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A phospholipase A_2 gene is linked to Jack bean urease toxicity in the Chagas' disease vector *Rhodnius prolixus*

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ABSTRACT

Background: Ureases are multifunctional enzymes that display biological activities independent of their enzymatic function, including exocytosis induction and insecticidal effects. The hemipteran *Rhodnius prolixus* is one of the known susceptible models for this toxicity. It has been shown that Jack bean urease (JBU) has deleterious effects on *R. prolixus*, and these effects are modulated by eicosanoids, which are synthesized in a cascade involving phospholipase A_2 (PLA_2) enzymes.
Methods: R. prolixus genome was screened for putative PLA_2s and matching transcripts were cloned. Predicted amino acid sequences were analyzed and transcript distribution among tissues was determined by qPCR. RNAi techniques were used and subsequent JBU toxicity assays were performed.
Results: Two PLA_2 genes were identified, *Rhopr-PLA2III* and *Rhopr-PLA2XII*. The transcripts are widely distributed in the tissues but at different levels. The analyses fit the putative proteins into groups III and XII of secretory PLA_2s. After 70% of *Rhopr-PLA2XII* expression was knocked down, JBU's toxicity was decreased by more than 50% on 5th instars *R. prolixus*.
Conclusions: Rhopr-PLA2XII gene is linked to JBU's toxic effect in *R. prolixus* and our findings support previous studies demonstrating that eicosanoids modulate this toxicity.
General significance: Besides identifying and characterizing two PLA_2 genes in the major Chagas' disease vector *R. prolixus*, we have shown that the potent toxicity of JBU is linked to one of these genes. Our results contribute to the general comprehension of urease's mechanisms of action in insects, and, potentially, to studies on the control of the Chagas' disease parasite transmission.

1. Introduction

Ureases are metallo enzymes that catalyze urea hydrolysis and are synthesized by plants, fungi and bacteria [12,28]. These enzymes have other biological properties that are independent from their catalytic function, such as entomotoxic activity and exocytosis induction [18,19,44]. Urease isoforms isolated from the seeds of the legume *Canavalia ensiformis* display strong insecticidal effects, partly due to the processing of urease and release of internal peptides inside the insects' gut. Nymphs of the hemipterans *Rhodnius prolixus*, *Dysdercus peruvianus* and *Oncopeltus fasciatus* are examples of susceptible insects [11,14,38]. It was recently demonstrated in nymphs of *R. prolixus* that within hours after feeding on a urease solution, intact molecules are detected in the hemolymph, and when urease is directly injected into the hemolymph it displays insecticidal effects [46]. These findings indicate that` urease's entomotoxic activity is also due to actions of the intact molecule. When tested *ex vivo* on individual *R. prolixus* tissues, urease interferes with different cell signaling pathways and these effects are modulated by eicosanoids [45,46]. The same happens in different mammalian models, where plant and bacterial ureases induce exocytosis modulated by eicosanoids [3,21,40,52,53].

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) are enzymes on the beginning of the eicosanoid biosynthetic pathway, where they catalyze the hydrolysis of the fatty acid ester at the *sn*−2 position of glycerophospholipids, releasing both unsaturated fatty acids and lysophospholipids [7,8]. These enzymes are remarkably diverse and are found in viruses, bacteria, plants and animals. They are classified into 18 different groups and several subgroups based on their sequence homology and biochemical characteristics [7,8]. Arachidonic acid, like other polyunsaturated fatty acids with 20 carbon chains, is released by PLA_2 hydrolysis reaction and it can be subsequently oxygenated by three different enzymes:

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cyclooxygenase (yielding prostaglandins), lipoxygenase, or cytochrome P 450 — 'epoxygenase'. Most work on insect systems has focused on the prostaglandins, which act as central mediators of the insects' immune response, particularly in cellular reactions [48]. In insects PLA_2s also act in digestion, reproduction and general metabolism [47]. Previous studies characterized the genes encoding PLA_2s in *Drosophila melanogaster* [41], in the bumblebee *Bombus ignitus* [54] and in *Tribolium castaneum* [43].

Rhodnius prolixus is a major vector of human Chagas' disease, a chronic parasitic disease caused by the protozoan *Trypanosoma cruzi*. It is endemic in all Latin American countries, where approximately 100 million people are at risk and 16 million people are estimated to be infected [13,15]. *Rhodnius prolixus* feeds exclusively on vertebrate blood, ingesting up to ten times its own body mass per meal. After ingestion, it must rapidly eliminate large volumes of salts and water, defecating on the host's skin and thereby transmitting the parasite [2,34]. Phospholipids and lysophospholipids (products of PLA_2 hydrolysis) have been shown to be present in the saliva of *R. prolixus*, where lysophosphatidylcholine acts as an antihemostatic molecule facilitating the feeding process [20]. The activity of PLA_2s has also been detected in *R. prolixus* hemolymph and hemocytes, where PLA_2s play roles in cellular immune reactions [16,17]. More recently, it was demonstrated that the lysophosphatidylcholine present in saliva modulates the transmission and infection of *T. cruzi*, along with being present in the feces of *R. prolixus* [27].

Although urease toxicity is modulated by eicosanoids both in insects and in mammals, the oxygenation pathway of arachidonic acid seems to be different in each case. In *R. prolixus* the effect is modulated by prostaglandins, products of cyclooxygenase [46], while in different mammal models the effects are modulated by lipoxygenase products [3,53]. Considering that the common step for both cases is the release of arachidonic acid by PLA_2s, we have investigated the connection between urease toxicity and a PLA_2 gene in the well known model *R. prolixus*.

Here we have identified two PLA_2 genes from *R. prolixus* and cloned the complete coding sequence of the two transcripts. After isolating and identifying the transcripts we have analyzed their expression profiles in different tissues by real time qPCR. We have phylogenetically compared the two *R. prolixus* PLA_2 sequences with other PLA_2 sequences from vertebrates and invertebrates available through GenBank. Also, we have inferred some of the properties of the proteins, which suggest that they are both secretory PLA_2s but possibly with distinct functions. Based on function predictions for the two PLA_2s and their distribution among tissues, we have chosen to knockdown the *Rhopr-PLA2XII* transcript in order to study effects on urease toxicity. We have tested urease on knockdown insects and observed a significant decrease in mortality when compared to control insects, suggesting that in *R. prolixus* urease toxicity is linked to *Rhopr-PLA2XII* gene.

2. Methods

2.1. Screening of the predicted peptidome and the transcriptome of R. prolixus for the identification of putative PLA_2s

In order to identify putative PLA_2s present in *R. prolixus*, the predicted peptidome (available at rprolixus.vectorbase.org/GetData/Downloads/) was screened using the search tool in the program Geneious Basic [25]. The PLA_2 conserved pattern present in all known functional PLA_2s, and characterized by the sequence C-C-{P}-x-H-{LGY}-x-C (accordingly to the online tool PROSITE, prosite.expasy.org, x means any residue, {} means any residue but the ones indicated and the histidine indicates the active site), was used as a reference for the search. After the identification of putative amino acid sequences, the relative mRNA sequences were used to design primers and clone the transcripts.

