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A pathway-focused RT-qPCR array study on immune relevant genes in rainbow trout (*Oncorhynchus mykiss*) harboring cecropin P1 transgene

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ABSTRACT

Recently, our laboratory had produced five families of transgenic rainbow trout harboring cecropin P1 transgene, and via repeated challenge studies these fish exhibited a significant elevation of resistance to infection by microbial pathogens. By cDNA microarray and mRNA deep sequencing (mRNA-seq) analyses on two of the five families of cecropin P1 transgenic fish, differentially expressed genes (DEGs) relevant to the innate and adaptive immune pathways in three different immune-related tissues, (i.e. spleen, kidney and liver) were profiled. These results supported our hypothesis that in addition to its direct microbicidal activity, the transgene product of cecropin P1 induces immunomodulatory activity in the transgenic host. Here, we have adapted the technique of quantitative reverse transcription real time PCR (RT-qPCR) array to analyze the expression of genes relevant to the innate and adaptive immune pathways in the rest three families. A RT-qPCR array was constructed with oligonucleotide primers of fifty-two innate/adaptive immune relevant DEGs shown to be the most perturbed by cecropin P1 transgene product in previous studies. Messenger RNA isolated from the spleen, kidney and liver of transgenic fish and non-transgenic fish control were studied on this array. Results of RT-qPCR array revealed that statistically significant perturbations of gene expression were detected in pathways of cytokine/chemokine signaling, Toll-like receptor signaling, complement cascade, antigen processing/presentation, lysosomal phagocytosis and leukocyte trans-endothelial migration in the transgenic spleen; extracellular matrix (ECM) organization and leukocyte trans-endothelial migration pathways in the transgenic kidney; lysosomal activity pathway in the transgenic liver. Furthermore, genes related to the pathways of the peroxisome proliferator-activated receptors (PPAR) signaling, lipid metabolism process and arachidonic acid metabolism were also impacted in the transgenic liver. Findings of the current study are in good agreement with those discoveries in previous two transgenic families by cDNA microarray and mRNA-seq analyses.

1. Introduction

Outbreak of fish diseases as the consequence of infection by microbial pathogens causes serious financial losses in the aquaculture industry worldwide annually [1–3]. In the past decades, approaches, such as development of vaccines, treatment of diseased fish with chemicals or antibiotics, and selection of fish strains with resistant characteristic to infectious pathogens by traditional method of cross-breeding have been applied to control fish diseases with significant success. While several effective vaccines have been developed in recent years for some important fish pathogens, the current vaccination practice is labor intensive and high economic cost [4,5]. Although controlling fish diseases via utilizing antibiotics is effective, the appearance of increasing number of antibiotic resistant pathogenic microorganisms in the aquatic environment has challenged the effectiveness of this approach [6]. Furthermore, the number of antibiotics approved for treating diseased fish caused by bacterial pathogens is rather limited [7]. In addition, genetic selection of fish strains with resistant characteristic to bacterial infection, based on traditional cross-breeding techniques, is time consuming and the result is frequently unpredictable or disappointing due to lacking the desired genetic traits [4]. Therefore, developing effective techniques to control fish diseases are still in great demand. Since fish rely heavily on innate immune system to protect themselves from microbial infection [8], effective manipulation of the fish innate immune system may provide an opportunity to protect fish from contracting diseases caused by microbial

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pathogens.

Cecropin B, first discovered in the hemolymph of diapausing cecropia (*Hyalophora cecropia*) pupae following inoculation with bacterial debris, is one of the antimicrobial peptide (AMP) family member proteins playing an essential role in the innate immunity of insects [9]. Since then, many cecropin-like AMPs have been identified and characterized in a wide variety of organisms ranging from nematode [10] to invertebrates, vertebrates [11] and plants [12]. Due to the unique structural features of cecropins and cecropin-like peptides, these 31 to 39 amino acid-residue amphipathic peptides can be readily incorporated into the plasma membrane of many pathogenic microorganisms, and result in the formation of pores on the plasma membrane of the pathogenic microorganisms leading to inevitable lysis of cells [13]. Genes (cDNAs and their genomic sequences) encoding cecropins and cecropin-like peptides have been cloned from insects [14,15], nematode [10], shrimp [16,17], and vertebrates [18]. By transgenic technologies, these cDNAs have been used to produce transgenic plants [19–22] exhibiting increased resistant characteristic to infection by bacterial or fungal pathogens. Cecropin P1, identified in nematode inhabiting in the pig small intestine, is known to be more potent against Gram-negative bacteria than Gram-positive bacteria [23], and, therefore, was chosen as a transgene for production of disease resistant rainbow trout by Chiou et al. [24] in our laboratory. By utilizing the sperm-mediated gene transfer method, five families of transgenic rainbow trout bearing cecropin P1 transgene was produced. Moreover, through repeated challenge studies, cecropin P1 transgenic rainbow trout (F2 and F3 generations) exhibited resistant characteristic to infection by *Aeromonas salmonicida*, infectious hematopoietic necrosis virus (IHNV) and, *Ceratomyxa shasta* (a trout parasitic pathogen) [24,25].

