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Transcriptional analysis of olive flounder lectins in response to VHSV infection

Young Mee Lee, Jae Koo Noh†, Hyun Chul Kim, Choul-Ji Park, Jong-Won Park, Gyeong Eon Noh, Woo-Jin Kim and Kyung-Kil Kim

Genetics and Breeding Research Center, National Institute of Fisheries Science (NIFS), 81-9, Geojenamseo-ro, Nambu-myeon, Geoje-si, 53334, Gyeongnam, Korea

Lectins play significant roles in the innate immune responses through binding to pathogen-associated molecular patterns (PAMPs) on the surfaces of microorganisms. In the present study, tissue distribution and expression analysis of olive flounder lectins were performed after viral hemorrhagic septicemia virus (VHSV) challenge. Fish egg lectin and serum lectin were found to be predominantly expressed in the gills and liver, these results indicate that the transcript expression of olive flounder lectins is concentrated in immune-related tissues. Following a VHSV challenge, an overall increase in the transcript levels of the genes was observed and the expression patterns were distinctly divided into early and later responses during VHSV infection. In conclusion, olive flounder lectins are specifically expressed in immune-related organs and induced in both the immediate and long-lasting immune responses to VHSV in the olive flounder. These results indicate that lectins may be play important roles in the host defense mechanism and involved in the innate and adaptive immune response to viruses in fish.

Key words: Gene expression, Olive flounder, *Paralichthys olivaceus*, Lectin, Viral hemorrhagic septicemia virus (VHSV), Adaptive immune response

Fish larvae are free-living in the aquatic environment following fertilization and are frequently exposed to pathogenic microorganisms. Innate immunity constitutes the first line of defense against microbial invasion based on pattern recognition; thus, maternal immunity is particularly important in fish (McGreal *et al*, 2004; Holmskov *et al*, 2003; Vasta *et al*, 2004; Fujita *et al*, 2004). Lectins are carbohydrate-binding proteins that act as central components of the innate immune response in animals. They are able to recognize and bind exposed carbohydrates on the cell surface or sugar moieties on glycoproteins and glycolipids. Additionally, lectins exhibit a variety of biological functions in innate immunity, including self and non-self recognition (Vasta *et al*, 1994; Arason, 1996), promotion of tumor metastasis (Kim *et al*, 1998), stimulation of respiratory burst (Gasparini *et al*, 2008), agglutination of microbial cells (Argayosa and Lee, 2009; Fock *et al*, 2000) and in the cell adhesion, migration, apoptosis (Kasai *et al*, 1996; Ni and Tizard, 1996), encapsulation (Koizumi *et al*, 1999) and opsonization through binding to pathogen-associated molecular patterns (PAMPs) (Yu *et al*, 2013). In particular, fish lectins identify and stimulate the uptake of pathogens by phagocytes and facilitate complement-mediated cell lysis and elimination through natural killer cells (Hoffmann *et al*, 1999).

†Corresponding author: Jae Koo Noh
Tel: 82-55-639-5813; Fax: 82-55-639-5809
E-mail: jae9noh@korea.kr

Dendritic cells (DCs) exhibit important functions in the initiation and differentiation of immune responses and serve as a link between the innate and adaptive immune systems, and some lectins are known to be involved in the adaptive immune response (Wassaman *et al*, 1986; van Vliet *et al*, 2008; Zhu *et al*, 2013). In adaptive immunity, lectins function as regulators by recognizing bacterial or viral pathogens on DCs, which involves cytokine signal transduction, lymphocyte maturation and polarization against the invading pathogens (den Dunnen *et al*, 2010).

Recently, several members of the lectins have been studied in various defense mechanisms related to pathogen infection. C-type lectins have been reported in zebra fish (*Danio rerio*) (Zheng *et al*, 2015), roughskin sculpin (*Trachidermus fasciatus*) (Yu *et al*, 2013), grass carp (*Ctenopharyngodon idellus*) (Liu *et al*, 2011), *Scophthalmus maximus* (Zhang *et al*, 2010), orange-spotted grouper (*Epinephelus coioides*) (Wei *et al*, 2010), Japanese flounder (*Paralichthys olivaceus*) (Kondo *et al*, 2007), tongue sole (Zhou and Sun, 2015) and common carp (*Cyprinus carpio*) (Savan *et al*, 2004); fish-egg lectins have been reported in rock bream (*Oplegnathus fasciatus*) (Kim *et al*, 2011); galectins have been reported in roughskin sculpin (*Trachidermus fasciatus*) (Yang *et al*, 2013), Japanese flounder (*Paralichthys olivaceus*) (Liu *et al*, 2013), Atlantic cod (*Gadus morhua*) (Rajan *et al*, 2013), Korean rose bitterling (*Rhodeus uyekii*) (Kong *et al*, 2012) and sea bass (*Dicentrarchus labrax*) (Poisa-Beiro *et al*, 2009); and serum lectins have been reported in *Trichogaster trichopterus* (Fock *et al*, 2001) and spotted halibut (*Verasper variegatus*) (Hatanaka *et al*, 2008).