2.2. Identification of PLA_2 cDNA sequences from 5th instar R. prolixus CNS cDNA library

For the identification of the previously predicted PLA_2 cDNA sequences, a cDNA library from the CNS of 5th instar *R. prolixus* [36] was screened using a modified amplification of cDNA ends (RACE) PCR as outlined below. Gene-specific primers (GSPs) were designed based on two predicted PLA_2 mRNA sequences (Table S1). For the 3′ RACE PCR, GSPs were used successively combined with the cDNA library plasmid reverse primers (Table S1) in a semi-nested PCR approach, which was used to increase the specificity of the amplified products. Next, 5′ RACE GSPs (Table S1) were used successively combined with the cDNA library plasmid forward primers (Table S1), also in a semi-nested approach. After the first round of PCRs was completed, the products were column purified (Biobasic, Markham, ON, Canada) and used as a template for the second semi-nested PCRs. The conditions of all reactions were 3 min initial denaturation at 94 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 59–60 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. The final amplified PCR products were run on 1.5% agarose gels stained with ethidium bromide, and the desired bands were extracted. The amplified fragments extracted from the gels were column purified and cloned using the pGEM-T Easy Vector system, according to the manufacturer instructions (Promega, Madison, WI, USA). The clones expressing the desired products were submitted to column purification and the PLA_2 nucleotide sequences were determined at the Centre for Applied Genomics, at the Hospital for Sick Children in Toronto (MaRS Centre, Toronto, ON, Canada). Sequences were confirmed from at least three independent clones to ensure base accuracy.

2.3. Isolation of the complete PLA_2 ORF nucleotide sequences from 5th instar R. prolixus CNS cDNA library

In order to isolate the complete PLA_2 open reading frame (ORF) nucleotide sequences, the cDNA library from the CNS of 5th instar *R. prolixus* [36] was used as template for PCRs with GSPs (Table S2) designed from the sequences obtained with RACE PCRs. The conditions of all reactions were 3 min initial denaturation at 94 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60–61 °C, extension for 1 min at 72 °C, and a final extension for 10 min at 72 °C. The final amplified PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and the desired bands were extracted. The amplified fragments extracted from the gels were column purified and cloned using the pGEM-T Easy Vector system, according to the manufacturer instructions (Promega, Madison, WI, USA). The clones expressing the desired products were submitted to column purification and the PLA_2 nucleotide sequences were determined at the Centre for Applied Genomics, at the Hospital for Sick Children in Toronto (MaRS Centre, Toronto, ON, Canada). Sequences were confirmed from at least three independent clones to ensure base accuracy.

2.4. Prediction of PLA_2 gene structure and sequence analyses

The predictions of location and size of introns in the identified PLA_2 genes were performed using the nucleotide sequences in a local BLASTN search against the *R. prolixus* genome assembly database with Geneious Basic [25]. After selecting the high scoring hits, the PLA_2 cDNA sequences were compared to the assembled genomic sequences to predict the location and size of exons/introns. The sequences were analyzed for potential signal peptides using the SignalP 4.0 server (www.cbs.dtu.dk/services/SignalP) and TargetP 1.1 server (www.cbs.dtu.dk/services/TargetP), and the membrane topologies were predicted using the TMHMM Server v. 2.0 189 (http://www.cbs.dtu.dk/services/TMHMM/).

2.5. Phylogenetic analysis of PLA_2 proteins

The predicted protein sequences of Rhopr-PLA2III (JQ670894) and Rhopr-PLA2XII (KC896626), along with other arthropod and chordate PLA_2s (*Triatoma matogrossensis*, gi|307095164; *Anasa tristis*, gi|391226623; *T. castaneum*, gi|225543488; *Acyrthosiphon pisum*, gi|326368257; *Phlebotomus arabicus*, gi|242554316; *Drosophila virilis*, gi|195396407; *Drosophila erecta*, gi|194895667; *D. melanogaster*, gi|24641677; *Megachile rotundata*, gi|383849880; *Bombus impatiens*, gi|350406620; *Bombus terrestris*, gi|340721396; *B. terrestris*, gi|340725190; *Bombus lucorum*, gi|229890780; *Harpegnathos saltator*, gi|307198397; *Acromyrmex echinatior*, gi|332029748; *Glossina morsitans*, gi|289742203; *Camponotus floridanus*, gi|307187781; *A. echinatior*, gi|332031357; *Apis mellifera*, gi|328786047; *M. rotundata*, gi|383848342; *Apis florea*, gi|380021879; *Nasonia vitripennis*, gi|156542901; *Pediculus humanus corporis*, gi|242020505; *Branchiostoma belcheri*, gi|374675332; *Gallus gallus*, gi|50749318; *Salmo salar*, gi|209737974; *Sus scrofa*, gi|343183417; *Xenopus* (*Silurana*) *tropicalis*, gi|56118646; *Mus musculus*, gi|148680280; *Anolis carolinensis*, gi|327274146), were aligned using ClustalW [50], and the regions with conserved residues were used to construct the phylogenetic tree. In order to select the best-fit model of amino acid substitution for the data set, the Molecular Evolutionary Genetics Analysis 5 (MEGA5) [49] evaluation tool was employed, suggesting that the most appropriate model would be WAG + G. Two different phylogenetic trees were constructed with the WAG + G model, a maximum likelihood (ML) and a Bayesian inference. The ML trees were generated by MEGA5 with phylogeny test by bootstrap method with 1000 replications to calculate the consensus tree. In order to construct the Bayesian inference, the analyses were carried out with MrBayes [24] for 100,000 generations, sampling every 10 generations with a 'burn-in' of 25%. The obtained topologies were used to calculate the consensus tree, with the percentage of samples recovering any particular clade representing that clade's posterior probability.

2.6. Insects

Fifth instars *R. prolixus* were maintained at high relative humidity in incubators at 25 °C and were fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada). Tissues were dissected under nuclease-free phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada) and stored at −20 °C in RNAlater™ RNA stabilization reagent (Qiagen Inc., Mississauga, ON, Canada) until use for RNA isolation. Hemolymph samples were carefully collected from a cut leg with a 10 μl micropipette and immediately added to RNA extraction TRIzol Reagent (Invitrogen, Burlington, ON, Canada).

2.7. Quantitative tissue-specific expression analysis by real time qPCR

Quantitative expression profiles were conducted with starved fifth instars (3–4 weeks after feeding as fourth instars). Tissues were dissected as described above and pooled into eleven different groups: (1) central nervous system — CNS, (2) salivary glands, (3) foregut, (4) anterior midgut, (5) posterior midgut, (6) hindgut, (7) Malpighian tubules, (8) fat bodies and trachea, (9) immature testes, (10) immature ovaries and (11) hemocytes. Total RNA was isolated from the tissues with TRIzol Reagent (Invitrogen, Burlington, ON, Canada), according to the manufacturer's protocol, and quantified using a NanoDrop System (ND-1000, Thermo Scientific). cDNA was synthesized with 100 ng of RNA from each tissue using the cDNA iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) following the manufacturer recommendations. The cDNA synthesis reactions were diluted to the appropriate concentrations with nuclease-free water and 5 ng of cDNA per well was used as template for real time qPCR reactions. Primers for the amplification of *Rhopr-PLA2III* and *Rhopr-PLA2XII* (Table S2) were optimized to amplify target fragments of similar size across all experimental and reference genes (rp49, β-actin and α-tubulin) [35]. All qPCR reactions were carried out on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada) with reaction parameters as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation for 5 s at 95 °C, annealing for 5 s at 59.5 °C, and extension for 5 s at 72 °C, with data acquisition during the extension step. To validate the specificity of the SYBR Green detected products, melting curve analyses along with gel electrophoresis were performed. The expression levels were quantified using the ΔΔCt method [37] and quantities were normalized to the reference genes. Experiments were repeated for a total of three biological replicates with three technical replicates each.

