Cloning and characterization of the Argonaute-1 gene from *Macrobrachium rosenbergii*

Rattikon Thammason[1] and Amnat Phetrungnapha[2,*]
[1]Undergraduate student, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand
[2]Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand
*Corresponding author. E-mail: amnatp@nu.ac.th

ABSTRACT

RNA interference (RNAi) is a post-transcriptional gene silencing process mediated by a unique family of RNA-binding proteins named Argonaute (Ago). In this study, we identified and characterized the gene encoding Argonaute-1 from the giant freshwater prawn, *Macrobrachium rosenbergii*. The complete open reading frame of *M. rosenbergii* Ago1 gene (MrAgo1) consisted of 2823 nucleotides encoding a polypeptide of 840 amino acids with a predicted molecular weight of 105 kDa and an isoelectric point of 9.36. Analysis of the deduced amino acid sequence of MrAgo1 revealed the presence of two signature domains of Ago proteins, PAZ and PIWI. Phylogenetic analysis indicated that MrAgo1 is classified into Ago subfamily and shared the highest amino acid sequence identity (91%) with *Marsupenaeus japonicus* Ago1. MrAgo1 gene was highly expressed in lymphoid organ and gills. Further studies elucidating the function of MrAgo1 will be benefit in understanding the mechanism of RNAi in this economically important species.

Keywords: giant freshwater prawn, Argonaute-1, RNA interference, RACE-PCR

INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii*, is an economically important crustacean, being farmed on a large scale in many different countries. Its culture is mostly developed in Southern and South-Eastern Asian countries, such as continental China, India, Thailand, and Taiwan. The global production of farmed *M. rosenbergii* increases from year to year. The income in Asia alone is now worth 1 billion US$ per annum (New and Nair 2012). However, production of this prawn worldwide is facing a serious threat from fatal diseases caused by nodaviruses and bacteria, particularly from the *Vibrio* species (Khuntia et al. 2008, Pillai and Bonami 2012). The emergence of these pathogens has had a detrimental impact on the *M. rosenbergii* farming industry, causing considerable economic losses.

Therefore, in order to develop approaches to lessen the effects of these diseases for sustainable crustacean industry, there is much interest in understanding crustacean immune system. RNA interference (RNAi) emerges at a historic moment to be an important immune mechanism in eukaryote, and can be considered as a new promising therapeutic modality to combat pathogen infection in crustacean.

RNAi is a biological process whereby small RNA duplexes including short-interfering RNA (siRNA) and microRNA (miRNA) trigger post-transcriptional gene silencing. This mechanism is evolutionarily conserved and found in many eukaryotes. RNAi is initiated by the Ribonuclease III enzyme, Dicer, which cleaves long double-stranded RNA (dsRNA) and precursor miRNA into siRNA and miRNA, respectively. After incorporated into an RNA-induced silencing complex (RISC), these small RNA duplexes are unwound. The remaining strand, called guide strand, then guides RISC to the target mRNA by complementary base-pairing for subsequent nucleolytic cleavage or translation inhibition of the target mRNA (Wilson and Doudna 2013). RNAi regulates gene expression in several cellular processes such as cell cycle, cell death, cellular metabolism, signal transduction, and development. In invertebrates, RNAi is employed as a major defense system against viruses.

Argonaute (Ago) proteins are 100-kDa multidomain proteins which function as a key player in RNAi pathway. Ago proteins bind siRNA and miRNA to form the RISC, and then direct either target RNA degradation or translational inhibition. Ago proteins are evolutionarily conserved in a wide range of eukaryotes (Hutvagner and Simard 2008). All Ago proteins shares two common structural domains, including PAZ and PIWI domains. The PAZ domain exhibits oligonucleotide-binding fold which can recognize the 2-nt overhang at the 3′-end of the small RNA duplex. The PIWI domain is structurally similar to ribonuclease H (RNase H) enzyme, and consists of the conserved catalytic residues DDE/H motif which is crucial for the endonucleolytic “slicer” activity of the RISC (Meister 2013).