Olive flounder, *Paralichthys olivaceus*, is widely cultured in Korea and other Southeast Asian countries, but viral diseases have affected the aquaculture industry, causing significant economic losses. Viral hemorrhagic septicemia virus (VHSV) was identified as the most important pathogen infecting olive flounder in the last decade and is one of the major pathogens in olive flounder aquaculture. However, there is little available information based on integrated observations of flounder lectins in the immune responses against virus infection. In this study, we analyzed the tissue distribution and temporal expression profile of olive flounder lectins in the immediate and long-lasting immune response against VHSV infection.

Materials and Methods

Fish maintenance and tissue samples

Olive flounders were obtained from the Genetics and Breeding Research Center of the National Institute of Fisheries Science (NIFS, Geoje, Republic of Korea) and were maintained in a 10-ton flow-through tank at 20±1°C with continuous aeration under a natural photoperiod. The olive flounders were anaesthetized prior to experiments involving tissue collection and pathogen injection, and samples were collected under aseptic conditions. Abnormal and diseased fish were excluded in all of the experiments.

Tissue samples, including brain, eye, gill, intestine, kidney, liver, muscle, spleen and stomach samples, were dissected from ten healthy olive flounders (total length of approximately 10 cm, 4~5 months old) and immediately frozen in liquid nitrogen, followed by storage in a -80°C freezer until use.

VHSV challenge

The olive flounders were divided randomly into two groups for the VHSV challenge experiment: a control group and a challenged group. The control and challenged fish were injected with 100 μl of phosphate buffered saline (PBS) or a VHSV suspension ($10^{4.8}$ $TCID_{50}$ virus/ fish), respectively (Kong *et al*, 2009). The temperature to which the experimental fish were subjected was controlled at 15°C using a re-circulation system, without flow and feeding. The kidney, spleen, liver and gill were collected under aseptic conditions at 0, 1, 3, 6, 9, 12 and 24 hours

and at 2, 3, 4 and 5 days post-injection. Tissues from each fish were pooled together in equal amounts and frozen in liquid nitrogen. The pooled tissues were ground using a homogenizer, and equal amounts of the ground tissues from each fish were mixed and subjected to RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the ground fish tissues with TRI solution (BSK-Bio Co.) as described in the manufacturer's protocol. The total RNA was treated with DNase-I (Sigma-Aldrich) to remove genomic DNA contamination. The RNA concentration was measured spectrophotometrically (BioTek, Gen 5.2.), and RNA quality was assessed via electrophoresis in 1% agarose gels.

cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche) using an oligo $(dT)_{18}$ primer. To synthesize cDNA, the reverse transcription reaction was conducted as follows: a mixture containing 1 μg of total RNA, the oligo $(dT)_{18}$ primer and RNase-free dH_2O was held at 65°C for 5 min, then placed on ice for 5 min, after which 5x Transcriptor Reverse Transcriptase Reaction Buffer, Protector RNase Inhibitor, a dNTP mixture, Transcriptor Reverse Transcriptase and RNase-free dH_2O were added. The final reaction was carried out for 1 h at 50°C and 5 min at 85°C. Specific primers for olive flounder lectins and β-actin were designed using the Primer 3 program. The primers used for qRT-PCR are shown in Table 1.

Quantitative real-time PCR

Expression analysis of olive flounder lectins was conducted using quantitative real-time PCR with specific primers, and the mRNA levels of β-actin were used as an internal control. Quantitative real-time PCR was conducted using the ABI 7500 Real-time Detection System (Applied Biosystems) according to the manufacturer's instructions. The final reaction volume contained 10 μl of Fast SYBR Green PCR Master Mix (Applied Biosystems), 100 ng of cDNA, 0.3 μl of each of the forward and reverse primers. The amplification procedure consisted of an initial denaturation step for 20 seconds at 95°C, then 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C, followed by a final dissociation stage. Dissociation curve analysis of the amplification products was performed after quantitative real-time PCR to confirm the specificity of the PCR products.

