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Temperature-dependent regulation of gene expression in poly (I:C)-treated Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

Gene expression profiling of poly (I:C)-treated Japanese flounder, *Paralichthys olivaceus*, under different temperatures was investigated using microarray analysis. The response was analyzed in spleen tissue at 3 and 24 h post injection (hpi) at 15 °C and 25 °C. A large number of genes in fish treated with poly (I:C) at 25 °C were expressed at 3 hpi, whereas the expression profiles at 24 hpi appeared to be similar to those of the controls. Cluster analysis of the different expression profiles showed three distinct groups of up-regulated genes in fish reared at 15 °C. These were early (3 hpi), early-to-late (3 and 24 hpi), and late (24 hpi) up-regulated genes. These genes included type I IFN-related genes and inflammatory genes. Among the up-regulated genes, most of the type I IFN-related genes played early-to-late- and late-responding genes at 15 °C but early-responding genes at 25 °C. Thus, several up-regulated genes in these groups from the microarray result were further verified by qPCR. These results indicate that the type I IFN gene expressions of *P. olivaceus* treated with poly (I:C) can be regulated in a temperature-dependent manner.

1. Introduction

Temperature is an environmental factor that affects productivity in fish aquaculture. Several studies have addressed the relationship between temperature modulation and susceptibility of fish to pathogens [1–4]. For example, rearing Japanese flounder at 20 °C reduces the mortality caused by viral hemorrhagic septicemia virus (VHSV) compared with those reared at 14 °C [1]. Temperature changes have been shown to modulate immune-related gene expression [5–7]. The influence of temperature on immune-related gene expression has also been investigated in fish upon exposure to pathogens, such as viruses and formalin-killed pathogenic bacteria, or treatment with pathogen-associated molecular patterns (PAMPs), such as molecule including poly (I:C) and lipopolysaccharides (LPS) [8–14].

Poly (I:C) has been widely used for mimic viral infection when studying is associated with immune responses to virus in several fish species, including the sevenband grouper (*Epinephelus septemfasciatus*), large yellow croaker (*Pseudosciaena crocea*), and Atlantic cod (*Gadus morhua*) [10,15–17]. Recently, Dios et al. [15] demonstrated that zebrafish (*Danio rerio*) treated with poly (I:C) have different expression profiles of immune-related genes such as Toll-like receptor 3 (TLR3), Mx, and interferon regulatory factor 3 (IRF3) between low (15 °C) and high (28 °C) temperatures. Interferon stimulatory gene 15 (ISG15) expression in Atlantic cod after poly (I:C) injection was higher at 16 °C than 10 °C at 6 hpi [9]. Likewise, we previously reported that Mx expression in sevenband grouper injected with poly (I:C) was regulated by temperature [10]. These discoveries indicated that temperature regulates immune-related gene expression. However, gene expression changes in response to poly (I:C) and temperature changes in teleosts have not been comprehensively analyzed.

Microarray analysis is a powerful tool that has been used to extensively study global gene expression in several organisms [9,16,18–20]. A number of genes were identified after stimulation with various stimuli [9,21–25]. This approach provides valuable information for understanding and discovering gene functions, gene expression patterns, and signal transduction pathways. Consequently, in this study, we performed microarray analysis on the gene expression in Japanese flounder treated with poly (I:C) and reared at 15 °C or 25 °C using a Japanese flounder oligomicroarray containing more than 13,000 unique probes developed in a recent study [25]. The microarray result was further validated by qPCR. This study will provide information on immune-relevant

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genes that responded to poly (I:C) treatment and elucidate how expression of immune genes are regulated and affected by temperature.

2. Material and methods

2.1. Fish specimens and poly (I:C) treatment

Japanese flounder with an average size of 8 cm in total length were acclimated and reared at 15 °C or 25 °C for a week prior to the start of the experiment. Poly (I:C) was dissolved in DEPC-treated water and fish were intramuscularly injected with 100 μg/100 μl poly (I:C) or 100 μl DEPC-treated water as a negative control. At 3 and 24 hpi, spleen samples were collected for microarray ($n = 3$) and qPCR ($n = 4$) analyses as described below.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using RNAiso Plus (Takara Bio, Japan) and purified with an RNeasy® Mini Kit (Qiagen, USA). RNA quality was evaluated using a 2200 TapeStation (Agilent, USA). Qualified RNA (750 ng in a 20 μl reaction) was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). All procedures were performed following the manufacturer's instructions.

2.3. Microarray analysis

The cDNA microarray used in this study contained more than 13,000 unique probes [25]. Microarray analysis was carried out using three samples in each group, and each sample was selected based on RNA quality. The cDNA was synthesized from 200 ng of qualified RNA and labeled with cyanine 3-CTP using a one-color microarray-based gene expression analysis following the manufacturer's instructions (Agilent, USA). The labeled cDNA was subsequently hybridized at 65 °C for 17 h.