Accumulating evidence demonstrates that RNAi plays a pivotal role in crustacean innate immunity. A number of RNAi-related proteins were identified and characterized, especially in penaeid shrimp (Sagi et al. 2013). Moreover, the existence of miRNA in crustacean has been recently demonstrated (Huang and Zhang 2012, Xi et al. 2015). Nevertheless, the information of the RNAi-based mechanism in the giant freshwater prawn is still largely elusive. Identification of the RNAi-related proteins is not only important for elucidating their functions, but also provides more insights into RNAi mechanism and innate immune response in these economically important species. Here, we

reported the identification of the Argonaute-1 gene from the giant freshwater prawn (MrAgo1). In addition, the molecular evolution and tissue distribution of MrAgo1 were also described.

METHODOLOGY

RNA extraction and cDNA synthesis

Giant freshwater prawns, *Macrobrachium rosenbergii* (~30 g body weight) were purchased from a local restaurant in Phitsanulok, Thailand. Hemolymph was drawn out from the ventral sinus using a sterile syringe containing anticoagulant (19.3 mM NaCl, 239.8 mM sodium citrate, 182.5 mM glucose, 0.5 M EDTA, pH 7.0). Hemocyte was immediately separated from the plasma by centrifugation at 3000 g for 3 min at 4°C. Total RNA was then extracted from hemocyte by using Ribozol™ RNA Extraction Reagent (Amresco), according to the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Reverse transcription was performed with PRT-oligo-dT_{12} primer using SuperScript III® reverse transcriptase (Invitrogen), following the manufacturer's instruction.

Cloning of the MrAgo1 cDNA

To amplify a partial sequence of the MrAgo1 gene, a pair of degenerate primers, dAgo1-F and dAgo1-R (Table 1) was designed based on the conserved regions of the PIWI domain of Ago1 proteins from *Litopenaeus vannamei* (GenBank accession number: ADK25180.1), *Penaeus monodon* (GenBank accession number: ABC68592.1), and *Marsupenaeus japonicus* (GenBank accession number: ADB44074.1). PCR was performed according to the following condition: denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s. The final extension was carried out at 72 °C for 7 min. The PCR products were analyzed using agarose gel electrophoresis. The target band was then purified from the gel by using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience) and cloned into the pTA2 vector (Toyobo) for DNA sequencing.

Amplification of the 5′ and 3′ end sequences

Rapid amplification of cDNA ends (RACE) was used for identifying the 5′ and 3′ end sequences. Primers for RACE-PCR were designed from a partial sequence of MrAgo1 cDNA. For 3′RACE, PCR was performed with 3R-AGO1-F1 and 3R-AGO1-R. The PCR products were then diluted and used as a template for nested PCR with 3R-AGO1-F2 and 3R-AGO1-R. For 5′RACE,

addition of A-tail to the 3′-end of cDNA was performed using terminal deoxynucleotidyl transferase (TdT) (New England Biolabs). The A-tailed cDNA was then purified by using HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience), and used as a template for PCR with 5R-AGO1-R1 and PRT-oligo-dT_{12} primers. The nested PCR was then performed with 5R-AGO1-F and 5R-AGO1-R2. The PCR products from 5' and 3'RACE-PCR were purified, cloned into the pTA2 vector (Toyobo), and sequenced. All primers used for cloning were listed in Table 1. Schematic diagrams showing RACE-PCR are demonstrated in Supplementary figure 1 and 2.

Sequence analysis of MrAgo1

Molecular weight and isoelectric point of the putative MrAgo1 protein were predicted by tools in Expasy (www.expasy.org). Motifs of MrAgo1 were identified by using ScanProsite. The deduced amino acid sequence of MrAgo1 was compared with other known Ago sequences available in GenBank by using Blastp. Ago protein sequences from other species were retrieved from GenBank and used for multiple sequence alignment and phylogenetic analysis. Multiple sequence alignment was performed by using ClustalW. Phylogenetic tree was constructed by using MEGA 5.05 based on the Neighbor-joining method. Bootstrap values of 1000 replicates were used for the consensus tree. Argonaute proteins used for multiple sequence alignment and phylogenetic analysis are shown in Supplementary table 1.

Tissue distribution study of MrAgo1

Tissues including hemocyte, gills, stomach, hepatopancreas, lymphoid organ, and muscle were collected and dissected from prawns (n = 3). Total RNA was then extracted from tissues using Ribozol™ RNA Extraction Reagent (Amresco), according to the manufacturer's protocol. RT-PCR was then performed to detect the expression of MrAgo1 gene in various tissues. β-actin was used as an internal control gene. Primers used for detection of MrAgo1 expression are listed in Table 1.