The relative expression ratio of the target gene *versus* the β-actin gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). All samples were analyzed with three duplicates, and all data are presented in terms of relative mRNA levels, expressed as the mean ± SE (n = 3). Statistical analyses were performed with SPSS 17.0 software (SPSS Inc.) and the data were subjected to one-way analysis of variance (ANOVA).

Table 1. PCR primers used in this study

Target gene	GenBank acc. no.	Product length	Sense primer	Antisense primer	Ta[a]
β-actin	HQ386788	218	GAGCGTGGCTACTCCTTCAC	AGGAAGGAAGGCTGGAAGAG	60
CD8	AB082957	108	TAAGGGCAACACTAACACAGG	ATGAGGAGGAGGAGAAGGAG	56
Caspase 3	JQ394697	115	ACATCATGACACGGGTGAAC	TCCTTCGTCAGCATTGACAC	58
C-type lectin	BR000404	214	AGAATGGAAATGGGTGGACA	TGAAGGTTGGTGTCAGTGGA	60
Fish-egg lectin	FJ211414	169	GGAGGTGATGGTCAGGTTGT	CAATTCCCCAGCATCCATAC	60
Galectin	AF220550	150	AAGGTCGGACAGACCATGAC	GTTGCACACCACTGCATTCT	60
Serum lectin	GU165767	171	CAAAACCCTGGTCAGCAACT	TTGTTGTCAGCACAGCCTTC	60

[a]Ta: annealing temperature.

Differences were considered significant at P <0.05 (*) and extremely significant at P <0.01 (**).

Results

Tissue distribution of olive flounder lectins mRNA under normal physiological conditions

To examine the expression profile of lectins in various tissues of olive flounder, real-time PCR analysis was employed using β-actin as a control. For both the olive flounder lectins and the internal control β-actin, there was a single peak at the corresponding melting temperature observed in the dissociation curve analysis, which confirmed that PCR amplification was specific. To compare the relative expression levels of the mRNAs in different tissues, the lectin transcript levels in the tested tissues were normalized to that in the tissue showing the lowest expression (set as 1).

As with previous studies, our results shows that the expression of C-type lectin mRNA was observed to be much higher in the liver (Fig. 1A). The highest galectin transcript level was observed in muscle, followed by the intestines, stomach, gills, spleen, eyes, brain, and kidneys, while the expression level was lowest in the brain. The levels of galectin mRNA in the muscle, intestine, and stomach samples were all significantly higher than in the liver (Fig. 1C) (Jang *et al*, 2013).

In the present study, the mRNA level of serum lectin was very high in the liver and low in the spleen and intestine (Fig. 1D). The expression pattern of fish egg lectin was similar to that of C-type and serum lectin, being predominantly expressed in the gills, also

