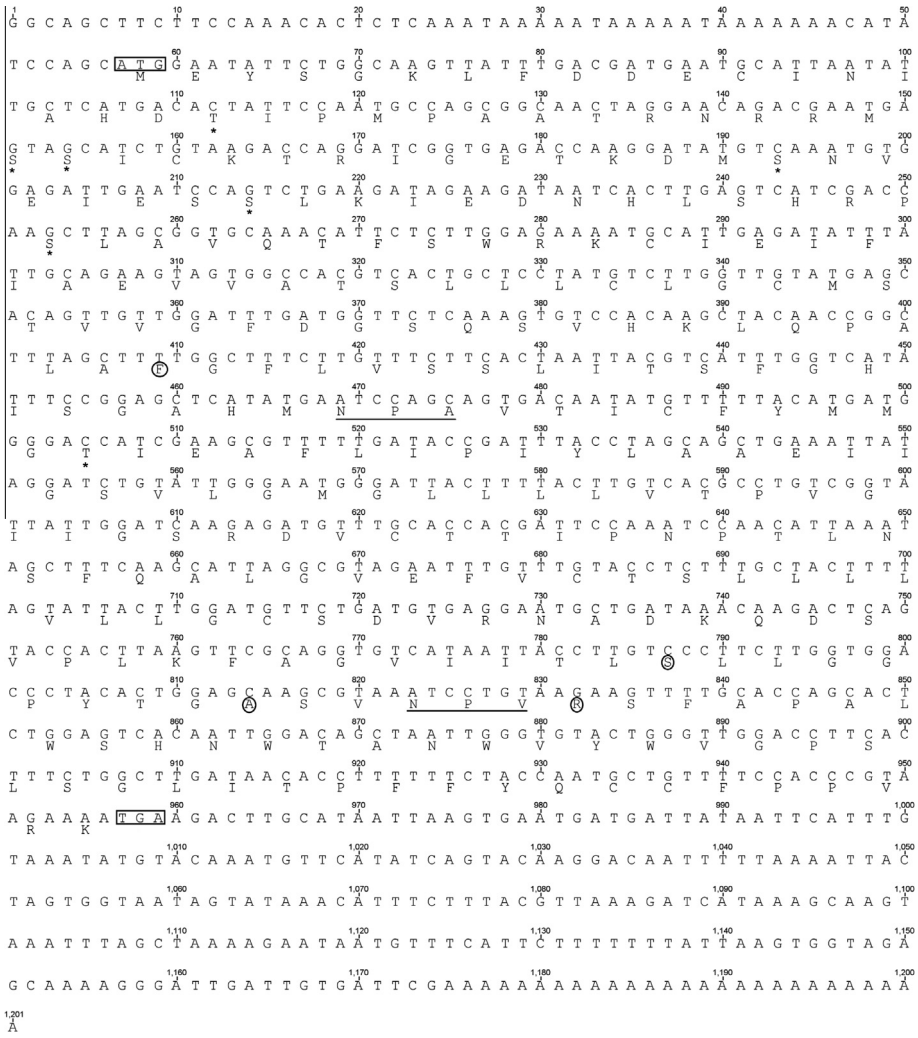

Fig. 2. RhoprMIP-A cDNA and predicted protein sequences. Start and stop codons are boxed. NPA and NPV motifs are underlined. Residues from the selective filter are circled. Predicted phosphorylation sites are denoted by "*".

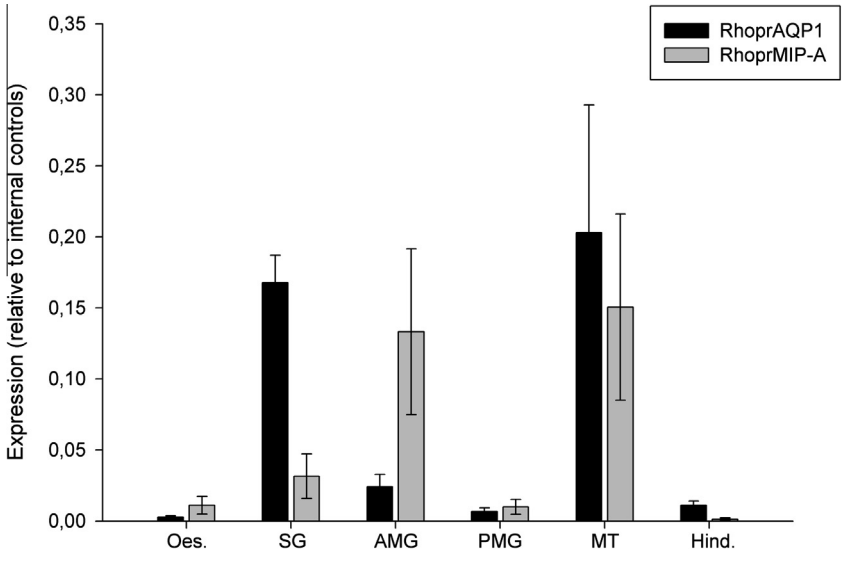

Fig. 3. RhoprAQP1 and RhoprMIP-A mRNA quantification in different tissues of unfed *R. prolixus*. RT-qPCR was performed using specific primers (see Methods). Oes., oesophagus; SG, salivary glands; AMG, anterior midgut; PMG, posterior midgut; MT, Malpighian tubules; Hind., hindgut. Each point is mean ± s.d. of two independent experiments performed in duplicate.