Microarrays were scanned with an Agilent G2565CA Microarray Scanner, and the images obtained from scanning were analyzed with Feature Extraction Software v9.5.3.1 (Agilent, USA). The data were normalized and analyzed using GeneSpring GX v11.5.1 Software (Agilent, USA). The average intensity of each sample was normalized to the median intensity of all groups injected with DEPC-treated water. The data were subsequently filtered based on expression, flags, error, and fold increase. Genes with a fold change of at least 4.0 were considered being differentially expressed. The microarray data were deposited in the GEO database under accession number GSE66692.

2.4. Validation of the microarray result by qPCR analysis

To confirm the microarray results, mRNA expression levels of the genes of interest were subsequently analyzed by qPCR. qPCR primers were designed based on Japanese flounder EST sequences that were obtained by next-generation sequencing and spotted onto microarray chips (Table 1). qPCR was carried out ($n = 4$) using THUNDERBIRD SYBR *qPCR* mix (Toyobo, Japan). The expression of all examined genes was normalized to the EF-1α expression [26].

3. Results

3.1. Microarray analysis of genes modulated by temperature and poly (I:C)

Temperature influence on gene expression was studied in poly (I:C)-injected fish reared at 15 °C and 25 °C. A total of 253 genes responded to poly (I:C) and temperature and are shown in Table 2. At 25 °C, a large number of the genes (174) were up-regulated at 3 hpi and then declined at 24 hpi, whereas eight genes remained highly expressed until 24 hpi (Fig. 1 and Table 2). The gene expression profile at 24 hpi was similar to that of the controls. However, among up-regulated genes at 15 °C (62 genes at 3 hpi and 139 genes at 24 hpi; Table 2), clear clustering in the expression profiles was observed, and the expression profiles clustered into three groups (Fig. 1). The first group, whose expression levels were up-regulated at 3 hpi and dramatically decreased at 24 hpi, was considered early-responding genes. Several genes in this cluster were inhibitor of NF-κB signaling (IκB)-delta, granulocyte colony-stimulating factor (GCSF), mitogen-activated protein kinase kinase kinase 8 (MAP3K8), and early growth response protein 1 (EGR1) (Table 3). The second group, whose expression levels were up-regulated at 3 hpi and remained relatively constant until 24 hpi, was considered early-to-late responding genes. The genes in the group included type I IFN-related genes such as Mx, IRF3, ISG15, DExD/H, interleukin 27 (IL-27) subunit beta-like, and IRF (Table 3). The third group, whose expression levels were only up-regulated at 24 hpi, was considered late-responding genes; the genes included suppressor of cytokine signaling 1 (SOCS1)-like, signal transducer and activator of transcription 1 (STAT1), ISG56, NLRC5-like, and interferon-induced very large GTPase 1-like (Table 3).

3.2. Verification of gene expression by qPCR

Two representative genes of each cluster with the same expression profile were selected for validation of microarray results by qPCR. IκB-delta and GCSF of the early-responding group had expression profiles similar to the heat map in the microarray result, which showed early up-regulation at 3 hpi and a dramatic reduction in expression at 15 °C (Fig. 2). Likewise, the qPCR results of IRF3 and Mx (early-to-late group), as well as SOCS1-like and STAT1 (late group) were similar to the expression profiles obtained by microarray analysis (Table 3 and Fig. 2).

4. Discussion

Immune-related gene expression modulated by temperature and pathogen infection or PAMPs stimulation has recently been studied in various fish species [8,9,12,14,15]. However, few studies have investigated the gene expression profiling after poly (I:C) stimulation and temperature modulation. In the present study, we investigated the global gene expression profile of poly (I:C)-injected Japanese flounder reared at either 15 °C (low) or 25 °C (high). The microarray result showed clear distinctive patterns of gene expression at 15 °C, in which the genes were clustered into three groups; early-, early-to-late-, or late-responding genes. However, these genes did not cluster in the same pattern at 25 °C.

After poly (I:C) stimulation, a large number of genes from fish reared at 25 °C were up-regulated at 3 hpi and dramatically declined at 24 hpi. The number of up-regulated genes at 3 hpi in the 15 °C-reared group was lower than that in fish reared at 25 °C (Fig. 1 and Table 2). High levels of gene expression at 25 °C were detected at 3 hpi, while at 15 °C, high levels of gene expression were clearly observed at 24 hpi (Fig. 1). These results are consistent with those of previously published data on the transcriptome response of the Atlantic cod, which showed that an earlier maximum response was observed at 16 °C than at 10 °C, where the maximum response was detected late [9]. Similar results were also observed in rainbow trout (*Oncorhynchus mykiss*) injected with *Yersinia ruckeri* bacterin, in which high levels of expression of immune-related genes such as IL-1β and IFN-γ were detected earlier at 15 °C or 25 °C than at 5 °C [12]. Collectively, these results indicate that higher temperatures