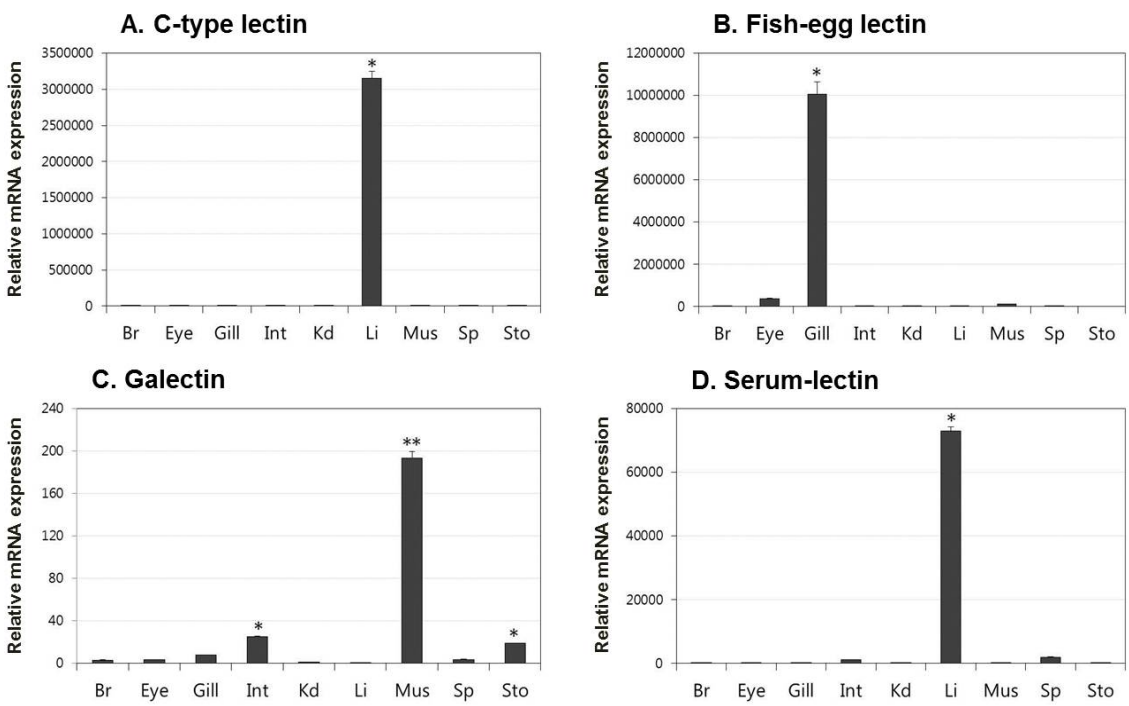

Fig. 1. mRNA expression of olive flounder lectins in various tissues of healthy fish. The transcript level was determined via quantitative real-time PCR, and olive flounder β-actin was chosen as an internal reference gene. Br, brain; Int, intestine; Kd, kidney; Li, liver; Mus, muscle; Sp, spleen; Sto, stomach. The results are reported as the mean ± standard deviation (SD) of triplicates. Significance was analyzed via one-way analysis of variance (ANOVA) using the SPSS 17.0 program.

known as other immune tissue, whereas it exhibited lower levels in the eyes and muscles (Fig. 1B).

Temporal expression profiles of olive flounder lectins in VHSV-infected olive flounder tissues

Expression patterns and timing are preferentially confirmed with VHSV-induced marker genes to verify a successful VHSV infection experiment. The mRNA level of CD8 was only induced in the early stage in VHSV-infected olive flounders, while caspase 3 expression was high in both the early and later stages in the kidneys; these results are consistent with previous findings (Avunje *et al*, 2012). To investigate whether olive flounder lectins were involved in the response to VHSV challenge, quantitative real-time PCR was performed to detect the lectin mRNA levels in the kidneys, spleen, liver and gills, which showed a close relationship with the immune response. To examine the effect of viral infection on olive flounder lectin expression, olive flounders were challenged with VHSV, and the resultant expression profile was analyzed from the early to the later response to pathogen infection.

As shown in Fig. 2, C-type lectin expression was induced in a time-dependent manner in response to VHSV challenge. However, the general induction patterns were different depending on the tissue. Following VHSV infection, C-type lectin expression was