AQPs, the media was supplemented with 2.5 mM H_2O_2 . The differences in growth phenotype were followed. The plates were photographed using a Gel Doc™ XR System (BioRad).

2.7. Phylogenetic analysis

Sequence analyses of RhoprMIP-A and RhoprAQP1 were carried out using Geneious Pro (version 6.1.2) bioinformatic software and comparison against the insect protein database using the BLASTp search program. In general, only sequences from a single species within each genus were selected for phylogenetic analysis. For example, of all the species with protein database information available within the *Drosophila* genus, only *D. melanogaster* data were selected for the analysis. Sequences were imported into MEGA5 (Tamura et al., 2011), and were aligned for similarity using ClustalW. Phylogenetic trees were prepared using the neighbor-joining method (Saitou and Nei, 1987), Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Sneath and Sokal, 1973) and minimum evolution method (Rzhetsky and Nei, 1992); however, all produced trees having highly similar topology. The reliability of the relationships between taxa was tested using the bootstrap test with 1000 iterations (Felsenstein, 1985).

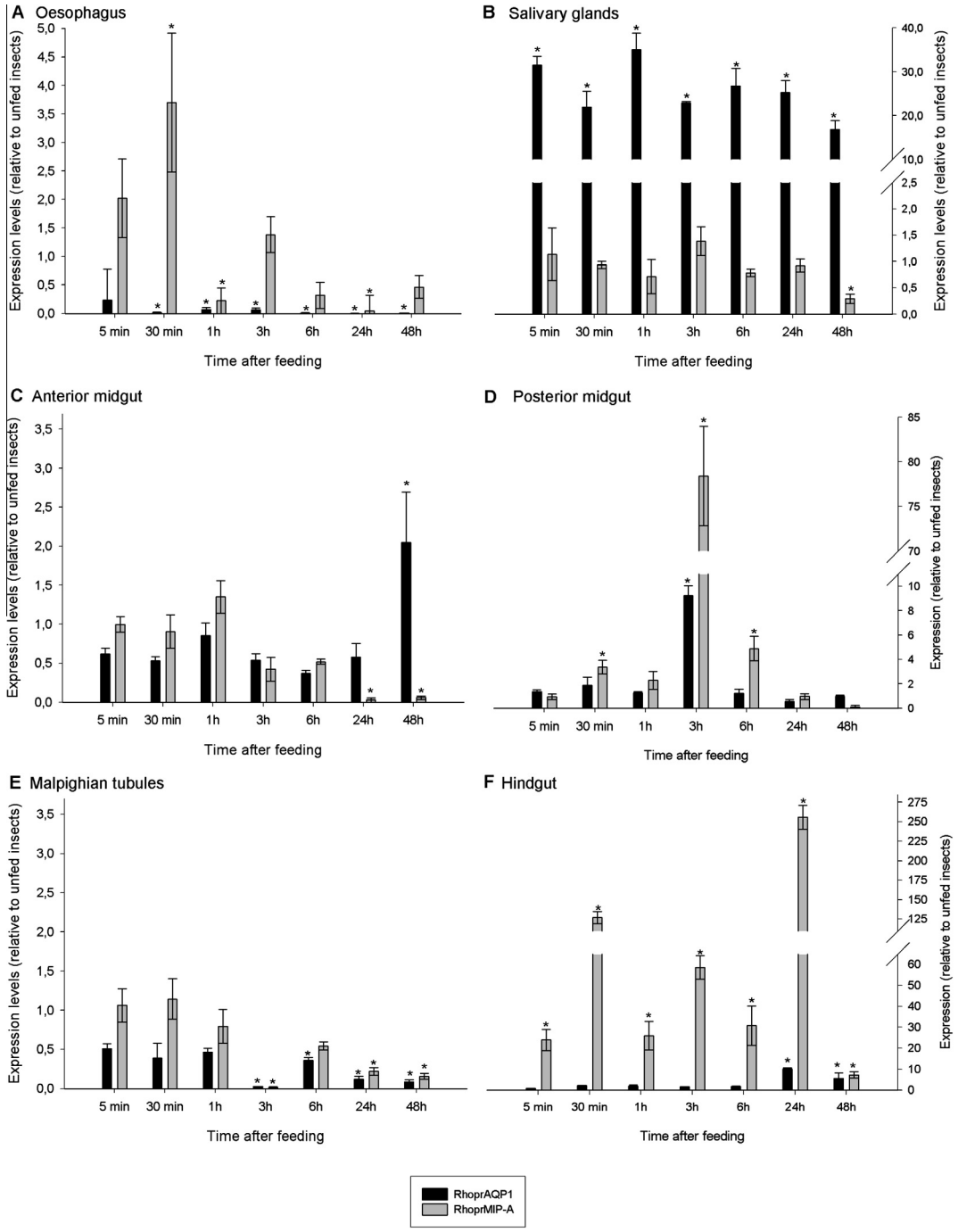

Fig. 4. RhoprAQP1 and RhoprMIP-A mRNA quantification in different tissues and times after feeding on a blood meal. RT-qPCR was performed using specific primers (see Methods). (A) oesophagus, (B) salivary glands, (C) anterior midgut, (D) posterior midgut, (E) Malpighian tubules, (F) hindgut. Each point is mean ± s.d. of two independent experiments performed in duplicate. ∗ indicates values statistically different from the expression in the unfed insects ($p \leqslant 0.05$).