Table 1. Primers used in this study**.**

Primers	Sequence (5'→3')	Experiments
PRT-oligo-dT	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTT TTTTTTTTTT	RT-PCR
dAgo1-F	GTGAAGCGAGTAGGTGATAC	RT-PCR
dAgo1-R	CTGCAATGTACGTGATACCAG	RT-PCR
3R-AGO1-F1	TCAACGAGCCAGTAATCTTC	3'RACE-PCR
3R-AGO1-F2	CCGTCCATTGCAGCTGTCGT	3'RACE-PCR
3R-AGO1-R	TTAAGCAAAGTACATGACTC	3'RACE-PCR
5R-AGO1-RT	TACTGGCTCGTTGAACACC	5'RACE-PCR
5R-AGO1-R1	CTTGACATTAATCTTAAGGC	5'RACE-PCR
5R-AGO1-R2	CGGACTGTACGCTGTGG	5'RACE-PCR
5R-AGO1-F	ATGTACCCTGTTGGGCAG	5'RACE-PCR
MrAgo1-F	CTCATTGTGGTGCAATGCG	Tissue distribution
MrAgo1-R	CTTAATCATGGTGGACGTC	Tissue distribution
Actin-F	TCTTCCAGCCTTCCTTCCTTG	Tissue distribution
Actin-R	GACGTCGCACTTCATGATGCT	Tissue distribution

RESULTS AND DISCUSSION

We have successfully cloned the cDNA encoding Argonaute-1 from the giant freshwater prawn, *M. rosenbergii* (MrAgo1). A partial MrAgo1 fragment of 572 bp was amplified by using degenerate primers designed from penaeid shrimp Ago1 genes. Then, 5′ and 3′ RACE-PCR were performed to obtain the cDNA encoding MrAgo1. The DNA fragments of 1620 bp and 817 bp were obtained from 5′ and 3′ RACE-PCR, respectively (data not shown). After combining the sequences, the complete open reading frame (ORF) of MrAgo1 was successfully identified. The complete ORF of MrAgo1 consisted of 2823 bp, encoding a polypeptide of 940 amino acids with a predicted molecular weight of 105 kDa and an isoelectric point of 9.36. Analysis of the deduced amino acid sequence of PmAgo3 revealed the presence of two signature domains of Ago proteins, PAZ (amino acid 282-402) and PIWI (amino acid 571-899). The complete ORF and domain organization of MrAgo1 protein are shown in Figure 1. PAZ domain is a small RNA-binding domain that is required for recognition of 3'-end of the guide RNAs (Ma et al. 2004). Studies on isolated PAZ domains from different organisms revealed that this domain contains oligonucleotide/oligosaccharide-binding fold that anchors the characteristic two-nucleotide 3' overhang that results from RNase III digestion (Lingel et al. 2003, Song et al. 2003). It has been recently demonstrated that even though PAZ domain is dispensable for Ago loading of slicing-competent RISC, it plays an important role in RISC activation (Gu et al. 2012).