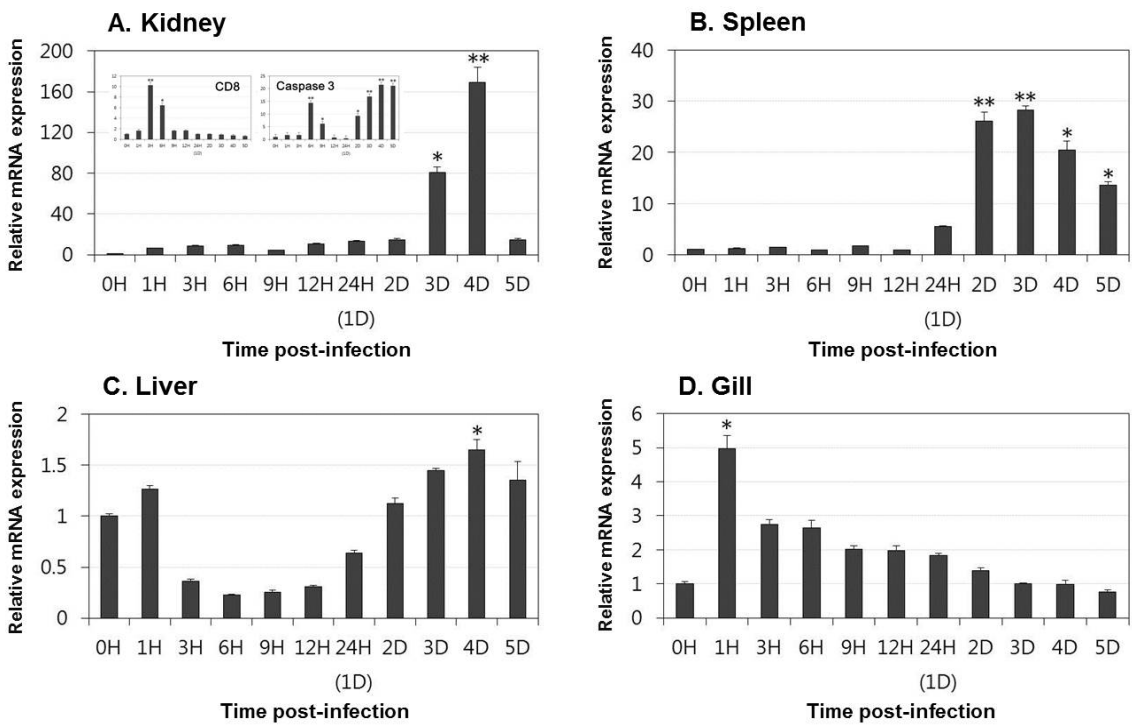

Fig. 2. The temporal expression pattern of C-type lectin after VHSV infection in the olive flounder. The expression patterns of olive flounder lectins were determined in the (A) kidney, (B) spleen, (C) liver, and (D) gill through quantitative real-time PCR. (A) CD8 and caspase 3 were used as markers to ensure a successful VHSV infection experiment (inset). The samples were analyzed at 0, 1, 3, 6, 9, 12 and 24 (1D) hours and at 2, 3, 4 and 5 days post-injection. The expression of β-actin was used as internal control for quantitative real-time PCR, and each experiment was performed in triplicate. An asterisk indicates a statistically significant difference (*$p<0.05$; **$p<0.01$) compared with 0 h (set as 1). The results are reported as the mean ± standard deviation (SD) of triplicates. Significance was analyzed via one-way analysis of variance (ANOVA) using the SPSS 17.0 program.

dramatically increased, reaching its peak level at 1 hour post-infection in the gills (4.97-fold) (Fig. 2D). In contrast, VHSV infection induced no apparent expression during the early response and was significantly up-regulated during the later response in the kidneys and spleen (169-fold at 4 days post-infection and 28.3-fold at 3 days post-infection, respectively) (Fig. 2A and B). In the liver, C-type lectin was initially significantly down-regulated from 3 h to 12 h post-challenge, then returned to normal levels at 2 days post-infection and gradually increased in response to VHSV infection in the later response stage (Fig. 2C). The variation of the expression of C-type lectin was not great compared with other tissues, as this lectin is already expressed at much higher basal levels in the liver.

When fish egg lectin expression was assessed during the response to pathogenic infection, all three patterns were observed, including the early response, later response and both responses. In particularly, fish egg lectin transcript levels were increased in both the early and later response stages in the kidney infection, which is referred to as a two-peak expression pattern. Fish egg lectin mRNA was primarily up-regulated, reaching a peak at 3 hours post-infection and then dropping to below the basal level at 24 hours post-infection. Thereafter, the fish egg lectin mRNA level was up-regulated again and peaked at 4 days post-infection (Fig. 3A). Fish egg lectin showed a one-peak pattern in the spleen and the liver; in the spleen, its