3. Results

3.1. Identification and localization of AQPs in R. prolixus

Using primers based on the putative AQP sequences from *R. prolixus* Trace Archives, we cloned a new AQP from a MTs cDNA library. This AQP was named RhoprAQP1 (GenBank ID: HQ711952). The full length RhoprAQP1 cDNA is 1087 bp (Fig. 1). Comparison of the cloned cDNA to the preliminary assembly of *R. prolixus* genome shows that the RhoprAQP1 gene seems to comprise 6 exons, spanning ~42 kb of the genome.

Also, a variant from the previously described RpMIP (Echevarría et al., 2001) was found. This variant was named RhoprMIP-A (GenBank ID: HQ711954). The full length cDNA of RhoprMIP-A is 1201 bp (Fig. 2). The preliminary assembly of the *R. prolixus* genome suggests that the RhoprMIP-A gene comprises 8 exons, spanning ~27 kb of the genome. This *in silico* analysis of the gene structure of RhoprMIP confirmed a single gene copy in the genome.

In order to investigate the presence of the AQPs in different tissues of *R. prolixus*, unfed insects were dissected and qPCR was performed on cDNA prepared from isolated RNA. Both RhoprAQP1 and RhoprMIP-A are present in all tissues, albeit at different levels (Fig. 3). RhoprAQP1 is most abundant in the salivary glands and MTs, while RhoprMIP-A is enriched in the anterior midgut and MTs. Comparatively, less mRNA for the AQPs is present in the oesophagus, posterior midgut or hindgut of unfed insects.

3.2. Post-feeding mRNA levels analysis

To investigate the influence of feeding on the expression levels of the AQPs in *R. prolixus*, the relative abundance of the transcripts in different tissues at several times after feeding was determined by RT-qPCR (Fig. 4). The expression was normalized against each of the reference genes, and standardized to the level of the transcript expression in unfed insects. As a control, RT-qPCR was performed with cDNA synthesized with RNA isolated from the rabbit's blood used to feed the insects, but no amplification was observed (data not shown).

Thirty minutes after feeding, RhoprMIP-A expression is increased in the oesophagous, while RhoprAQP1 expression is diminished in all times tested (Fig. 4A). In contrast, the expression of RhoprAQP1 is increased at all points in the salivary glands, while