Via a series of *in vitro* studies, Chiou et al. [26] revealed that cecropin B and a synthetic cecropin analogue (CF17) expressed inhibitory effect on the propagation of important fish viruses such as infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV), and infectious pancreatic necrosis virus (IPNV). Furthermore, by *in vitro* treating RTS-11 cells (a macrophage cell line of rainbow trout) with cecropin B, fish pleurocidin and CF17, Chiou et al. [26] observed an enhancement on the expression of two pro-inflammatory related genes, i.e. *interleukin-1β* (*il1β*) and *cycloxygenase-2* (*cox2*). By combining these observations with the results of studies that cecropin P1 transgenic rainbow trout manifested elevated resistance to infection by *Aeromonas salmonicida*, IHNV and *Ceratomyxa shasta* [1,24], we hypothesized that transgene product of cecropin P1 not only kills fish pathogens directly but also stimulated the host innate/adaptive immunity through its immunomodulatory activity, to account for the increased resistance to infection by pathogens in the transgenic fish. To address this hypothesis, the techniques of cDNA microarray analysis [27] and mRNA deep sequencing (RNA-seq) [28] were employed to analyze RNA samples isolated from the spleen, liver and kidney of two cecropin P1 transgenic fish families and one non-transgenic control. In the cDNA microarray analysis conducted on a 44k custom-made salmonid chip [29], tissue specific immune relevant DEGs were identified in the transgenic spleen, liver and kidney, and direct functional perturbations were also observed in pathways, e.g. lysosomal phagocytosis, complement cascade, antigen processing and leukocyte migration in the spleen; leukocyte migration and translational machinery in the kidney [27]. In addition, disturbance of indirect effects to host immunity pathways were observed as well, such as ECM organization in the kidney and lipid metabolic process in the liver [27]. Results of cDNA microarray analysis were confirmed by the RNA-seq analysis of Han et al. [28]. For instance, immune relevant pathways of chemokine and cytokine signaling, complement cascade, Toll-like receptor (TLR), leukocyte migration and the high affinity IgE receptor (Fc ε RI) signaling and IgG receptor (Fc γ RI)-mediated phagocytosis were significantly perturbed in the transgenic spleen; peroxisome proliferator-mediated receptor (PPAR) signaling, hematopoietic cell lineage and leukocyte migration in the kidney; PPAR signaling, fatty acid biosynthesis, phagosomal activity and complement cascade in the liver. Unfortunately, cDNA microarray and RNA-seq analyses were only conducted in two of the five transgenic families, and there are three remaining families of transgenic fish exhibiting consistent elevation of disease resistance awaiting to be analyzed. To complete the analyses of these families via cDNA microarray and RNA-seq, a substantial financial resource and time will be required. Alternatively, the three families of fish could be analyzed by the technique of reverse transcription real time quantitative poly chain reaction (RT-qPCR) array built on the pre-defined DEGs from the results of RNA-seq analysis [28].

Traditionally, RT-qPCR is a method of preference widely used to measure levels of gene expression due to its high sensitivity and reliability [30]. By combining the performance of RT-qPCR with the gene profiling capability of microarray, RT-qPCR array was developed as a highly reliable and sensitive gene expression profiling technology for analyzing focused panels of genes in signal transduction, biological processes, microRNAs and disease research [30–34]. In comparison of the technologies of cDNA microarray and RNA-seq, the RT-qPCR array is far more financial and time saving. Using the data of Lo et al. [27] and Han et al. [28], we have designed a custom-made pathway-focused RT-qPCR array for analysis of gene expression profiles in the spleen, liver and kidney of the remaining three families of cecropin P1 transgenic rainbow trout. Here we report the results of the study. According to comparing the results of RT-qPCR obtained from the remaining three transgenic fish families with those of the cDNA microarray of Lo et al. [27] and the mRNA-seq analysis of Han et al. [28], the profiling of DEGs in immune relevant pathways of the spleen and kidney, and the energy metabolism correlated pathways of the liver are statistically consistent. By combining these facts with the results of pathogene challenges described by Chiou et al. [24], three families of cecropin P1 transgenic fish, which exerted high consistency in both disease resistance characteristic and DEG expression profiles, can be inferred. Therefore, results reported in the current study support our hypothesis and the developed RT-qPCR array platform may serve as a preferred method to fast screening of markers for selecting disease resistant fish strains.

2. Materials and methods

2.1. RNA preparation

In this study, all fish are one year of age with compatible body weights, and they are F_2 heterozygous males generated from a hybridization of YY homozygous transgenic males (described by Chiou et al. [24]) and wild type females. Three families of these heterozygous fish (i.e., F231, F509 and F695) are described in current study and two families (i.e., F073 and F180) were reported in studies by Lo et al. [27] and Han et al. [28]. A total of 36 tissue samples of the spleen, liver and kidney were harvested from three transgenic families (F231, F509 and F695) and one non-transgenic family of rainbow trout maintained in the Salmon Disease Laboratory at the Oregon State University (OSU protocol #4282). Prior to tissue collection, fish were euthanized by treatment with MS222, Tricaine mesylate (Sigma-Aldrich, St. Louis, Mo), following the specification in OSU protocol #4282. For each family, one batch of tissue sample was pooled from three individuals and totally three batches of tissue samples were collected. RNA samples were prepared from these pooled tissues with the TRIzol reagent (Invitrogen, Carlsbad, CA) following the protocol provided by the manufacturer. The resulting RNA samples were treated with RNase free DNase-I (M610A, Promega, Madison, WI) to remove the genomic DNA contamination. The quality and the concentration of each RNA sample was assessed by an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2. cDNA synthesis and quality control