2.8. Rhopr-PLA2XII double-stranded RNA synthesis

Two fragments, of 653 and 589 nucleotides, from different but overlapping regions in the *Rhopr-PLA2XII* transcript were amplified by PCR from the *R. prolixus* CNS cDNA library [36] using gene specific primers (Table S3). The amplification of two distinct fragments from the same transcript was performed in order to use one of the fragments as a positive control for the gene expression knockdown. As previously validated in the lab, a fragment of the ampicillin resistance gene (ARG) from the pGEM-T Easy Vector system (Promega, Madison, WI, USA) was amplified by PCR with specific primers (Table S3) and used as a negative control throughout the experiment. After the first PCR amplification round, each reaction product was used as template for a PCR with gene specific primers conjugated with the T7 RNA polymerase promoter at the 5′ end (5′-TAATACGACTCACTATAGGGAGA-3′) (Table S3). All PCR amplification conditions were as follow: initial denaturation for 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 60 s at 72 °C, and final extension for 10 min at 72 °C. The resulting products of the second round of PCRs were used as templates for the double-stranded RNA (dsRNA) synthesis using the T7 Ribomax Express RNAi System (Promega, Madison, WI, USA). After synthesis the dsRNAs were precipitated with isopropanol and resuspended in nuclease-free water. Subsequently, the dsRNAs were quantified by a 260 nm wavelength using a NanoDrop System (ND-1000, Thermo Scientific) and the quality of products was verified by 1% agarose gel electrophoresis. The dsRNA products were stored at −80 °C in aliquots, which were independently thawed and diluted with nuclease-free water, to a 1 μg/μl concentration, immediately before use.

2.9. Injections of Rhopr-PLA2XII double-stranded RNA into unfed 5th instar R. prolixus and gene expression knockdown quantification

Injections of the two different *Rhopr-PLA2XII* dsRNAs and the ARG dsRNA were performed using a 10 μl volume capacity Hamilton Syringe (Hamilton Company, Reno, NV, USA) into the hemocoel of starved fifth instars (3–4 weeks after feeding as fourth instars) *R. prolixus*, where 1 μl of nuclease-free water containing 1 μg of dsRNA was injected per bug. The bugs were divided into 3 groups of 20 to 30 individuals each after injections: (1) dsRhopr-PLA2XII injected, (2) positive control dsRhopr-PLA2XII injected and (3) negative control dsARG injected. In order to quantify *Rhopr-PLA2XII* gene expression knockdown, different tissues of bugs from each of the three groups were dissected every day, from day 1 to day 5 after injections. Tissue dissection, RNA extraction and quantitative qPCR were performed as described above, using gene specific primers for *Rhopr-PLA2XII* (Table S2) and reference genes for normalizing expression (rp49, β-actin and α-tubulin) [35]. The *Rhopr-PLA2XII* transcript expression knockdown in dsRhopr-PLA2XII injected bugs, and in positive control dsRhopr-PLA2XII injected bugs, was quantified relatively to the expression of the transcript in dsARG

injected bugs. The expression knockdown screening was repeated to a total of three biological replicates.

2.10. Rhodnius prolixus saline and Jack bean urease

Rhodnius prolixus saline was prepared as follows: NaCl 150 mM, KCl 8.6 mM, $CaCl_2$ 2.0 mM, $MgCl_2$ 8.5 mM, $NaHCO_3$ 4.0 mM, glucose 34.0 mM, HEPES 5.0 mM, and pH 7.0 [26]. Crystalline Jack bean urease type III (JBU) was obtained from Sigma-Aldrich (Mississauga, ON, Canada) and diluted into *R. prolixus* saline to the appropriate concentrations.

2.11. Injections of JBU into Rhopr-PLA2XII knockdown 5th instars R. prolixus

Two days after the injections of 5th instars *R. prolixus* with dsRhopr-PLA2XII and dsARG, 20 to 25 bugs from each group were injected into the hemocoel with JBU diluted in *R. prolixus* saline to a final dose of 0.14 μg per mg of insect. Experiments were repeated for a total of three biological replicates. A negative control group was injected solely with saline along with each toxicity experiment to ensure that the injections were not killing the insects.

2.12. Statistical analysis

Results are expressed as mean ± standard error. Significance of differences between means was determined using one-way ANOVA followed by Holm–Sidack method (SigmaPlot software), and results were considered statistically different when $P < 0.001$.

3. Results

3.1. Identification, cloning and characterization of two PLA_2 transcripts from 5th instars R. prolixus

Based on predicted features of the two identified PLA_2 transcripts, we have named the genes *Rhopr-PLA2III* and *Rhopr-PLA2XII*, because they match with secretory PLA_2s from groups III and XII, respectively. We have cloned and sequenced 862 base pairs of *Rhopr-PLA2III* cDNA, where the coding sequence starts at base 55 and ends at base 768, yielding a protein of 238 amino acids (Fig. 1A). When comparing the cloned cDNA sequence with the genome assembly of *R. prolixus*, using a local BLASTN search on Geneious Basic [25], the corresponding gene exhibited 4 exons and 3 introns, ranging at least 12 kb of the whole