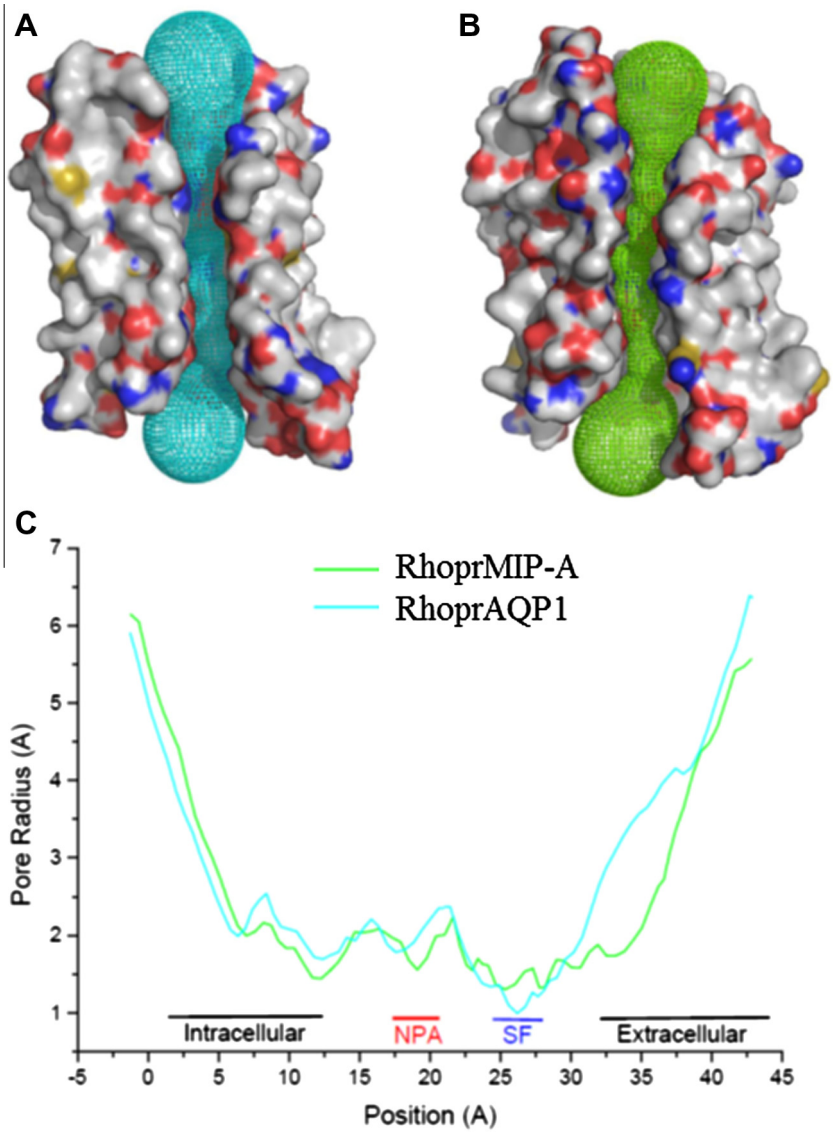

Fig. 5. Molecular models of RhoprAQP1 (A) and RhoprMIP-A (B) showing the pore surface. Pore radius for *R. prolixus* AQPs (C) were calculated using Mole software.

RhoprMIP-A expression remains almost unaltered (Fig. 4B). In the anterior midgut, a slightly increased level of RhoprAQP1 mRNA is observed 48 h after feeding (Fig. 4C). Significant differences were observed in the expression of both transcripts in the posterior midgut 3 h after feeding (Fig. 4D), with the expression of RhoprMIP-A mRNA increasing over 70 fold at 3 h, when compared to the expression in unfed insects. In the MTs, 3 h after feeding, the expression of the transcripts is almost abolished (Fig. 4E). RhoprMIP-A expression is elevated in the hindgut at almost all the times evaluated, with their peak at 24 h after feeding (Fig. 4F).

3.3. Characteristics of the proteins and phylogenetic analysis

The predicted translation products of the identified cDNA consist of 243 and 300 amino acid residues for RhoprAQP1 (Fig. 1) and RhoprMIP-A (Fig. 2), respectively. RhoprAQP1 possesses the classical NPA motifs at residues 77–79 and 193–195 (Fig. 1), and 6 predicted transmembrane helices. RhoprMIP-A possesses a NPA and a NPV motif at residues 138–140 and 256–258 (Fig. 2) and have 6 predicted transmembrane helices. RhoprAQP1 has 3 predicted phosphorylation sites: Ser14 in the intracellular N-terminus, Ser49 in extracellular loop A and Tyr241 in the intracellular C-terminus (Fig. 1). RhoprMIP-A has 7 predicted phosphorylation sites: Tyr19, Ser32, Ser33, Ser46, Ser53 and Ser66 in the intracellular N-terminus, and Tyr150 in the loop B (Fig. 2).

To investigate the structure and possible function of the predicted proteins, homology models were generated and the pore features of each AQP were analyzed. The models for RhoprAQP1 and RhoprMIP-A are shown in Fig. 5A and B. The RhoprAQP1 pore is narrower than that of RhoprMIP-A at the selective filter (Fig. 5C). The predicted RhoprAQP1A ar/R selective filter residues were identified as Phe-57 at helix 2, His-181 at helix 5, Ser-190 at loop E_1, and Arg-196 at loop E_2 (Fig. 6A). Similarly, the predicted residues of the ar/R selective filter for RhoprMIP-A are Phe-118 at helix 2, Ser-244 at helix 5, Ala-253 at loop E_1, and Arg-259 at loopE_2 (Fig. 6B).

It was important to verify if the genes studied here codified functional proteins. A yeast complementation assay was used to this end, where the cells were exposed to a high osmolarity medium (supplemented with 1 M NaCl). When a functional water channel is expressed, the cells are not able to survive in this environment. It was confirmed here that both genes codify functional AQPs (Fig. 7B). In order to increase our understanding of the possible physiological roles of these AQPs, the next step was to identify if substrates other than water were transported by these proteins. Several AQPs were shown to transport H_2O_2, including the human AQP8 and the plant AQPs AtTIP1; 1, AtTIP1; 2 from *Arabidopsis thaliana* (Bienert et al., 2007). Here, it was demonstrated that RhoprAQP1 did not transport H_2O_2, in contrast to what was observed for RhoprMIP-A (Fig. 7C).

Phylogenetic analyses of the deduced RhoprAQP1 and RhoprMIP-A amino acid sequences together with those identified or annotated previously in other insects reveal the existence of at least four main subfamilies of insect AQPs (Fig. 8). These include the *D. melanogaster* integral protein subfamily (DRIPs), the *P. rufa* integral proteins (PRIPs) and the *D. melanogaster* Big Brain gene-related proteins (BIBs) (for a review see Campbell et al., 2008) and a fourth unclassified subfamily. RhoprAQP1 clusters within the DRIPs subfamily, whereas RhoprMIP-A clusters within the unclassified AQP subfamily.