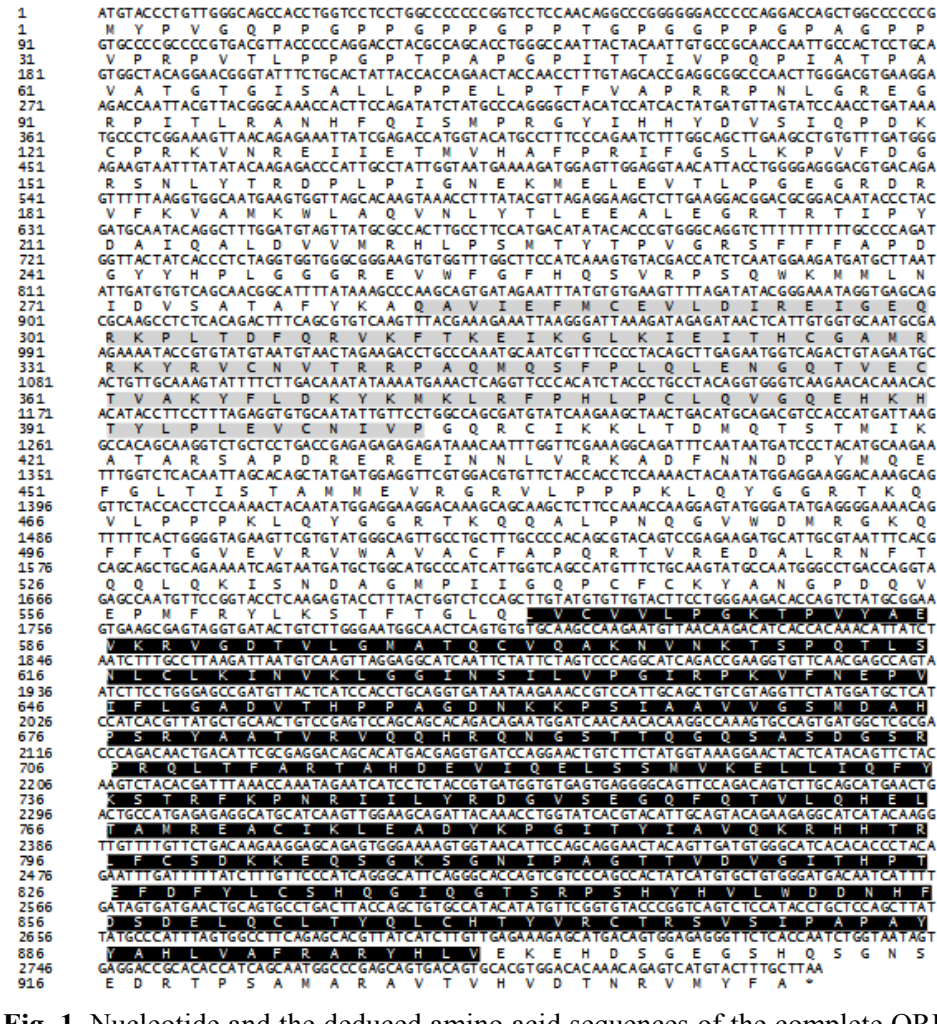

Fig. 1. Nucleotide and the deduced amino acid sequences of the complete ORF of MrAgo1. The deduced amino acid sequence of MrAgo1 is shown in a single letter under the respective codon. The PAZ and PIWI domains are highlighted in light gray and black, respectively.

PIWI domain is a catalytic domain of Ago protein. This domain shows extensive homology to RNase H (Hutvagner and Simard 2008). Figure 2 shows multiple sequence alignment based on PIWI domain of MrAgo1 and shrimp Ago proteins. The alignment revealed several conserved motifs within the PIWI domain. These motifs were predicted to be involved in an anchoring of the 5′-end of the small RNA to target RNA degradation. The conserved catalytic triad composing of DDH motif was also identified. It is also known that Ago can

directly bind to other proteins. It has been demonstrated that the PIWI box (a motif located within the PIWI domain) of human Ago2 binds to one of the RNase III domains of Dicer (Tahbaz et al. 2004). This interaction is important for the selection of the active strand of siRNAs and miRNAs (Hutvagner and Simard 2008). PIWI domain of the fly Ago1 directly interacts with GW182, a protein that is characteristic of cytoplasmic processing bodies (P-bodies), and also has a role in miRNA-mediated gene regulation (Eulalio et al. 2008).