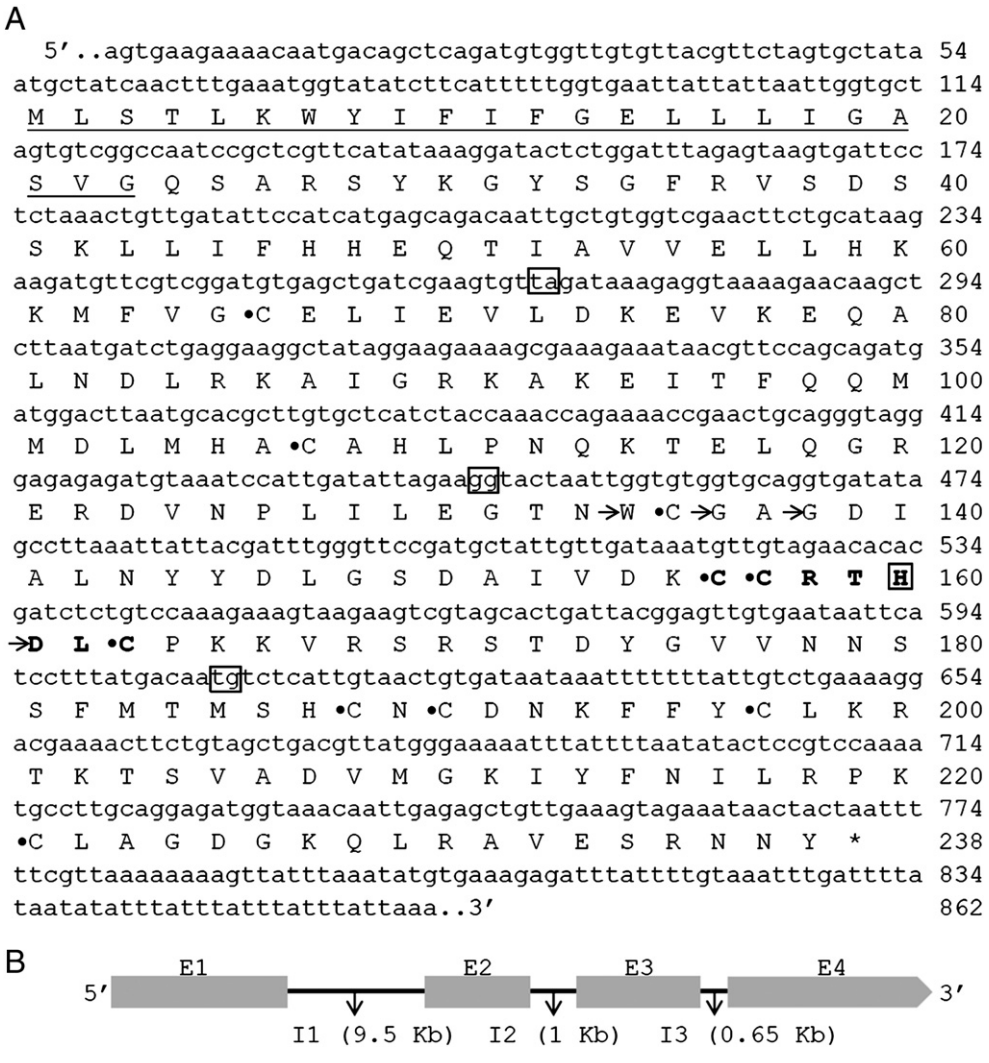

Fig. 1. Nucleotide sequence and predicted structure of *Rhopr-PLA2III* gene in *R. prolixus*. (A) Nucleotide sequence of *Rhopr-PLA2III* and deduced amino acid sequence of the protein. The numbering for each sequence is shown at right. The underlined region from residues 1 to 23 indicates the signal peptide. The characters highlighted in bold indicate the PLA_2 consensus pattern with the histidine residue on the active site inside the box. The dots indicate 10 cysteine residues that form 5 disulfide bonds and the arrows indicate the Ca^{2+} binding loop residues, both characteristic features to the group III sPLA_2s in arthropods. The nucleotides in squares indicate exon–exon boundaries. (B) Schematic diagram of predicted structure of the *Rhopr-PLA2III* gene, with exons and introns represented as boxes and lines, respectively.

genome (Fig. 1B). The putative protein has a signal peptide predicted with the cleavage site between residues 23 and 24, according to SignalP v4.1. The online tool TargetP 1.1 also predicted a signal peptide for the sequence, along with indicating that the protein is secreted to the extracellular environment. According to TMHMM Server v. 2.0, residues 1 to 6 stay inside the cell, residues 7 to 26 comprise a transmembrane helix and residues 27 to 238 are outside the cell, reaffirming the presence of a signal peptide in the N-terminal region of the protein. The putative Rhopr-PLA2III protein has the conserved catalytic histidine residue inside the PLA_2 signature, defined by the residues 156–163 (C-C-R-T-**H**-D-L-C). As specific features for the group III of $sPLA_2$s, Rhopr-PLA2III has 10 cysteine residues that are likely to form 5 disulfide bonds in the mature enzyme, and the conserved Ca^{2+}-binding loop with tryptophan, glycine and aspartic acid residues, respectively, in positions 134, 136, 138 and 161 [32] (Fig. 1A).

For *Rhopr-PLA2XII* we have cloned and sequenced a length of 1016 base pairs of the cDNA, with the coding sequence ranging from base 114 to base 716 and resulting in a 201 amino acid protein (Fig. 2A). After performing a local BLASTN search with the sequenced transcript on Geneious Basic [25], against the genome assembly of *R. prolixus*, it was demonstrated that the correspondent gene has an estimated size of at least 1.2 kb and is composed of 4 exons and 3 introns (Fig. 2B). According to the online tool SignalP v4.1, the putative protein does not have a signal peptide; however, the online tool TargetP 1.1 predicts a signal peptide with a very low score, suggesting that the protein could be secreted. The TMHMM Server v. 2.0 predicts that residues 1 to 6 remain inside the cell, residues 7 to 29 comprise a transmembrane helix and residues 30 to 201 are outside the cell, along with suggesting that there is the possibility of a signal peptide in the N-terminal region of the protein. Again though, this prediction has low scores. The putative Rhopr-PLA2XII protein shows the consensus sequence present in the invertebrate group XII PLA_2 functional domain, with conserved cysteines, asparagine, histidine and aspartic acid in the histidine-catalytic site, denoted by residues 114–121 (**C**-**C**-**N**-E-**H**-**D**-I-**C**). For the Ca^{2+} binding site, Rhopr-PLA2XII has the conserved glycine (residue 95) and the aspartic acid residue in the HD dyad inside the catalytic site, like other invertebrate group XII PLA_2s [31]. Also, as another conserved feature of group XII PLA_2s, the protein has 14 cysteine residues that will form 7 disulfide bonds [7] (Fig. 2A).