4. Discussion

Aquaporins have been identified in animals, plants and microorganisms. The knowledge of AQPs in insects has increased over

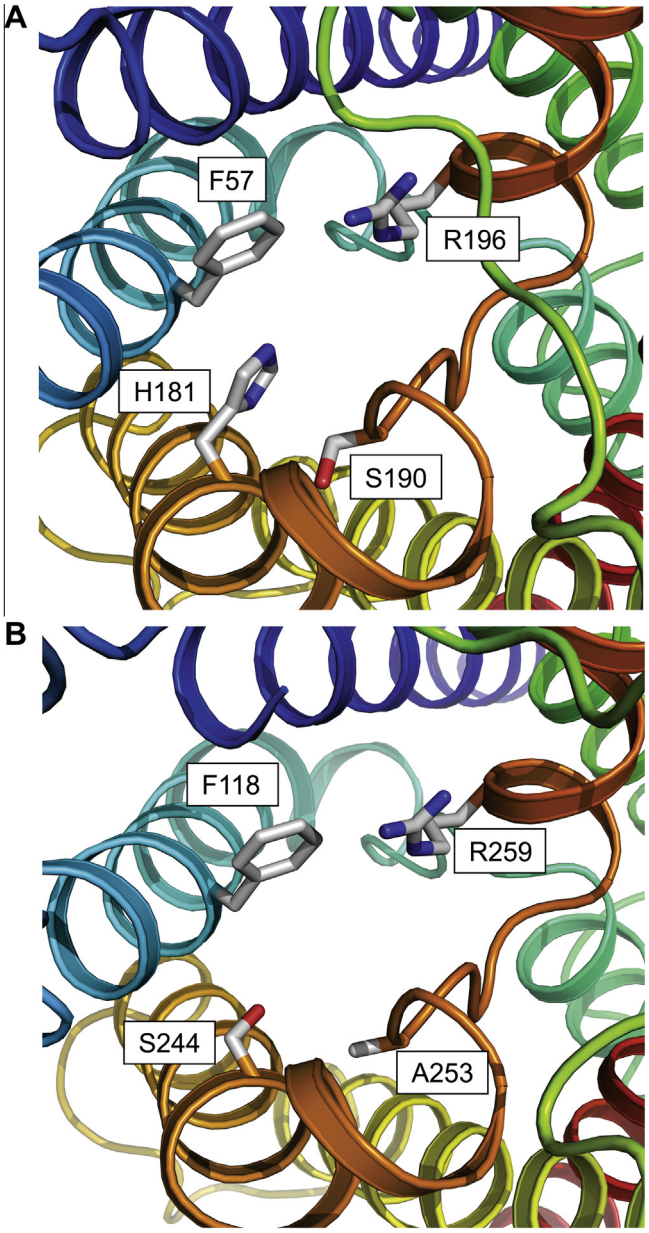

Fig. 6. Selective filter composition of RhoprAQP1 (A) and RhoprMIP-A. (B) Residues of the selective filter are labeled.

the years (Duchesne et al., 2003; Echevarría et al., 2001; Kambara et al., 2009; Kataoka et al., 2009; Kaufmann et al., 2005; Kikawada et al., 2008; Le Cahérec et al., 1996; Marusalin et al., 2012; Pietrantonio et al., 2000; Shakesby et al., 2009; Yanochko and Yool, 2002; Izume et al., 2006; Philip et al., 2011; Nagae et al., 2013). Here, we describe a new cDNA for AQP in *R. prolixus* (RhoprAQP1). Also, we describe a new unique variant (RhoprMIP-A) of the previously described RpMIP (Echevarría et al., 2001). The existence of alternatively spliced variants of AQPs genes has been described in other organisms (Ma et al., 1996; Amiry-Moghaddam et al., 2005; Moe et al., 2008; Goto et al., 2011). For the rat AQP4 gene, six cDNA isoforms have been identified (Moe et al., 2008) and for the *B. antarctica* AQP1, three transcript variants were produced (BaAQP1 A, B and C), most likely by alternative splicing (Goto et al., 2011).

At the protein level, RhoprAQP1 presents 52%, 50% and 50% identity with AQPcic (Le Cahérec et al., 1996), CfAQP (Kambara et al., 2009) and AeaAQP (Pietrantonio et al., 2000) respectively. In AQPcic, the selective filter is determined by Phe at helix 2, His