Superscript III reverse transcriptase (18080–044, Life Technology) and oligo-$(dT)_{18}$ were used to reverse transcribe two μg of genomic DNA-free RNA samples into first strand cDNA following conditions provided by the manufacturer. The resulting cDNA products were diluted with DNase-free water into a final volume of 100 μL. Qualities of the resulting cDNA samples were examined by the traditional PCR as described below. The PCR reaction was carried out in a reaction mixture (50 μL) containing 200 μM of dNTP, 0.1 μM of forward and reverse primers of housekeeping gene (*gapdh* and *actb*), 1.25 units/50 μL of iTaq™ DNA polymerase (170–8870, Bio-Rad) and 5 μL of each template cDNA. The amplification program consisted of initial denaturing for 1 min at 94 °C, followed by 35 cycles of 10 s at 94 °C for denaturation, 20 s at 50 °C for annealing and 15 s at 72 °C for synthesis. Afterwards, the PCR products were analyzed by electrophoresis (40 mV, 1.5 h) on 1% agarose gels and visualized after staining with ethidium bromide.

2.3. PCR arrays

The PCR array layouts were designed by modifying the standard RT^2 profiler layout described by Arikawa et al. [30]. Each 96-well plate contained control wells containing two housekeeping genes (*gapdh* and *actb*), one non-template control (NTC), one non-reverse transcription control (NRC), one negative control, serial dilutions of *gapdh* (1x to 10x to 100x to 1000x) and testing genes. The PCR array reaction was carried out in a final volume of 20 μL containing 1 μL of each synthesized cDNA, 1 μL of gene specific primers (with stock concentration of 10 μM) (supplementary file 1), 10 μL of 2x SsoFast EvaGreen Supermix (172–5201, Bio-Rad) and fluorescein (170–8780, Bio-Rad) to final concentration of 0.01 μM. The reaction was conducted in the C1000 thermal cycler/CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The amplification program consisted of initial denaturing for 2 min at 98 °C, followed by 40 cycles of 5 s at 98 °C and 30 s at 59 °C for annealing and synthesis. For quality control of the amplified products, a melting curve, 65 °C–95 °C with 0.5 °C increments every 5 s, was performed after each amplification. Finally, the threshold cycle (Ct) values were collected by using the CFX manager software (Bio-Rad) and analyzed by the standard method [35] of 2^(-ΔΔCt) as described below.

2.4. Analysis of real-time PCR array data

Each array contained two separate housekeeping genes (*gapdh* and *actb*), and *gapdh* was used to normalize each sample data point. Normalization was performed by computing differential threshold cycle (ΔCt) of gene of interests (GOI) and housekeeping gene (HKG), ΔCt = Ct of GOI – Ct of HKG. According to the methodology of Livak et al. [35], the commonly agreed method of ΔΔCt was, then, calculated, as well as standard deviations of each ΔCt. By averaging triplicates (biological) normalized expression levels (ΔCt) for each differentially expressed gene, the difference (ΔΔCt) between the control group (non-transgenic tissues) and the experimental group (transgenic tissues) was determined. Afterwards, the Student's t-test was performed by two-tail distribution and assuming equal variances to two samples in between two data sets based on the triplicate ΔCt values for each DEG in the transgenic group compared to non-transgenic group, and p-values were collected. The fold change of each DEGs was defined as 2^(-ΔΔCt), and standard deviations of ΔΔCt were defined as SD' = SQRT ((SD of $\Delta Ct_{experiment}$)^2 + (SD of $\Delta Ct_{control}$)^2). For the inter-fish and inter-family variations, the standard deviations of each ΔCt and ΔΔCt were involved, respectively, in the supplementary file 2.

3. Results

3.1. Construction of the custom-layout RT-qPCR array

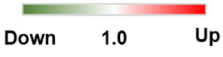
In order to analyze the expression pattern of immune relevant genes in the transgenic fish, tissue samples of three immune competent organs, i.e. the spleen, liver and kidney, were harvested from three families (F231, F509 and F695) of cecropin P1 transgenic rainbow trout and one non-transgenic control fish family of one year of age with the same gender. Within each family, pooling tissues from three individuals into one batch, and triplicating batches of RNA samples were obtained and assayed on the designed RT-qPCR array. The layout of the RT-qPCR array was modified from that of Arikawa et al. [30], containing housekeeping genes (*gapdh* and *actb*) as normalizer, non-template control (NTC), non-reverse transcription control (NRC), negative control, three serial dilutions of *gapdh* (1x to 10x to 100x to 1000x) and the pre-defined DEGs which significantly perturbed and highly correlated to specific immune functions in each tissue of the transgenic fish. In the transgenic spleen, DEGs involved in the chemokine and cytokine mediated Janus kinase (JAK) to signal transducer and activators of transcription (STAT) signaling pathway (*cxcl11/12*, *ccr8/9*, *cxcr3/4*, *il6st*, *il21r*, *stat1/6*, *irf7* and *c4*), and the Toll-like receptor pathway (*tlr2/3/7*, *cxcl11*, *myd88*, *irf3/irf7* and *cd40*) are incorporated in the construction of the RT-qPCR array. In addition, DEGs relevant to pathways of complement cascade (i.e. *c4/6/7* and *mbl2*), antigen processing/presentation (i.e. *mhc1/2*, *b2m*, *hspa4/5*, *calr* and *cd4*), lysosomal phagocytosis activity (i.e. *ctsb*, *ctsl* and *lamp2*) and leukocyte trans-endothelial migration (i.e. *cxcl12*, *itgb1/7*, *itga4*, *cxcr4* and *mmp9*) are also included in the array layout. In the transgenic kidney, DEGs related to pathways of ECM organization (i.e. *itgb1*, *fn1* and *lamb2*) and leukocyte trans-endothelial migration pathway (i.e. *itgb1/7*, *itga4*, *cxcl12* and *mmp9*) are included in the array layout. For the transgenic liver, DEGs which directly or indirectly affect the immune response are included in the RT-qPCR array. These are lysosomal activity related DEGs (i.e. *ctss*, *ctsd* and *lamp2*), PPAR/lipid metabolism processes relevant DEGs (i.e. *slc27a2*, *fabp1*, *ppar-α*, *rxr-γ*, *cyp27a1*, *pltp* and *c1qc*), and arachidonic acid metabolism regulatory DEGs (i.e. *cyp2j2*, *pla2g10* and *pla2g12b*). The experimental details of tissue preparation, RNA isolation and purification, cDNA synthesis and conditions of qPCR amplification are described in the "Materials and Methods".