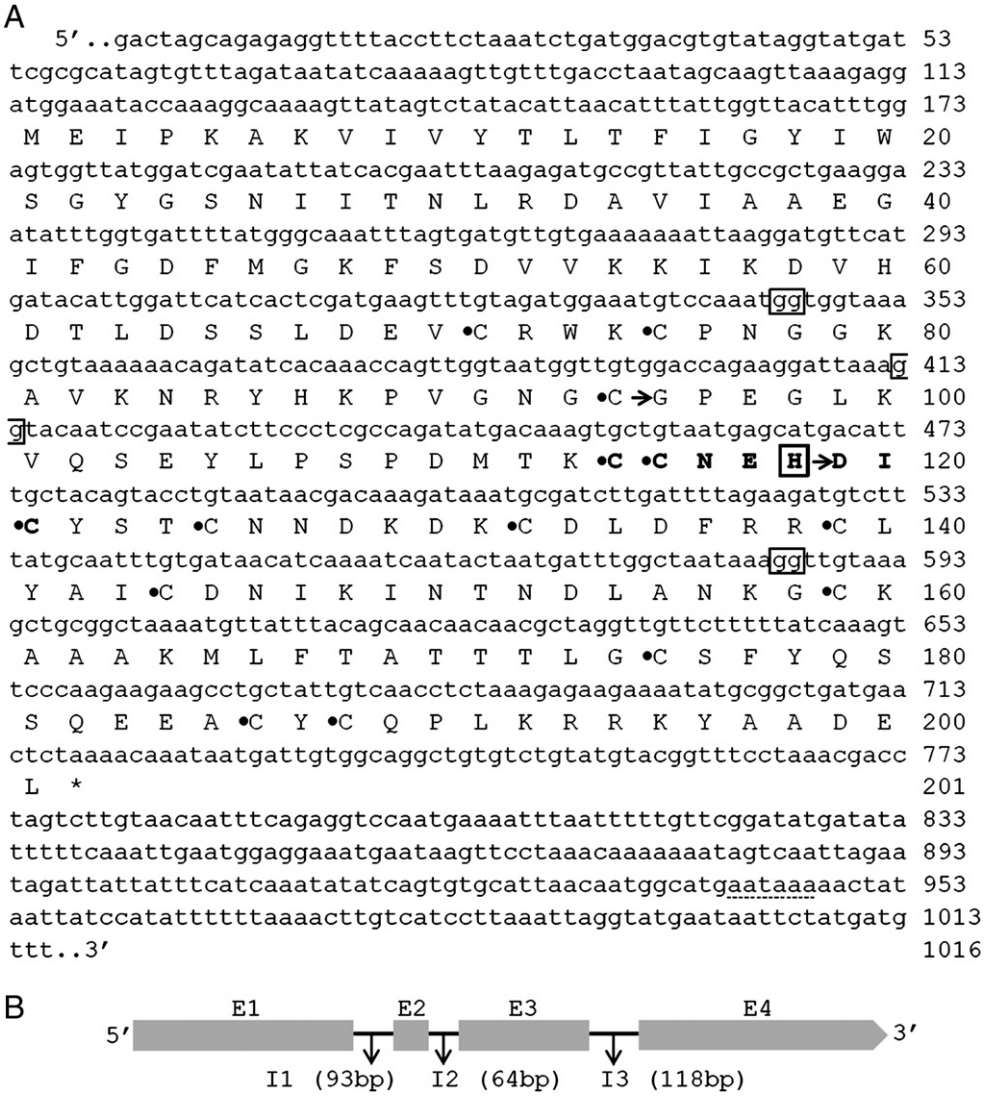

Fig. 2. Nucleotide sequence and predicted structure of *Rhopr-PLA2XII* gene in *R. prolixus*. (A) Nucleotide sequence of *Rhopr-PLA2XII* and deduced amino acid sequence of the protein. The numbering for each sequence is shown at right. The characters highlighted in bold indicate the PLA_2 consensus pattern with the histidine residue on the active site inside the box. The dots indicate 14 cysteine residues forming 7 disulfide bonds and the arrows indicate the Ca^{2+} binding loop residues that are conserved in group XII $sPLA_2$s. The nucleotides in squares indicate exon–exon boundaries. (B) Schematic diagram of predicted structure of the *Rhopr-PLA2XII* gene, with exons and introns represented as boxes and lines, respectively.

3.2. Characterization of Rhopr-PLA2III and Rhopr-PLA2XII expression profiles in unfed 5th instars

The spatial distribution pattern of *Rhopr-PLA2III* and *Rhopr-PLA2XII* was investigated using qPCR. *Rhopr-PLA2III* transcript is observed in most tissues with relatively low expression levels, except for the CNS, the salivary glands and the hindgut where it is highly expressed (Fig. 3). *Rhopr-PLA2XII* transcript is expressed in all tissues examined with similar abundant levels. The highest expression, observed in the salivary glands and in the immature testis, is only 3 fold higher than the lowest, observed in the anterior midgut and in the hemocytes (Fig. 4). In order to compare the expression of both transcripts, we quantified *Rhopr-PLA2XII* relative to *Rhopr-PLA2III*, where we observed that in all tissues scanned, except for the hindgut, *Rhopr-PLA2XII* has a higher expression rate. The anterior midgut has the biggest difference between the two transcripts, with *Rhopr-PLA2XII* being expressed almost 120 fold higher than *Rhopr-PLA2III* (Fig. 5).

3.3. Phylogenetic analyses of PLA_2s

The alignments of the PLA_2 sequences were performed with ClustalW and the conservative region around the active site was used to construct the phylogenies (Fig. 6). Two types of phylogenetic trees were constructed, a Bayesian inference and a maximum likelihood (ML). The Bayesian inference was used to generate trees and measure nodal support for the relationship among sampled sequences. The analyses were carried out with MrBayes for 1,000,000 generations, sampling every 10 generations, with a 'burn-in' of 25%. The obtained topologies were used to calculate the consensus tree, with the percentage of samples recovering any particular clade representing that clade's posterior probability (Fig. 7). The ML trees were generated with MEGA5 using phylogeny test by bootstrap method and 1000 replications to calculated the consensus tree (Fig. S1). The two PLA_2s from *R. prolixus* group separately into two distinct branches. As expected, Rhopr-PLA2XII grouped with other arthropod's group XII PLA_2s, while Rhopr-PLA2III grouped with a different set of sequences, where some of them have already been identified as possible group III $sPLA_2$s, like *T. castaneum* PLA_2 [43] and *H. saltator* PLA_2 [5].

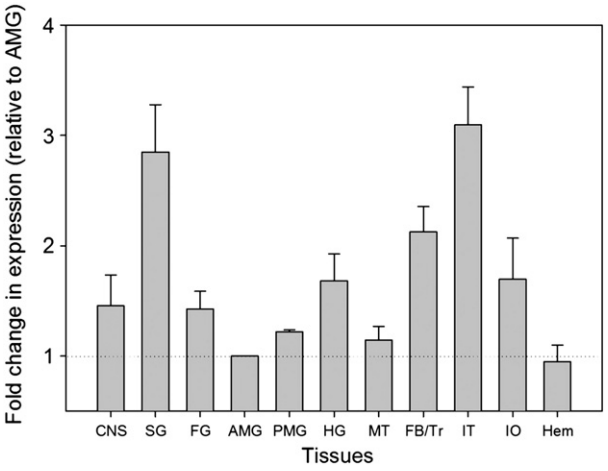

Fig. 4. Expression pattern of *Rhopr-PLA2XII* in various tissues of 5th instar *R. prolixus*. Tissues were dissected from at least 10 insects and pooled together into different categories: CNS — central nervous system, SG — salivary glands, FG — foregut, AMG — anterior midgut, PMG — posterior midgut, HG — hindgut, MT — Malpighian tubules, FB/Tr — fat body and trachea, IT — immature testes, IO — immature ovaries and Hem — hemocytes. The expression was quantified by qPCR and fold difference is relative to the anterior midgut (AMG), which is the tissue with lowest expression levels for *Rhopr-PLA2XII* transcript. Results are shown as means of 3 biological replicates with standard error.