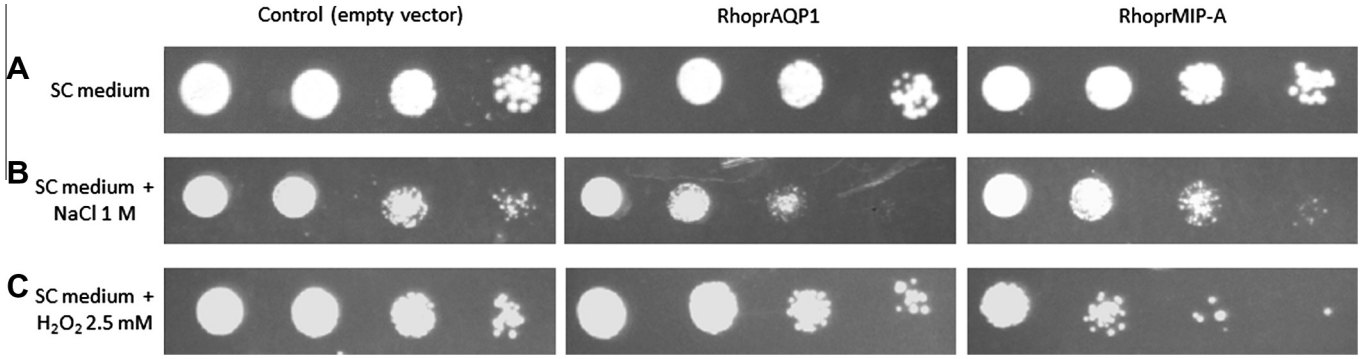

Fig. 7. Functional complementation of *Saccharomyces cerevisiae*. The *S. cerevisiae Δfps1* mutant, lacking one endogenous AQP, was complemented by the expression of RhoprAQP1 or RhoprMIP-A. Cell cultures were grow overnight on SC medium supplemented with 2% raffinose, and the absorbance adjusted to 0.5 A_{600} with fresh medium. Serial dilutions (1, 1:10, 1:100, 1:1.000) of cell cultures were plated on SC medium (A) without uracil, containing 1% raffinose and 2% galactose (to induce the expression of the genes cloned in the pYES vector), supplemented with 1 M NaCl (B) or 2.5 mM H_2O_2 (C). The plates were incubated at 28 °C for 3 days and differences in the growth were followed. Cells expressing a functional water channel are not able to grow in the medium supplemented with 1 M NaCl . Cells expressing a channel capable of transporting H_2O_2 are not able to grow in the medium supplemented with 2.5 mM H_2O_2 , due to its toxicity at this concentration.

at helix 5, Ser at loop E_1, and Arg at loop E_2 (Thomas et al., 2002) and AQPcic and AeaAQP were shown to transport water but not glycerol (Le Cahérec et al., 1996; Duchesne et al., 2003). Considering these data and the characteristics of RhoprAQP1 pore observed in the present work, it was plausible to assume that this protein is a strict AQP, responsible for water transport but not other molecules. This is further supported by the functional assays, where RhoprAQP1 was not able to transport H_2O_2, and by the phylogenetic analyses which indicate that RhoprAQP1 clusters within the DRIPs subfamily of insect aquaporins. Members of this subfamily, such as the *D. melanogaster* DRIP (Kaufmann et al., 2005) and *B. tabaci* AQP1 (Mathew et al., 2011), have been functionally tested and have characteristics including water permeability and mercury sensitivity. In contrast, RhoprMIP-A has a higher primary sequence identity (40%) with AQP-Bom2, an aquaglyceroporin (Kataoka et al., 2009). RhoprMIP-A's broader pore and the presence of a serine residue in the helix 5 instead of a histidine residue is suggestive that RhoprMIP-A might be an aquaglyceroporin, capable of transporting molecules other than water. Echevarría et al. (2001) demonstrated that RpMIP is able to transport water when expressed in oocytes, but with low osmotic water permeability. This also seems to be the case for RhoprMIP-A, since a lower sensitivity on the osmotic stress assay was observed for cells expressing this protein than for those expressing RhoprAQP1. The transport of other solutes by RpMIP was not tested by Echevarría et al. (2001). Here, it was shown that RhoprMIP-A is capable of transporting H_2O_2, further supporting the classification of RhoprMIP-A as an aquaglyceroporin. It is worth mentioning that the sequence deposited in Pubmed for RpMIP (Genebank ID: AJ250342) has important differences from the one characterized in the present work (Fig. 9). The most important difference is that RpMIP has two NPV motifs, instead of one NPA and one NPV motif that is present in the transcript variant identified in this study and as predicted in the preliminary assembly of *R. prolixus* genome. In support of these characteristics, the RhoprMIP-A identified herein clusters closely with a number of other insect AQP related proteins that do not belong to the major subfamilies denoted by Campbell et al. (2008), namely DRIPs, PRIPs and BIBs, which include some examples of functionally validated water-specific AQPs. Further work will be required on the AQPs identified in *R. prolixus* as well as other species to more fully understand the functional characteristics of insect AQPs.

Short-term regulation of cell membrane water permeability has been shown to be effected through phosphorylation of AQPs, causing gating of the AQP pore or translocation from the cytosol to the plasma membrane or *vice versa* (Ball et al., 2009). The main difference observed at the protein phosphorylation level between RhorpMIP-A and RhoprAQP1 is the number of phosphorylation sites in the intracellular N-terminus. In this region there are six predicted phosphorylation sites in RhoprMIP-A, and only one in RhoprAQP1. This could indicate a distinct regulation of the two AQPs. Also, considering the differences in expression levels of the AQPs in the tissues evaluated, one can speculate that these proteins may participate in distinct physiological processes in this insect.