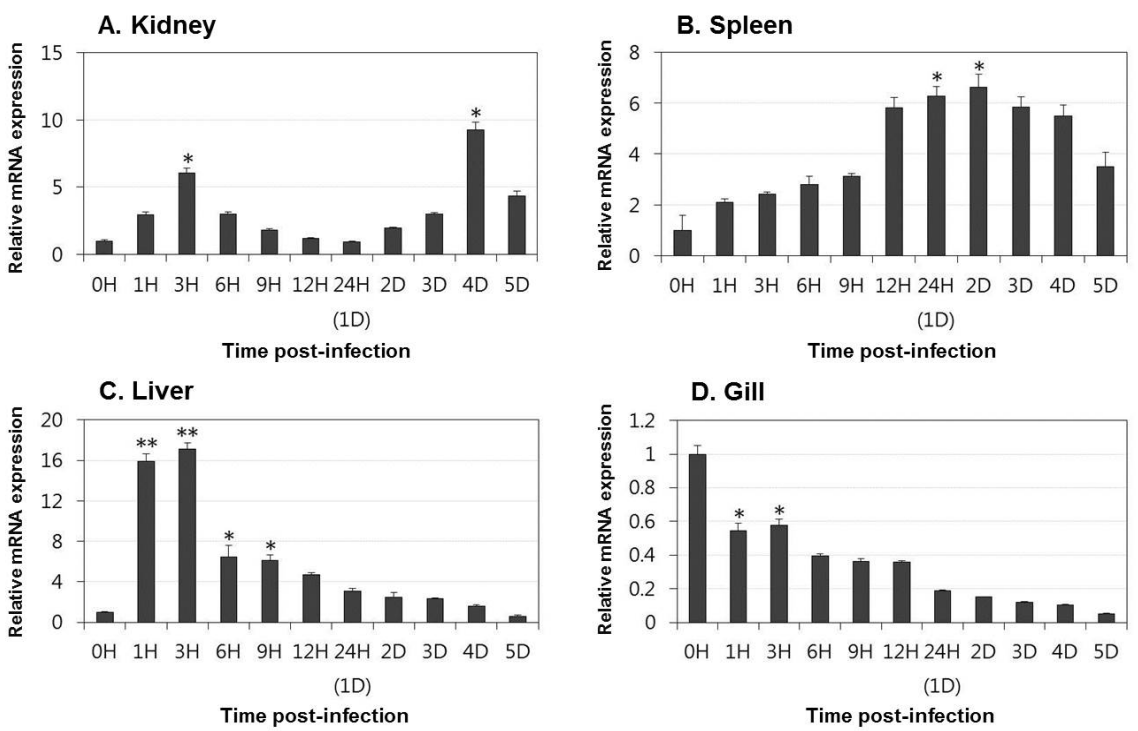

Fig. 3. The temporal expression pattern of fish egg lectin after VHSV infection in the olive flounder. The expression patterns of olive flounder lectins were determined in the (A) kidney, (B) spleen, (C) liver, and (D) gill through quantitative real-time PCR. The samples were analyzed at 0, 1, 3, 6, 9, 12 and 24 (1D) hours, and 2, 3, 4 and 5 days post-injection. The expression of β-actin was used as an internal control for quantitative real-time PCR, and each experiment was performed in triplicate. An asterisk indicates a statistically significant difference (*$p<0.05$; **$p<0.01$) compared with 0 h (set as 1).

expression increased gradually to up to 6.6-fold at 2 days post-infection, after which apparent down-regulation took place in the later response period (Fig. 3B). Additionally, the fish egg lectin transcript level in the liver increased rapidly up to 17.1-fold at 3 hours post-challenge, then dramatically decreased and returned to a basal state (Fig. 3C). In contrast to the previous results, fish egg lectin transcripts were greatly down-regulated as the infection continued in the gills (Fig. 3D).

The galectin transcript levels in the spleen, liver and gills were observed to increase rapidly at 1 hour post-challenge, then progressively decreased to the lowest level in the later response period (Fig. 4B-D). No significant up-regulation was observed in the kidney in the VHSV infection experiment during the early response stage, and galectin transcript levels temporary declined at 2 days post-infection and then increased again (Fig. 4A). Overall, the expression level of galectin was lower than that of the other lectins, with galectin expression being mainly concentrated in the initial response stage, while no apparent galectin expression was induced during the late infection stage.

Temporal expression analysis revealed that serum lectin was significantly elevated from 1 hour post-infection and reached its maximum level at 6 hours post-infection in the spleen (10.4-fold), then gradually declined to the basal level. In addition, serum lectin mRNA was slightly increased during the early in-