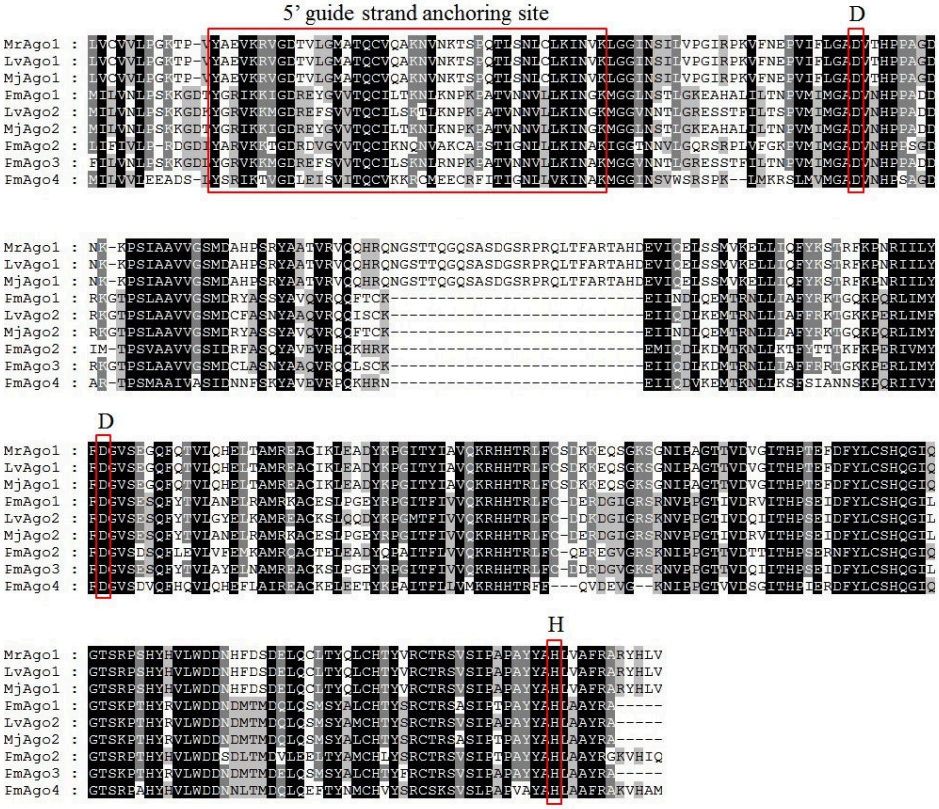

Fig. 2. Multiple sequence alignment of the PIWI domains of MrAgo1 and shrimp Ago proteins by ClustalW. The 5'guide strand anchoring site and DDH motif are boxed. MrAgo1, *Macrobrachium rosenbergii* Argonaute-1. Argonaute proteins used for multiple sequence alignment are shown in Supplementary table 1.