Gorging upon a blood meal triggers several responses in *R. prolixus*, in particular those needed to lower its mass and concentrate the nutrients of the meal, while preserving the volume, ionic and osmotic balance of the hemolymph (Orchard, 2009). Ion and water absorption across the anterior midgut epithelium into the hemolymph is the first process triggered. Little is known about the fluid transport in the anterior midgut, and it is postulated that the active transport of Na^+ from the lumen would lead to a passive transport of water and Cl^- (see Orchard, 2009). We find here that AQP genes are expressed in the anterior midgut and this data supports the prediction that these proteins may be involved in the fluid transport across this epithelium. We did not observe a significant change in the expression levels of the AQP transcripts in the anterior midgut shortly after feeding, when a rapid diuresis is triggered. Thus, future research will investigate if post-translational modifications of the AQP proteins, such as phosphorylation at the sites predicted herein, are involved in facilitating the rapid transport of water that accompanies the transport of ions. Aquaporins, as described for human AQP2, can be stored in intracellular vesicles and upon stimulation may be translocated to the apical plasma membrane (Takata et al., 2008). It is reasonable to speculate that the rapid increase (within seconds) in fluid transport in response to feeding in *R. prolixus* could be mediated by proteins already stored in the insect, and not by increasing gene expression. The slight increase in expression observed 24 and 48 h for RhoprAQP1 transcripts may reflect the synthesis of new molecules to restock those intracellular vesicles.

Interestingly, we observed an almost complete abolishment of expression of the AQP transcripts in the MTs 3 h after feeding, a time when the cessation of diuresis is observed (Maddrell, 1964). Paluzzi and Orchard (2006) suggested the release of an anti-diuretic hormone in *R. prolixus* beginning 3–4 h after feeding. This peptide, RhoprCAPA-2, has potent anti-diuretic activity, inhibiting both secretion by the MTs (Paluzzi et al., 2008) and absorption by the anterior midgut (Orchard and Paluzzi, 2009; Ianowski et al., 2010) following stimulation with the diuretic hormone, serotonin. Martini et al. (2004) demonstrated that serotonin stimulates an increase in RpMIP expression in MTs. It is possible that RhoprCAPA-2