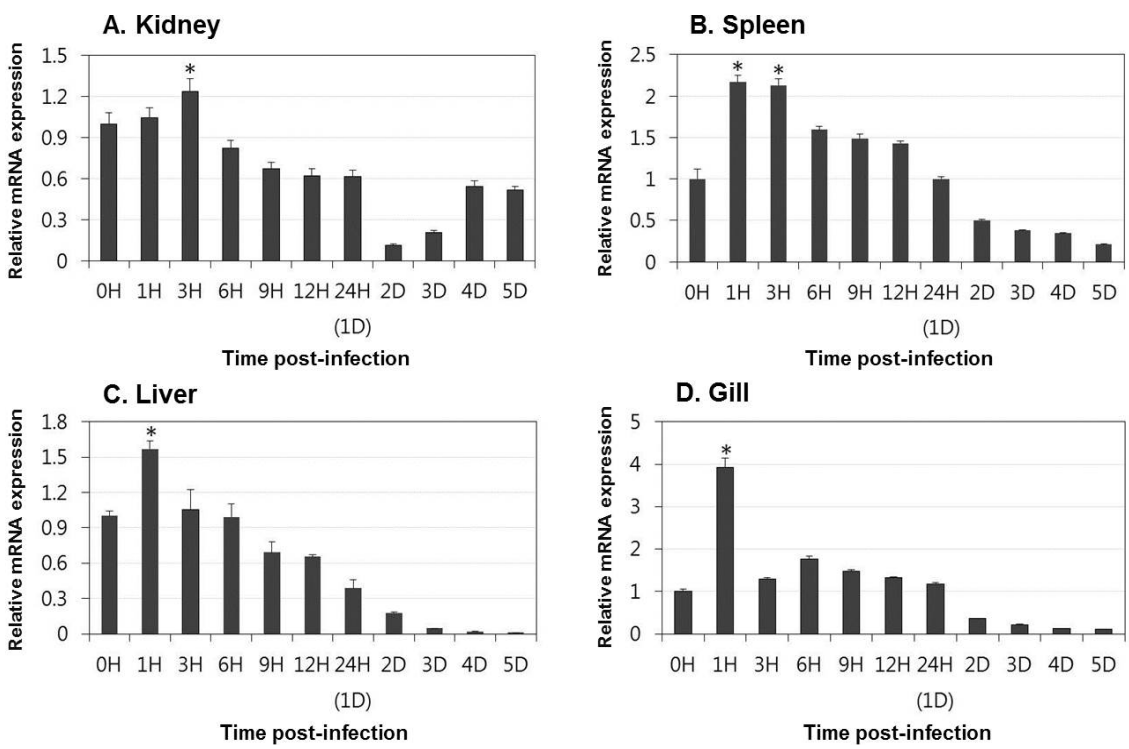

Fig. 4. The temporal expression pattern of galectin after VHSV infection in the olive flounder. The expression patterns of olive flounder lectins were determined in the (A) kidney, (B) spleen, (C) liver, and (D) gill through quantitative real-time PCR. The samples were analyzed at 0, 1, 3, 6, 9, 12 and 24 (1D) hours and at 2, 3, 4 and 5 days post-injection. The expression of β-actin was used as an internal control for quantitative real-time PCR, and each experiment was performed in triplicate. An asterisk indicates a statistically significant difference (*$p<0.05$; **$p<0.01$) compared with 0 h (set as 1).

fection stage following the injection of the viral pathogen in the gills (3 hours post-infection, 1.9-fold) (Fig. 5B and D). In the kidneys and liver, serum lectin expression showed almost no change in the early response period, while it was obviously increased later in the infection experiment in VHSV-infected tissue. The liver of olive flounder infected with VHSV exhibited a 12.5-fold elevation of serum lectin expression at 3 days post-infection; a similar pattern was found in the kidney at 4 days post-infection (2.4-fold). There was little difference in expression observed during the early response period (Fig. 5A and C). In conclusion, the expression pattern of lectins varied depending on the VHSV-infected tissue and type.

Discussion

Fish are vertebrates, but exhibits intrinsic inefficient acquired immune process due to the low state of evolution. The innate immune system of fish is thought to be of greater importance in combating microbial infection than higher vertebrates. Moreover, the innate and acquired immune response encompassing immune-related factors were more required in fish. Lectins are an enormous superfamily that consists of a great number of members throughout almost all living creatures. Lectins serving as the first line of defense against microbial invasion based on pattern recognition and pivotal components of innate immune response in animals (Magnadóttir, 2006; Gao *et al*,