3.4. Knockdown of Rhopr-PLA2XII expression in 5th instars and influence on urease toxicity

Based on what is known about urease toxic activity in *R. prolixus* [45,46] and the observed characteristics and distribution of both PLA_2 transcripts described here, we decided to investigate the involvement of the toxin with *Rhopr-PLA2XII* gene. We have tested the influence of Jack bean urease (JBU) on the expression of the transcript, where we observed that it does not alter transcript expression following injection with JBU (data not shown). In order to further investigate the connection of JBU toxicity with *Rhopr-PLA2XII* we used RNA interference to silence the expression. *Rhopr-PLA2XII* transcript in the knockdowns was always quantified relative to the expression of the transcript in the bugs injected with ARG dsRNA. We quantified

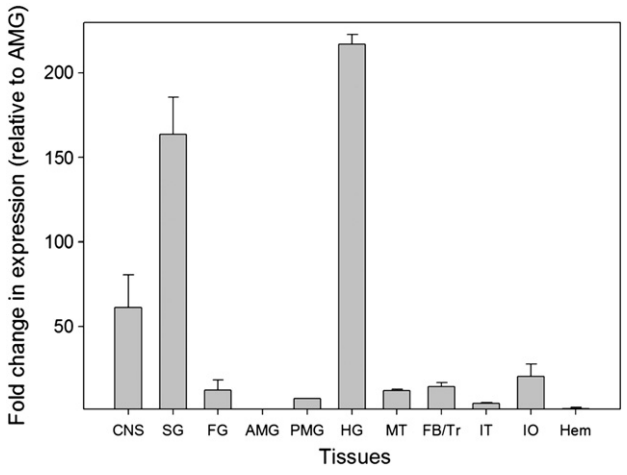

Fig. 3. Expression pattern of *Rhopr-PLA2III* in various tissues of 5th instar *R. prolixus*. Tissues were dissected from at least 10 insects and pooled together into different categories: CNS — central nervous system, SG — salivary glands, FG — foregut, AMG — anterior midgut, PMG — posterior midgut, HG — hindgut, MT — Malpighian tubules, FB/Tr — fat body and trachea, IT — immature testes, IO — immature ovaries and Hem — hemocytes. The expression was quantified by qPCR and fold difference is relative to the anterior midgut (AMG), which is the tissue with lowest expression levels for *Rhopr-PLA2III* transcript. Results are shown as means of 3 biological replicates with standard error.

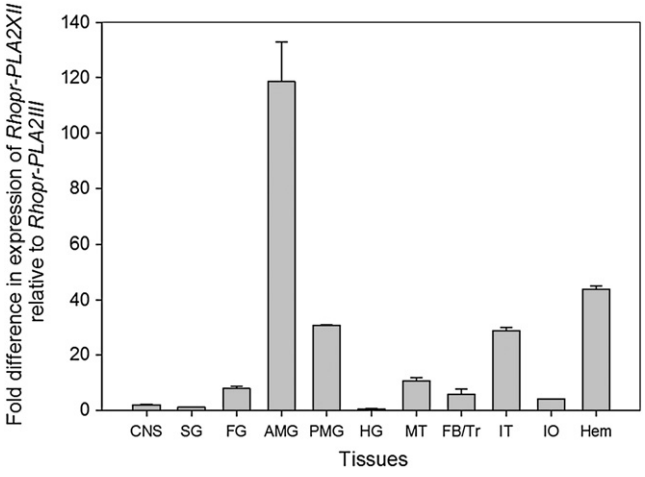

Fig. 5. Expression pattern of *Rhopr-PLA2XII* relative to *Rhopr-PLA2III* in various tissues of 5th instar *R. prolixus*. Tissues were dissected from at least 10 insects and pooled together into different categories: CNS — central nervous system, SG — salivary glands, FG — foregut, AMG — anterior midgut, PMG — posterior midgut, HG — hindgut, MT — Malpighian tubules, FB/Tr — fat body and trachea, IT — immature testes, IO — immature ovaries and Hem — hemocytes. The expression was quantified by qPCR and fold difference in *Rhopr-PLA2XII* transcript expression is relative to the expression *Rhopr-PLA2III* in the indicated tissues. Results are shown as means of 3 biological replicates with standard error.

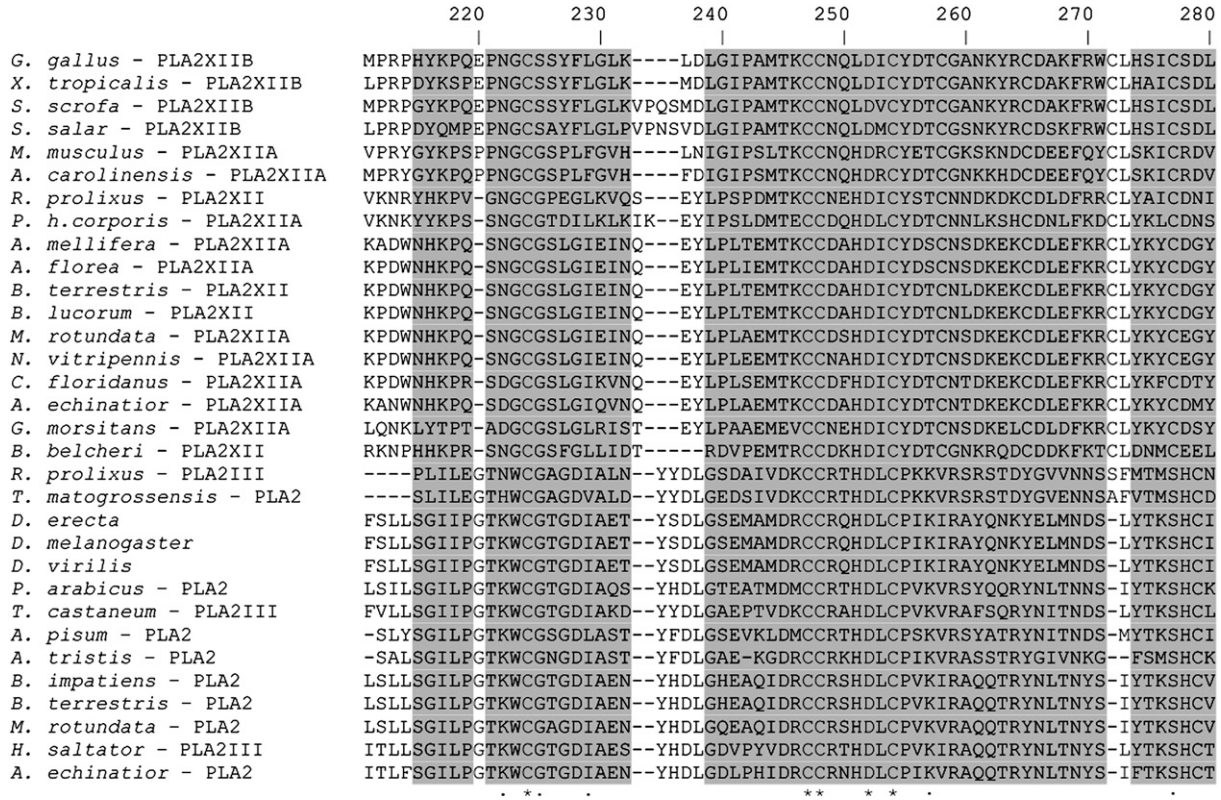

Fig. 6. Alignment of different PLA_2 sequences from arthropods and chordates. ClustalW alignment of the sequences was used to construct the trees. The gray shadows represent the conservative region around the active site. "*" indicates highly conserved residues and "." indicates residues with similarity.

the knockdown of *Rhopr-PLA2XII* expression from 24 h up to 5 days after injection, and we observed an average reduction of 70% among tissues that was persistent throughout this period, with exception of the hemocytes, where we did not observe any reduction in expression. JBU toxicity was significantly reduced in *Rhopr-PLA2XII* knockdown insects when compared to insects that have normal expression of the transcript. 72 h after JBU injections, 90% of negative control bugs (ARG dsRNA injected) had died, in contrast to only 40% mortality in the *Rhopr-PLA2XII* knockdown group (Fig. 8).