3.2. Profiling of differentially expressed genes of immune relevant pathways in the transgenic spleen

Results of differential expression levels of immune relevant pathways in the spleen of transgenic fish families (F231, F509 and F695) determined by RT-qPCR array assay are summarized in Table 1. As shown in Fig. 1A, the expression of genes relevant to ligand-receptor binding leading to the chemokine/cytokine JAK-STAT signaling pathway, is consistently up-regulated in all of the three families; these genes include *ccr9, cxcr3, cxcr4, cxcl11, cxcl12, il6st* and *il21r* (Fig. 1A). Although the expression of STAT transcription factors (*stat1* and *stat6*) and the target gene (*c4*) of STAT1 are increased, another target gene (*irf7*) of STAT1 is significantly decreased. While the expression of *ccr8* showed suppression in the fish family F231, increased expression is illustrated in fish families of F509 and F695. In the pathway of the Toll-like receptor signaling, the expression of three different TLRs (*tlr2*, *tlr3* and *tlr7*), an important adaptor (*myd88*), an interferon regulatory factor (*irf3*) and its target gene (*cxcl11*) are all up-regulated among three families (Fig. 1B). However, the expression of *irf7* and the IRF3 target gene of *tnfrsf5* (*cd40*) are suppressed. In the complement & coagulated cascade pathway, consistent elevation of expression levels is observed in the genes of the binding ligand (*mbl*) and three complement components (*c4*, *c6* and *c7*) (Fig. 1C). In the antigen processing & presentation pathway (Fig. 1D), the RT-qPCR array data showed elevation of expression levels in *hspa4*, *hspa5*, *cd4*, *calr* and *b2m*, but a down-

Table 1
Expression profiling of DEGs relevant to immune pathways in the spleen of cecropin P1 transgenic rainbow trout.