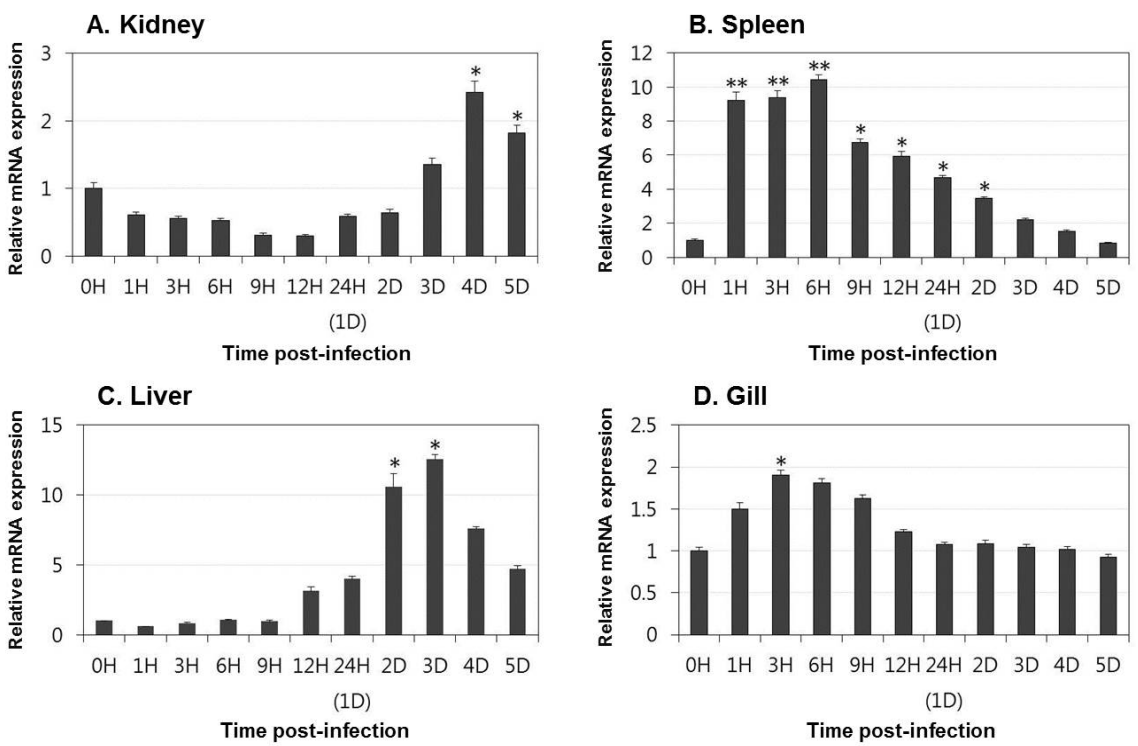

Fig. 5. The temporal expression pattern of serum lectin after VHSV infection in the olive flounder. The expression patterns of olive flounder lectins were determined in the (A) kidney, (B) spleen, (C) liver, and (D) gill through quantitative real-time PCR. The samples were analyzed at 0, 1, 3, 6, 9, 12 and 24 (1D) hours and at 2, 3, 4 and 5 days post-injection. The expression of β-actin was used as an internal control for quantitative real-time PCR, and each experiment was performed in triplicate. An asterisk indicates a statistically significant difference (*$p<0.05$; **$p<0.01$) compared with 0 h (set as 1).

2012). In recent years, the lectins have corresponding functions as regulators of adaptive immune responses by recognizing bacterial or viral components on dendritic cells (DCs) (den Dunnen *et al*, 2010). Therefore, it can be expected that a variety responses of lectins to microbial infection in particular organ. The studies on the lectins from a wide range of teleost species have been reported, no comprehensive expression analysis has been reported on the response with VHSV infections.

In present study, the spatial expression patterns of the lectins in various organs were different from each other under normal physiological conditions. Transcript of olive flounder lectins were specifically detected in the immune-related tissues, rather than being evenly distributed in various organs: lectins are mainly expressed in the liver and gill, except galectin. In particular, galectin was different from the other lectins, and our results suggest a functional difference for galectin. Gill is a unique organ in fish where the exchange of air and water functioning as the first mechanical and biochemical barrier. Water is a perfect medium for growth of bacteria and parasitic microbes, therefore, gills might be constantly involved in initial defense against the pathogens from water. Also, the liver is a major organ in response to inflammation and crucial for immunity as it is the main production site for immune-related genes. The tissue distribution of C-type lectin and galectin correspondence between previous studies and our research in similar size, although there are differences in growth depending on the breeding environment.

In the VHSV infection experiment, although lectins are known to be involved in the innate immune response, but expression was increased early as well as even later after VHSV infection. The transcripts of lectins were induced by viral pathogen infection and exhibited different regulation patterns in different tissues of olive flounder, which also depended on the type of lectin. After the olive flounders were challenged with VHSV, the mRNA expression of galectin increased significantly in the spleen, liver and gill in the early response. Unexpectedly, the expression of galectin was down-regulated in the kidney (the expression of fish egg lectin versa in the gill). Similar expression patterns of immune-relevant genes after microbe challenge have been reported. In conclusion, the immune-related tissue distribution and time-dependent upregulation against VHSV infection of olive flounder lectins, it is expected to be involved in the innate and adaptive immune response against this viral pathogen.

Acknowledgements

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