4. Discussion

In the present work we have identified two genes encoding PLA_2s in *R. prolixus*, *Rhopr-PLA2III* and *Rhopr-PLA2XII*. We have cloned the two respective transcripts and inferred characteristics of the putative proteins, which, according to the predictions, are both secretory PLA_2s, but from different groups. The first gene, *Rhopr-PLA2III*, encodes a protein of 238 amino acids with several conserved features of group III PLA_2s, while the second gene, *Rhopr-PLA2XII*, encodes a 201 residue protein with characteristics of group XII PLA_2s. When phylogenetically compared to other PLA_2s, including representatives of groups III and XII, the sequences from *R. prolixus* group separately in the respective branches of each group, supporting the classification based on structural predictions. *Rhopr-PLA2III* is expressed in most tissues of 5th instars *R. prolixus* in relatively low levels, with exception of the central nervous system, the salivary glands and the hindgut, where expression is relatively high. On the other hand, the *Rhopr-PLA2XII* transcript is more highly expressed, and in similar levels, in all tissues scanned. When comparing the expression of the two transcripts we observed that in all tissues, except for the hindgut, *Rhopr-PLA2XII* has the highest levels, especially in the anterior midgut and in the hemocytes. The presence of PLA_2s was reported in the saliva and salivary glands of arthropods, including the caterpillar *Manduca sexta* [51] and the tick *Amblyomma americanum* [6], where they are involved in digestive processes. Also, PLA_2 transcripts were identified in the salivary glands of the sand fly *P. arabicus* [23], the mosquito *Anopheles funestus* [9] and the dipteran 'Tsetse' *G. morsitans* [1]. Rhopr-PLA2III has a strongly predicted signal peptide, suggesting that the mature enzyme could act in digestion of phospholipids outside the cell, such as inside the salivary glands. It was demonstrated that group III secretory PLA_2s efficiently hydrolyse high density phospholipids releasing lysophosphatidylcholine (lysoPC) [42]. Phospholipids and lysophospholipids are present in *R. prolixus* saliva, where they play antihemostatic roles during the feeding process [20]. LysoPC is one of the lysophospholipids present in *R. prolixus* saliva, acting as a modulator of *T. cruzi* infection to the vertebrate host [27]. The prominent presence of *Rhopr-PLA2III* transcript in the hindgut, along with cell signaling, could be related to the digestion of the remaining phospholipids in the blood meal, and once the parasite is transmitted with the feces, the parasite could be taking advantage of the availability of these products for the infection process. In the CNS, *Rhopr-PLA2III* is probably linked to the breakdown of phospholipids from cell membranes and to cell signaling, as already observed for $sPLA_2$s in vertebrate models [8,30].

In contrast to Rhopr-PLA2III, Rhopr-PLA2XII does not have a signal peptide predicted by the online tool SignalP; however other tools suggest, with a low score, that there could be a signal peptide present in the N-terminal region of the protein. Rhopr-PLA2XII could be associated with cell membranes, or even with organelle membranes as shown for a murine group XII PLA_2 that is associated with Golgi/endoplasmic reticulum membranes. It was suggested that this same group XII PLA_2 provides arachidonic acid to cyclooxygenase 2 for the generation of prostaglandins in cells of the immune system of murines [22]. The biosynthesis of eicosanoids seems to be dependent on the cellular location of the enzymes involved in the process, which apparently occurs in perinuclear locations [4]. Since there is considerable conservation within the group XII PLA_2 catalytic functional domains across evolution from