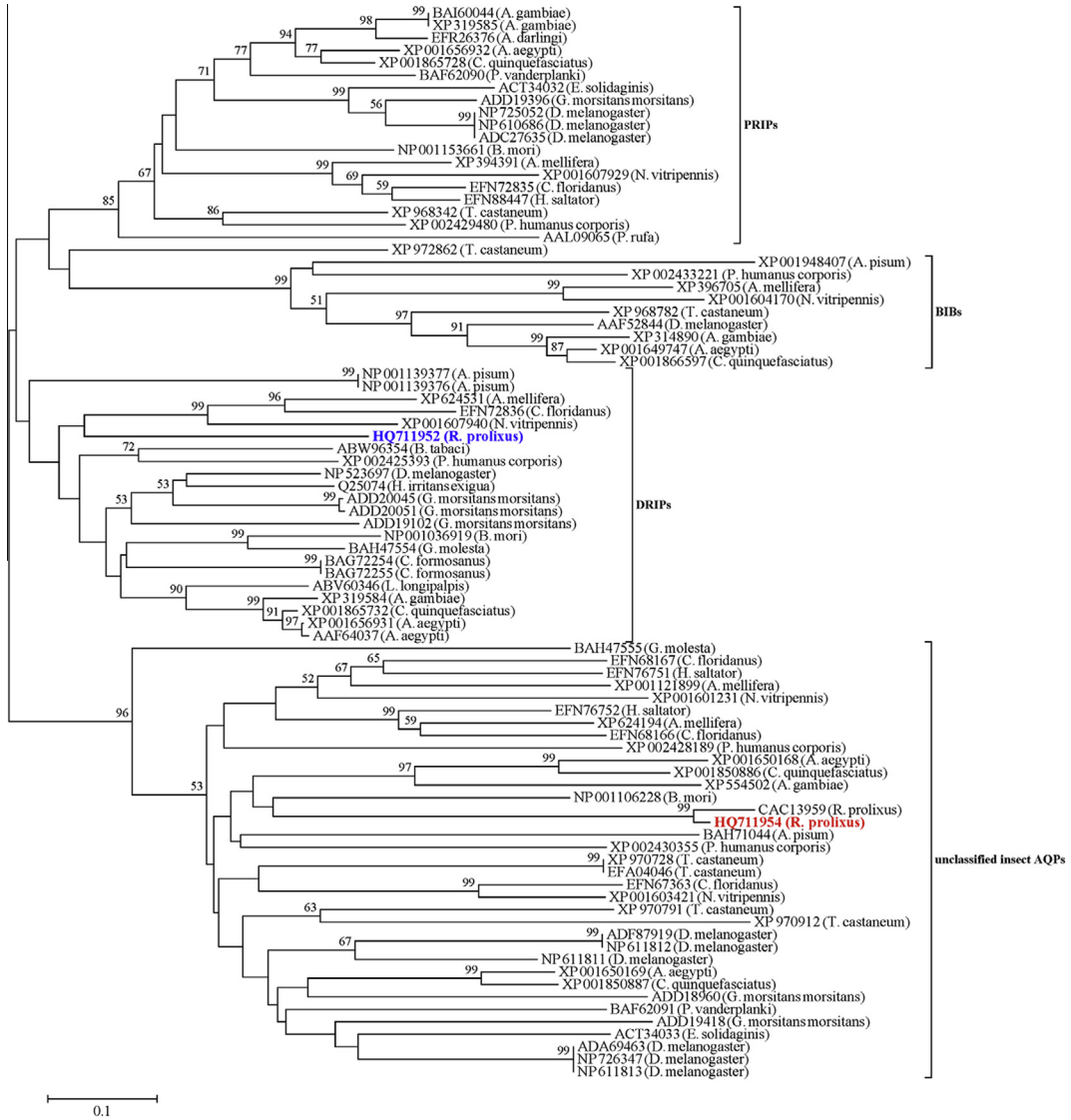

Fig. 8. Phylogenetic analysis illustrating the relationship of the aquaporins isolated in this study (RhoprAQP1 blue text and RhoprMIP-A in red text) to other known insect AQPs using neighbor-joining analysis (Saitou and Nei, 1987). In addition, analysis of the amino acid sequences using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and minimum evolution methods yielded trees with near-identical topology. Three sub-families were denoted as DRIP, PRIP and BIBs as suggested in a recent review of insect AQPs (Campbell et al., 2008) and a fourth sub-family was denoted as an 'unclassified insect AQPs' group. Branches are labeled with accession numbers and species name in parentheses. Branch-length units are representative of the number of amino acid substitutions per site. Numbers adjacent to the nodes denote bootstrap support for the clustering of associated sequences (Felsenstein, 1985). Genbank accession numbers for RhoprAQP1 and RhoprMIP-A are HQ711952 and HQ711954, respectively. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

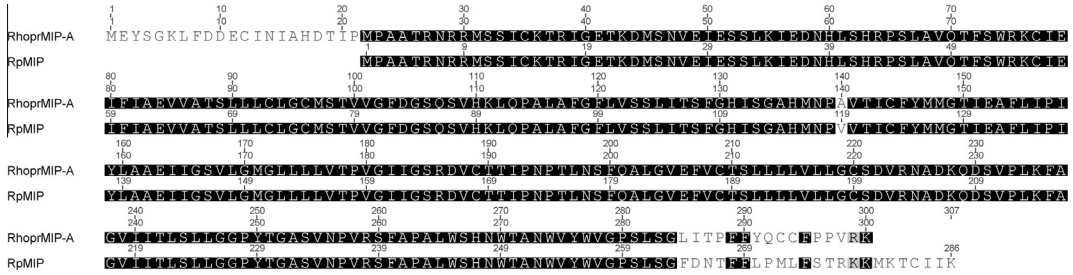

Fig. 9. RhoprMIP variants. RhoprMIP-A protein sequence was aligned with RpMIP sequence (GenBank accession: AJ250342.1) using Geneious software. Identical amino acids are highlighted in black.

blocks this stimulus, as part of the control of the diuresis in *R. prolixus*.

In many Lepidoptera, the hindgut contents proceed through a stepwise dehydration, which enables larvae to produce very dry faeces. Kataoka et al. (2009) demonstrated the expression of an AQP in the hindgut of *B. mori*, likely involved in the water retrieval function of the hindgut. In contrast, *R. prolixus* produces liquid faeces, and there is no evidence so far of water absorption through the

hindgut in this insect post feeding. We observed differences in the expression of RhoprMIP-A at all times evaluated in this work. Since it is suggested that RhoprMIP is an aquaglyceroporin, it is possible that this protein is involved in the transport of molecules other than water across the hindgut epithelium, such as urea and ammonium, during the process of urine and faeces elimination.

It is worth mentioning that the expression evaluation performed here is restricted to mRNA levels and it is possible that no correlation exists with the protein levels. Further studies are necessary to address this matter. Nevertheless, the variations observed here indicate relevant roles of AQPs in the post-feeding physiology of *R. prolixus*.

The handling of water from the ingested meal by insects is essential for maintenance of water balance. The AQPs described here may play important roles in water movement and osmoregulation in fluid-transporting epithelia of *R. prolixus*. Also, RhoprMIP-A has the characteristics of aquaglyceroporins and may be involved in the transport of other solutes physiologically important, such as H_2O_2. It has been suggested that blood digestion is a source of oxidative stress to hematophagous insects, such as *R. prolixus* (Dansa-Petretski et al., 1995; Oliveira et al., 1995), and H_2O_2 have been demonstrated to be produced in high quantities in *R. prolixus* digestive apparatus (Paes et al., 2001). Even though reactive oxygen species such as H_2O_2 are potentially toxic, these compounds also function in signaling, presenting the ability to cross membranes and move between different compartments. Aquaporin-mediated H_2O_2 diffusion has been described in plants and mammals (Bienert et al., 2007) and the data presented here suggests that this phenomenon may also occur in insects.

The high multiplicity of AQP isoforms found in several organisms, from plants to animals, is an indication of how much is to be discovered in the area of insect AQPs and this work is one more contribution in the path to a better understanding of the complex process of water homeostasis in insects.

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