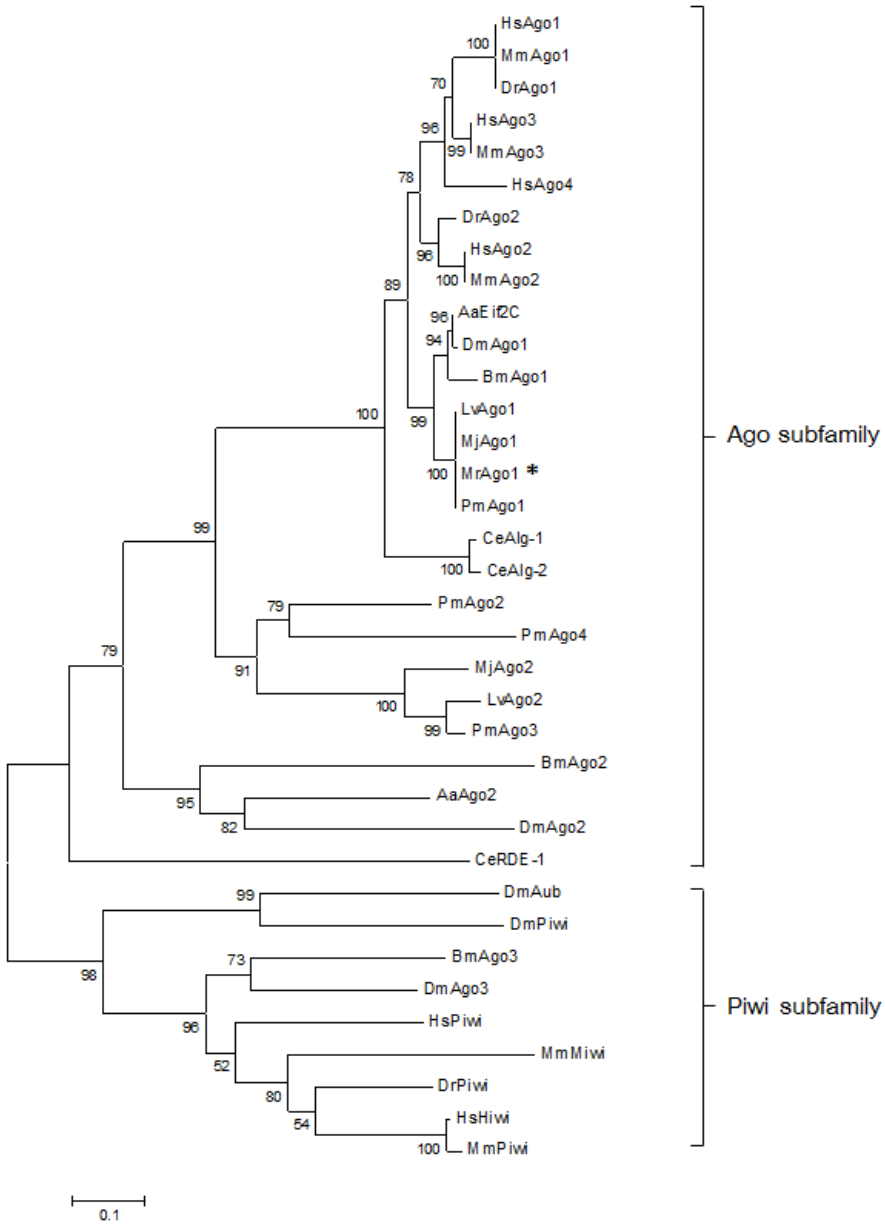

Fig. 3. Phylogenetic relationship of Argonaute proteins. The Neighbor-joining tree was constructed based on multiple sequence alignment of the PIWI domain with bootstrap value of 1000. The asterisk indicates MrAgo1 (*Macrobrachium rosenbergii* Argonaute-1). Argonaute proteins used for phylogenetic analysis are shown in Supplementary table 1.

Sequence comparison by using BlastP indicated that MrAgo1 was closely related to penaeid shrimp Ago1 proteins by sharing 90%, 90%, and 91% identity with *P. monodon*, *L. vannamei*, and *M. japonicus* Ago1, respectively. Phylogenetic analysis was performed to gain more insight in the molecular evolution of MrAgo1 protein (Figure 3). The bootstrapped NJ tree separated Ago1 proteins into two subfamilies, including Ago and Piwi subfamilies. MrAgo1 was classified into the Ago subfamily and clustered in invertebrate Ago1 cluster. In this cluster, it was located in the same group with penaeid shrimp Ago1 protein, including PmAgo1, LvAgo1, and MjAgo1. Tissue distribution study indicated that MrAgo1 gene was highly expressed in lymphoid organ and gills, similar to that seen in PmAgo1 and MjAgo1 (Figure 4) (Unajak et al. 2006, Huang and Zhang 2012). In *P. monodon*, PmAgo1 gene was highly in lymphoid organ. The expression of PmAgo1 gene in in lymphoid organ increased upon yellow head virus infection (Unajak et al. 2006). Silencing of PmAgo1 expression resulted in diminishing of dsRNA-mediated gene silencing (Dechklar et al. 2008, Phetrungnapha et al. 2013). In *M. japonicus*, three isoforms of MjAgo1, termed MjAgo1A, MjAgo1B, and MjAgo1C were highly expressed in lymphoid organ. The expression of MjAgo1A and MjAgo1B were up-regulated significantly upon white spot syndrome virus challenge. In addition, inhibition of MjAgo1A and MjAgo1B resulted in increase in viral loads in shrimp, indicating that MjAgo1A and MjAgo1B isoforms were involved in shrimp antiviral immunity (Huang and Zhang 2012). Taken together, MrAgo1 might be involved in antiviral immunity in *M. rosenbergii*. Further studies elucidating the function of MrAgo1 will be benefit in understanding the mechanism of RNAi in this economically important species

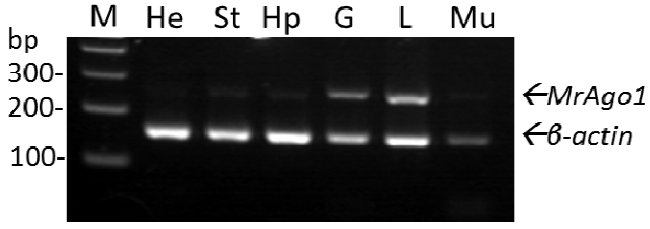

Fig. 4. Tissue distribution of MrAgo1. A representative gel represents RT-PCR products of MrAgo1 and β-actin. M, 100-bp DNA ladder; He, hemocyte; St, stomach; Hp, hepatopancreas; G, gills; L, lymphoid organ; Mu, muscle**.**

CONCLUSION

We have identified the cDNA encoding MrAgo1. The sequence features, molecular evolution and tissue distribution of MrAgo1 were also described. Further studies elucidating the mechanism of action of MrAgo1 will be benefit in understanding the mechanism of RNAi in this economically important species.