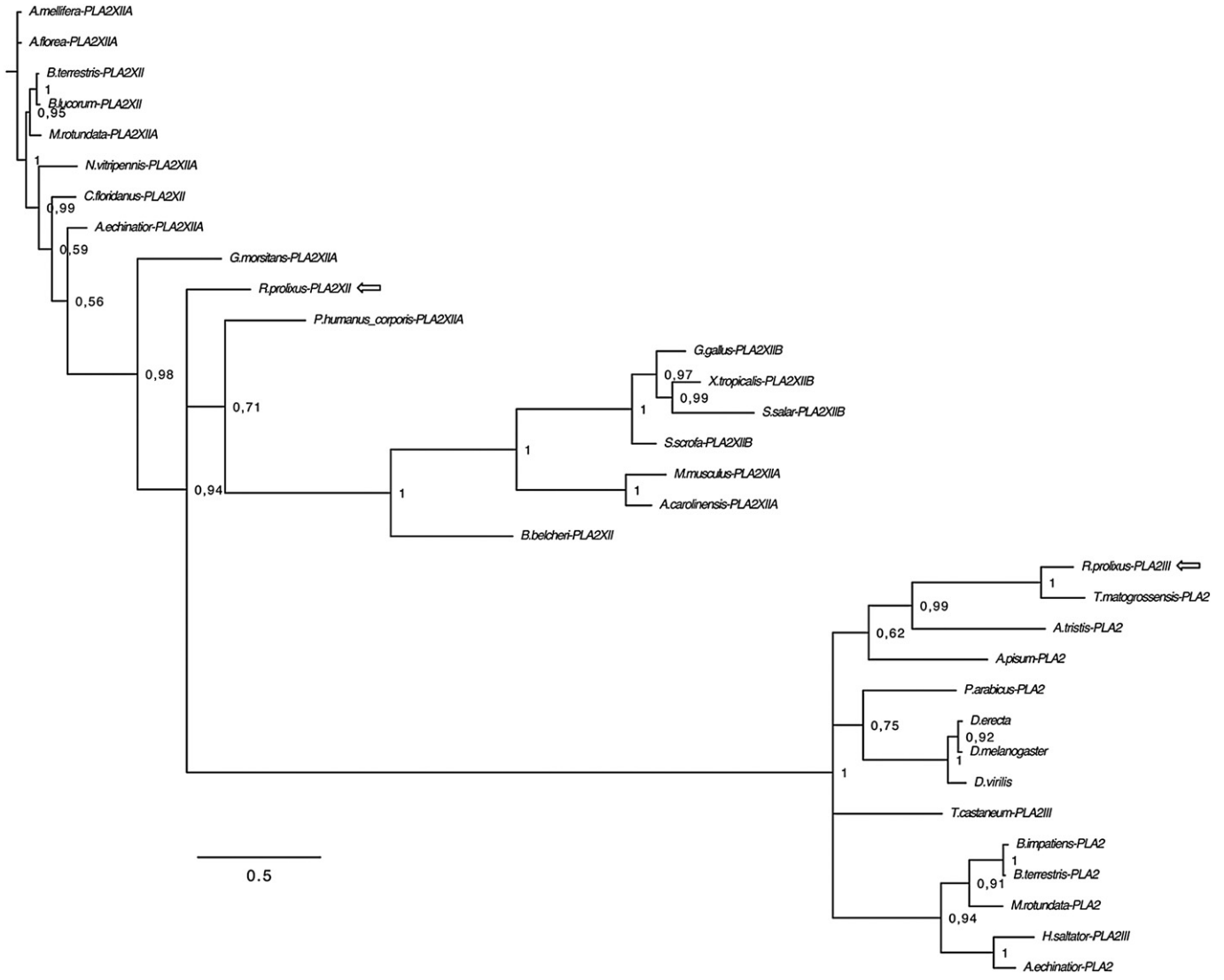

Fig. 7. Bayesian inference for phylogenetic analysis of different PLA_2 sequences from arthropods and chordates. Phylogenetic tree illustrating the relationships among 30 PLA_2s, Rhopr-PLA2III and Rhopr-PLA2XII, generated by the Bayesian inference using the WAG + G model. The analyses were carried out for 100,000 generations, sampling every 10 generations with a 'burn-in' of 25%. Rhopr-PLA2XII and Rhopr-PLA2III are indicated by arrows.

vertebrates to invertebrates [31], Rhopr-PLA2XII could also be an enzyme releasing arachidonic acid inside the cells for subsequent oxygenation and production of prostaglandins. The activity of PLA_2s, sometimes also along with presence of prostaglandins, has been detected in *R. prolixus* hemolymph and hemocytes, where they apparently play roles in cellular immune reactions. This activity has been identified in living insects as well as in isolated hemocytes [16,17], suggesting that the eicosanoid signaling for hemocyte functions can be locally produced in those cells. When Jack bean urease (JBU) was tested in preparations *ex vivo* on *R. prolixus* anterior midgut [46] and Malpighian tubules [45], it caused disturbing effects modulated by eicosanoids, indicating that these molecules can also be locally produced in those tissues.

Because of the relatively very low expression of *Rhopr-PLA2III* in the anterior midgut and other tissues, we have tested the influence of a JBU treatment on the expression of the gene, in order to check for a possible up-regulation after exposure to the toxin. 1, 6 and 18–20 h after injecting 5th instars *R. prolixus* with JBU, neither *Rhopr-PLA2III* nor *Rhopr-PLA2XII* expression levels changed in any tissues. These findings suggest that it is unlikely that *Rhopr-PLA2III* is linked to JBU's toxic activity, since the transcript is not significantly present in the anterior midgut (where JBU has a strong eicosanoid mediated effect), even when the insects are challenged with the toxin. The fact that *Rhopr-PLA2XII* does not have its expression altered is not enough to discard any linkage with JBU's toxicity, since the connection could be downstream with PLA_2 products.

To further investigate a possible interaction between *Rhopr-PLA2XII* presence and JBU's toxicity, we used RNA interference to knockdown the expression. The *Rhopr-PLA2XII* transcript was reduced by an average of 70% in the tissues throughout the 5 days of quantification. Subsequently we tested JBU on knockdown insects. Interestingly, JBU's toxicity was significantly decreased in knockdown insects, with more than 50% reduction in mortality when compared to those normally expressing the transcript. When 5th instars *R. prolixus* are injected into the hemolymph with 0.25 μg of JBU per mg of insect, a 96% mortality is observed 24 h after injection [46], but whether this lethal action was mediated by eicosanoids, as in isolated tissues, was unclear. With our results we can conclude that normal expression levels of *Rhopr-PLA2XII* are necessary for JBU's toxicity in living insects, and *Rhopr-PLA2XII* is likely to be responsible for the release of eicosanoids modulating this toxicity. We are not excluding the existence and involvement of more PLA_2s in the eicosanoid biosynthesis pathway in *R. prolixus*, and also we are not excluding the possibility that *Rhopr-PLA2III* produces

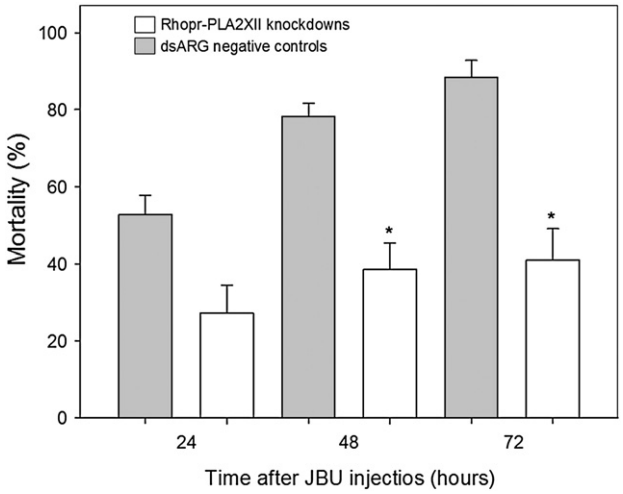

Fig. 8. Mortality of *Rhopr-PLA2XII* knockdown in 5th instar *R. prolixus* after JBU injections. Insects were injected into the hemocoel with 1 μl of nuclease-free water containing 1 μg of dsRhopr-PLA2XII or 1 μg of dsARG. 48 h after the dsRNA treatment, the same insects were injected with JBU to a final dose of 0.14 μg per mg of insect. Mortality was monitored for 72 h after JBU injections. Results are shown as means of 3 biological replicates with standard error. "*" indicates statistically significant difference.

eicosanoids in this insect. The fact that JBU's toxicity was not totally inhibited suggests that the cells that are still expressing *Rhopr-PLA2XII* could be sufficient to signal deleterious effects in *R. prolixus*. It is interesting to note that in the hemocytes we did not observe any reduction in expression, which could mean that those cells play a significant role in JBU's toxicity signaling. In order to better understand the interactions between *Rhopr-PLA2XII* and JBU, future experiments focusing on the PLA_2 protein and at the physiological level are necessary.

For the past 30 years ureases have been studied as potent toxins that display a variety of effects in different models, including fungi, insects and mammals [10,14,18,33,39]. Throughout the years many advances have been made in elucidating ureases' effects, such as the discovery of urease's internal insecticidal peptides, which led to the design of a recombinant peptide with high potential for biotechnological applications [29]. Other advances have been made in the fields of medically important organisms, as for example the bacterial urease from *Helicobacter pylori* that induces inflammation and exocytosis in different cell models modulated by eicosanoids [52,53]. The recent findings on the eicosanoid modulated toxic effects of JBU in *R. prolixus* opened the door for the present study, where, for the first time, an insect gene linked to urease's toxic effect was identified and characterized. Also, *Rhopr-PLA2III* and *Rhopr-PLA2XII* are the first phospholipase A2 genes to be identified and to have their transcripts cloned in a Chagas' disease vector, which may be helpful for future studies on the control of the transmission of the parasite *T. cruzi*.

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