Pathway	Gene	Description	Fold Change			T-test P-value	RefSeq
			F231	F509	F695		
Chemokine/Cytokine JAK-STAT Signaling	*ccr8*	C-C motif chemokine receptor type 8	0.6100	1.0832	1.0060	6.1677E-08	XM_021613380.1
	ccr9	C-C motif chemokine receptor type 9	1.9660	1.7083	2.1112	1.2626E-04	XM_021590079.1
	cxcr3	C-X-C motif chemokine receptor type 3	1.2559	2.2475	2.5244	8.6467E-06	NM_001124625.1
	cxcr4	C-X-C motif chemokine receptor type 4	1.2042	1.7062	1.0258	6.6199E-05	NM_001165293.1
	cxcl11	C-X-C chemokine ligand 11	1.5403	2.1205	1.9870	2.3759E-04	XM_021622222.1
	cxcl12	C-X-C chemokine ligand 12	1.0628	1.6740	1.3761	2.7565E-07	XM_014180105.1
	il6st	Interleukin 6 signal transducer	1.1547	1.5743	1.9282	2.9469E-06	NM_001281326.1
	il21r	Interleukin 21 receptor	1.2061	1.4204	1.0628	2.1979E-05	NM_001124623.1
	stat1	Signal transducer and activator of transcription 1	2.6121	1.5504	1.9020	1.1918E-05	NM_001141285.1
	stat6	Signal transducer and activator of transcription 6	1.0784	1.1782	1.3182	4.3773E-05	XM_021565805.1
	irf7	Interferon regulatory factor 7	0.1985	0.1805	0.2479	5.2917E-04	NM_001136548.1
	c4	Complement component 4	1.1773	1.5552	1.4550	3.6257E-06	XM_021582384.1
Toll-like Receptor Signaling	*tlr2*	Toll-like receptor 2	1.4287	1.3933	1.8450	5.0016E-08	XM_021578334.1
	tlr3	Toll-like receptor 3	1.1872	1.8848	1.0097	2.4358E-06	NM_001124578.1
	tlr7	Toll-like receptor 7	1.2759	1.1821	1.3376	3.8518E-06	XM_021598013.1
	myd88	Myeloid differentiation primary response protein	1.1571	1.3154	1.2672	5.7986E-06	NM_001136545.1
	irf3	Interferon regulatory factor 3	1.3560	1.6239	2.1789	2.5965E-05	NM_001257262.1
	irf7	Interferon regulatory factor 7	0.1985	0.1805	0.2479	5.2917E-04	NM_001136548.1
	cxcl11	C-X-C chemokine ligand 11	1.5403	2.1205	1.9870	2.3759E-04	XM_021622222.1
	cd40	Tumor necrosis factor receptor superfamily member 5	0.3696	0.5940	0.5425	8.5599E-05	NM_001124378.1
Complement & Coagulatory Cascade	*c4*	Complement component 4	1.1773	1.5552	1.4550	3.6257E-06	XM_021582384.1
	c6	Complement component 6	1.0510	1.5918	1.1508	4.6264E-04	NM_001124621.1
	c7	Complement component 7	1.2403	1.1876	1.0783	9.6032E-07	NM_001124618.1
	mbl2	mannose-binding lectin 2	1.0694	1.4652	1.0699	8.0408E-04	NM_001160481
Antigen Processing & Presentation	*hspa4*	Heat shock 70 kDa protein 4	1.0409	1.1270	1.4332	1.5816E-06	XM_021624134.1
	hspa5	Heat shock 70 kDa protein 5	1.1198	1.7713	1.6031	7.0413E-04	XM_021590926.1
	calr	Calreticulin	1.0674	1.6661	1.6654	1.9546E-06	NM_001124478.1
	cd4	CD4 antigen (P55)	1.1913	1.3900	1.1808	7.2745E-06	NM_001124539.1
	mhc1	Major Histocompatibility Complex, Class I	0.2329	0.4262	0.4912	7.1688E-05	XM_021586736.1
	mhc2	Major Histocompatibility Complex, Class II	0.2714	0.2207	0.2852	3.6517E-05	XM_014137042.1
	b2m	Beta-2-Microglobulin	1.5315	1.5806	2.3541	6.6449E-06	XM_021565716.1
Lysosome & Phagocytosis	*ctsb*	Cathepsin B	1.4278	1.3981	1.1651	3.6093E-07	NM_001124304.1
	ctsl	Cathepsin L	1.4263	2.0560	1.9010	2.7185E-03	NM_001124305.1
	lamp2	Lysosome-associated membrane glycoprotein 2	1.0106	1.6524	1.4761	4.0639E-05	XM_021561467.1
Leukocyte trans-endothelial migration	*itgb1*	Integrin beta 1	1.0379	1.3206	1.2412	1.0649E-04	XM_021620604.1
	itgb7	Integrin beta 7	1.3235	2.1969	2.6843	2.7178E-02	XM_021616851.1
	itga4	Integrin alpha 4	1.1329	1.2255	1.3670	8.3710E-07	XM_021597547.1
	cxcl12	C-X-C chemokine ligand 12	1.0628	1.6740	1.3761	2.7565E-07	XM_014180105.1
	mmp9	Matrix Metallopeptidase 9	1.5163	3.5219	2.0755	2.2447E-06	XM_021567121.1



The DEGs of the transgenic spleen considered significant (*t*-test p-value < 0.05) are shown. Fold changes are defined as 2^(-ΔΔCt). Heat map is defined as red, 2^(-ΔΔCt) > 1, = up-regulated; green, 2^(-ΔΔCt) < 1, = down-regulated. RefSeq is the mRNA from NCBI reference sequence database correlated to the analyzed DEGs.