ACKNOWLEDGEMENTS

This work is supported by the Naresuan University Research Grant (R2558C142 to A.P.).

REFERENCES

Dechklar, M., A. Udomkit and S. Panyim (2008). Characterization of Argonaute cDNA from *Penaeus monodon* and implication of its role in RNA interference. *Biochem Biophys Res Commun,* 367(4): 768-774.

Eulalio, A., E. Huntzinger and E. Izaurralde (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol,* 15(4): 346-353.

Gu, S., L. Jin, Y. Huang, F. Zhang and M. A. Kay (2012). Slicing-independent RISC activation requires the argonaute PAZ domain. *Curr Biol*, 22(16): 1536-1542.

Huang, T. and X. Zhang (2012). Contribution of the argonaute-1 isoforms to invertebrate antiviral defense. *PLoS One*, 7(11): e50581.

Huang, T. and X. Zhang (2012). Functional analysis of a crustacean microRNA in host-virus interactions. *J Virol*, 86(23): 12997-13004.

Hutvagner, G. and M. J. Simard (2008). Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol*, 9(1): 22-32.

Khuntia, C. P., B. K. Das, B. R. Samantaray, S. K. Samal and B. K. Mishra (2008). Characterization and pathogenicity studies of *Vibrio parahaemolyticus* isolated from diseased freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aquaculture Research*, 39(3): 301-310.

Lingel, A., B. Simon, E. Izaurralde and M. Sattler (2003). Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. *Nature*, 426(6965): 465-469.

Ma, J. B., K. Ye and D. J. Patel (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature*, 429(6989): 318-322.

Meister, G. (2013). Argonaute proteins: functional insights and emerging roles. *Nat Rev Genet*, 14(7): 447-459.

New, M. B. and C. M. Nair (2012). Global scale of freshwater prawn farming. *Aquaculture Research*, 43(7): 960-969.

Phetrungnapha, A., S. Panyim and C. Ongvarrasopone (2013). *Penaeus monodon* Tudor staphylococcal nuclease preferentially interacts with N-terminal domain of Argonaute-1. *Fish Shellfish Immunol*, 34(3): 875-884.

Pillai, D. and J. R. Bonami (2012). A review on the diseases of freshwater prawns with special focus on white tail disease of *Macrobrachium rosenbergii*. *Aquaculture Research*, 43(7): 1029-1037.

Sagi, A., R. Manor and T. Ventura (2013). Gene silencing in crustaceans: from basic research to biotechnologies. *Genes (Basel)*, 4(4): 620-645.

Song, J. J., J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon and L. Joshua-Tor (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat Struct Biol*, 10(12): 1026-1032.

Tahbaz, N., F. A. Kolb, H. Zhang, K. Jaronczyk, W. Filipowicz and T. C. Hobman (2004). Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. *EMBO Rep*, 5(2): 189-194.

Unajak, S., V. Boonsaeng and S. Jitrapakdee (2006). Isolation and characterization of cDNA encoding Argonaute, a component of RNA silencing in shrimp (*Penaeus monodon*). *Comp Biochem Physiol B Biochem Mol Biol*, 145(2): 179-187.

Wilson, R. C. and J. A. Doudna (2013). Molecular mechanisms of RNA interference. *Annu Rev Biophys*, 42: 217-239.

Xi, Q. Y., Y. Y. Xiong, Y. M. Wang, X. Cheng, Q. E. Qi, G. Shu, S. B. Wang, L. N. Wang, P. Gao, X. T. Zhu, Q. Y. Jiang, Y. L. Zhang and L. Liu (2015). Genome-wide discovery of novel and conserved microRNAs in white shrimp (*Litopenaeus vannamei*). *Mol Biol Rep*, 42(1): 61-69.