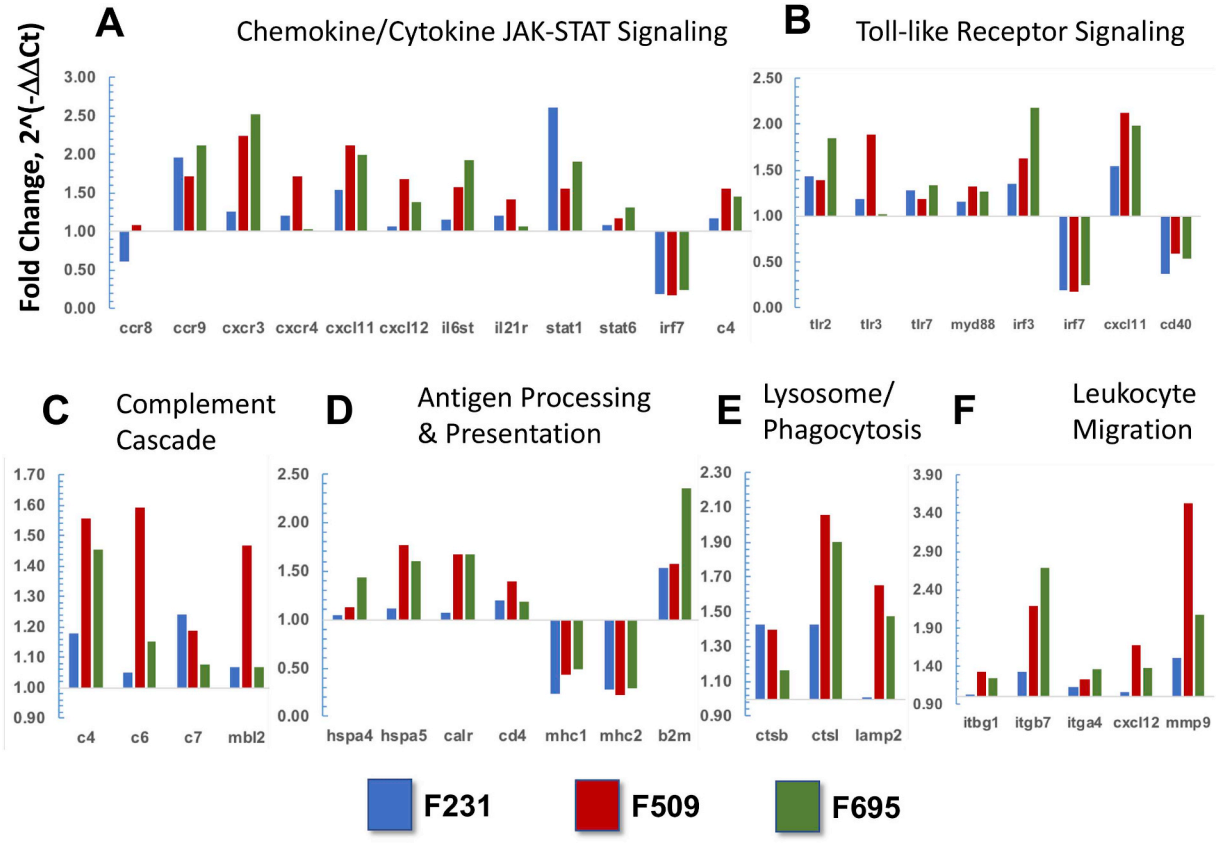


Fig. 1.

regulation of two major histocompatibility complex (MHC), i.e. *mhc1* and *mhc2* were observed. In addition, cathepsin B and L (*ctsb* and *ctsl*) and the essential membrane glycoprotein (*lamp2*) in the lysosome/phagocytosis pathway were consistently up-regulated (Fig. 1E). In the leukocyte trans-endothelial migration pathway, three important integrin (*itgb1*, *itgb7* and *itga4*) and adhesion ligand (*cxcl12*) were significantly stimulated, as well as the critical collagenase (*mmp9*) (Fig. 1F).

3.3. Profiling of differentially expressed genes of immune relevant pathways in the transgenic kidney

Results of expression levels of DEGs of the immune relevant pathways in the kidney of three transgenic fish families determined in the RT-qPCR array analysis are summarized in Table 2. Statistically significant (the p-value of student t-test < 0.05) alteration in levels of expression among immune relevant genes, tested in this tissue, were observed in all three fish families. While mRNA levels of the key integrin genes (*itgb1, itgb7 and itga4*) and the binding ligand gene (*cxcl12*)

Table 2
Expression profiling of DEGs relevant to immune pathways in the kidney of cecropin P1 transgenic rainbow trout.

Pathway	Gene	Description	Fold Change F231	Fold Change F509	Fold Change F695	T-test P-value	RefSeq
Leukocyte trans-endothelial migration	*cxcl12*	C-X-C chemokine ligand 12	0.7423	0.2325	0.4152	3.7219E-02	XM_014180105.1
	itgb1	Integrin beta 1	0.1544	0.0908	0.0589	2.6795E-04	XM_021620604.1
	itgb7	Integrin beta 7	0.8937	0.2801	0.8198	1.2196E-02	XM_021616851.1
	itga4	Integrin alpha 4	0.4146	0.2656	0.3655	1.1663E-04	XM_021597547.1
	mmp9	Matrix Metallopeptidase 9	0.3555	0.7446	0.5178	1.0203E-04	XM_021567121.1
ECM organization	*fn1*	Fibronectin 1	0.3036	0.2272	0.2107	6.9399E-05	XM_021608384.1
	lamb2	Laminin, beta 2	0.1390	0.2955	0.5735	3.0488E-03	XM_014145941.1

Down 1.0 Up

The DEGs of the transgenic kidney considered significant (*t*-test p-value < 0.05) are shown. Fold changes are defined as 2^(-ΔΔCt). Heat map is defined as red, 2^(-ΔΔCt) > 1, = up-regulated; green, 2^(-ΔΔCt) < 1, = down-regulated. RefSeq is the mRNA from NCBI reference sequence database correlated to the analyzed DEGs.

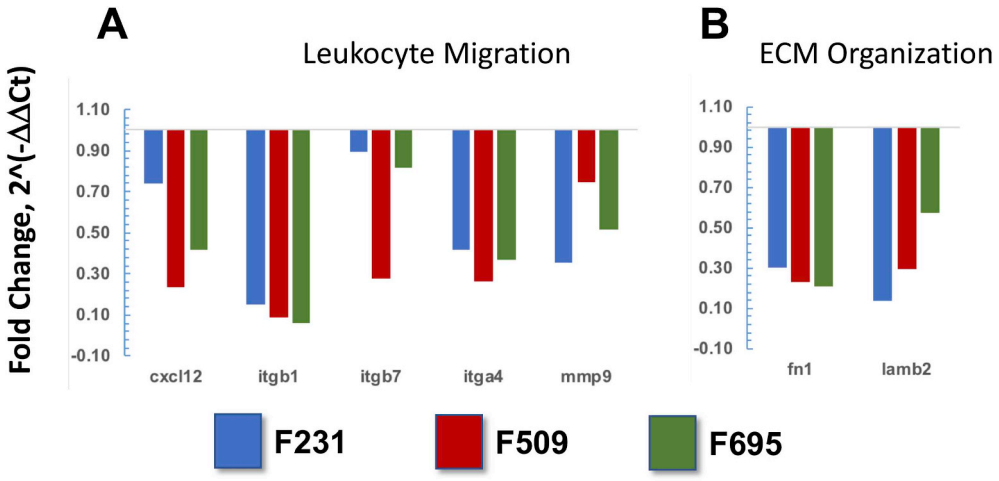


Fig. 2.

in the leukocyte trans-endothelial migration pathway were drastically elevated (Fig. 2A), and collagenase gene (*mmp9*) was also up-regulated, suppression of fibronectin (*fn1*) and laminin (*lamb2*) mRNAs, which are both regulating the ECM organization, was observed in the transgenic kidney among three families (Fig. 2B).

3.4. Profiling of differentially expressed genes of immune relevant pathways in the transgenic liver

Table 3 summarizes the results of RT-qPCR array analysis of genes directly and indirectly related to immune relevant pathways in the transgenic liver, and the results show that levels of several differentially expressed genes were statistically significant (the p-value of student t-

Table 3
Expression profiling of DEGs relevant to immune pathways in the liver of cecropin P1 transgenic rainbow trout.

Pathway	Gene	Description	Fold Change			P-value	RefSeq
			F231	F509	F695		
PPAR & lipid metabolic process	*slc27a2*	Very long-chain acyl-CoA synthetase	2.4773	1.3840	1.5284	2.7817E-03	XM_021596400.1
	fabp1	Fatty Acid-Binding Protein 1	0.0436	1.2833	1.2522	1.0066E-01	XM_021590970.1
	ppara	peroxisome proliferator-activated receptor alpha	0.7545	0.5929	0.5854	3.2628E-04	NM_001197211.1
	rxrg	retinoid X receptor	0.4335	0.3488	0.6557	2.5521E-02	XM_021589724.1
	cyp27a1	Cytochrome P450 27, mitochondrial precursor	0.8227	0.7354	0.6983	2.2375E-05	XM_021596960.1
	pltp	phospholipid transfer protein	1.2889	1.0320	2.8492	2.2628E-05	XM_021611178.1
Arachidonic acid metabolism	*cyp2j2*	cytochrome P450 family 2 subfamily J member 2	0.7188	0.5885	0.6860	7.1787E-05	XM_021599626.1
	pla2g12b	Group 12B secretory phospholipase A2-like protein	0.6527	0.4150	0.4774	4.8536E-03	XM_021568044.1
Lysosome & Phagocytosis	*ctsd*	Cathepsin D	0.9241	0.7791	0.8678	3.3367E-03	XM_021557499.1
	ctss	Cathepsin S	0.6331	0.6434	0.8894	8.7113E-03	NM_001123629.1
	lamp2	Lysosome-associated membrane glycoprotein 2	1.3954	0.7840	1.1711	1.3808E-03	XM_021561467.1

Down 1.0 Up

The DEGs of the transgenic liver considered significant (*t*-test p-value < 0.05) or borderline significant (*t*-test p-value $\cong 0.1$) are shown. Fold changes are defined as 2^(-ΔΔCt). Heat map is defined as red, 2^(-ΔΔCt) > 1, = up-regulated; green, 2^(-ΔΔCt) < 1, = down-regulated. RefSeq is the mRNA from NCBI reference sequence database correlated to the analyzed DEGs.

test < 0.05) or borderline significant (p-value $\cong 0.1$), by comparing to the control non-transgenic fish liver. As shown in Fig. 3A, the expression of the important membrane transporter/isozyme (*slc27a2*) and phospholipid transfer protein (*pltp*) genes in the PPAR signaling and lipid metabolic process pathways were differentially enhanced among the three transgenic fish families. In contrast, the mRNA levels of the signaling receptors, (*ppar-α* and *rxr-γ*), and their down-stream target gene (*cyp27a1*) in transgenic liver were suppressed. Interestingly, while the mRNA levels of the fatty-acid binding protein (*fabp1*) were up-regulated (considered as borderline up-regulation with p-value of student t-test $\cong 0.1$) in the transgenic fish families of F509 and F695, it was down-regulated in the family of F231. The DEGs related to arachidonic acid metabolism pathway were down-regulated, including *cyp2j2* and *pla2g12b* (Fig. 3B). Finally, cathepsin D and S (*ctsd* and *ctss*) in lysosome/phagocytosis pathway were consistently suppressed in all three transgenic families (Fig. 3C). Interestingly, the expression of the membrane glycoprotein gene (*lamp2*) is enhanced in transgenic fish families F231 and F695, however it was suppressed in the family F509. The inconsistency of *fabp1* and *lamp2* among families were further discussed in section 4.3.

4. Discussion

While the major function of cationic AMPs (e.g. cecropin P1), which directly eliminate bacteria and viruses through the cytotoxic activity, has been well studied [23,36], evidence is also available showing the immunomodulatory effects of AMPs via altering the expression of immune relevant genes in the host [36,37]. For example, Hilchie et al. reported that cationic AMPs may cause functional perturbations of chemoattraction, cytokine production and cell differentiation in monocytes and/or macrophages through altering corresponding transcription factors, e.g. SP-1, AP-1/2, NF-κB *etc.* [36]. Similar effects of AMPs that innate defense regulator (IDR) peptide stimulating the level of monocyte chemokines while suppressing pro-inflammatory cytokine responses were reported by Scott et al. [38] in mouse model. Previously, Chiou et al. [24] reported the production of five families of cecropin P1 transgenic rainbow trout. Through repeated challenge studies on F_2 and F_3 transgenic fish, they reported that these fish exerted elevation of resistance to infection by bacterial, viral and parasitic pathogens [24,25]. According to the analyses of two of the five families of the disease resistant transgenic fish via two genomics approaches, i.e. cDNA microarray and RNA-seq analyses, Lo et al. [27] and Han et al. [28] reported independently that genes in the immune relevant

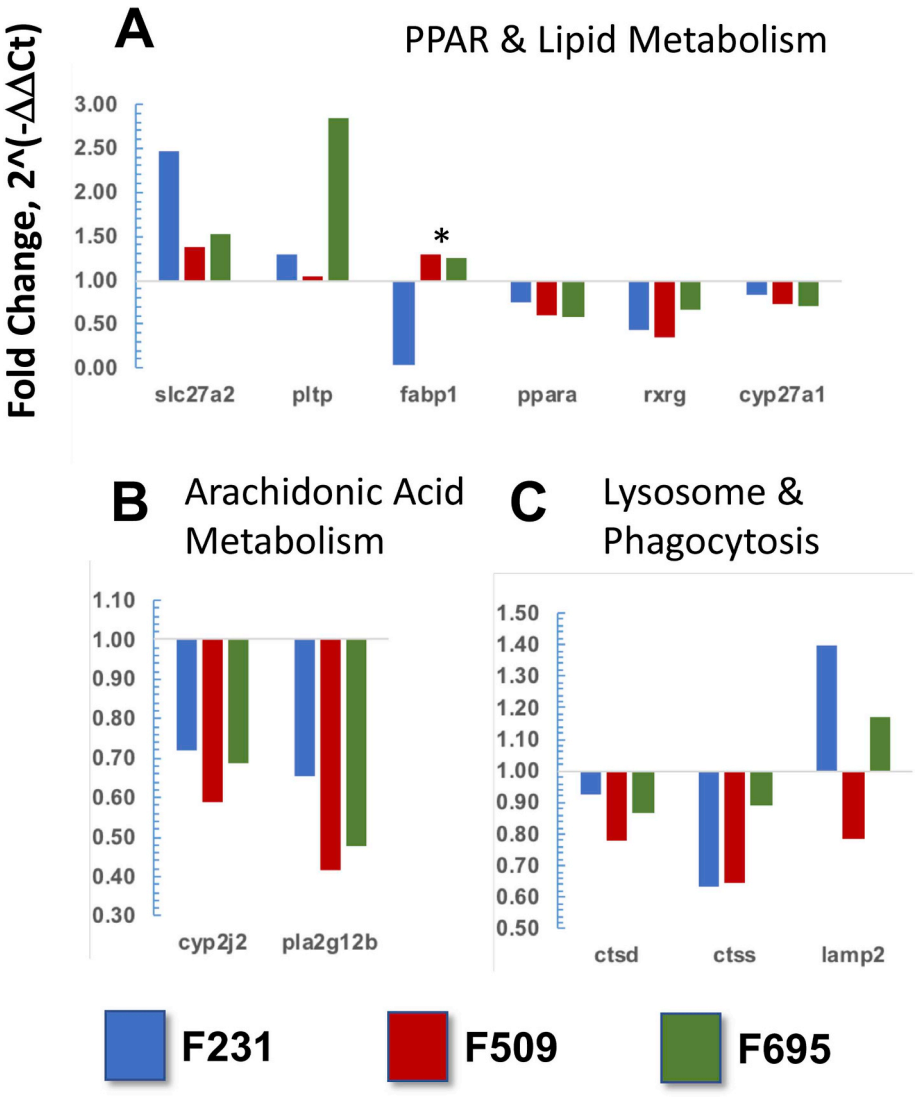


Fig. 3.

pathways of chemokine and cytokine signaling, complement cascade, Toll-like receptor, leukocyte migration, and the Fc ε RI signaling and Fc γ RI-mediated phagocytosis in the transgenic spleen; genes of PPAR signaling, hematopoietic cell lineage and leukocyte migration in the kidney, and PPAR signaling, fatty acid biosynthesis, phagosomal activity and complement cascade in the liver were significantly perturbed. These results supported the hypothesis that cecropin P1 transgene product may not only directly kill pathogens, but also modulate host innate/adaptive immunity. Via challenge studies, Chiou et al. [24] showed that all five families of cecropin P1 transgenic fish exhibited similar extent of resistance to infection by microbial pathogens. It would be of great interest to assess whether all these families of transgenic fish exert the same immunomodulatory effect. For economical utilization of financial resource and time, a RT-qPCR array was constructed with pre-defined immune relevant DEGs [28] for the analysis of the remaining three transgenic fish families (F231, F509 and F695). Similar patterns of expression of immune relevant DEGs were observed in the spleen, kidney and liver of these transgenic fish families. Here, we discuss the consistency among families and compatibility between research platforms to illustrate the correlations with disease resistant feature of the transgenic fish.

4.1. Spleen: the primary innate and adaptive immune competent tissue

As reviewed by Zapata et al. [39] and Uribe et al. [40], the spleen plays an essential role in both innate and adaptive immunity in teleost fish. As reported recently that the spleen of the cecropin P1 transgenic rainbow trout exhibited enhanced JAK-STAT signaling pathway, which may cause enhancing productions of down-stream chemokines and cytokines [28]. Moreover, since it is known that the spleen forms complexes of splenic ellipsoids, melanomacrophages centers (MMCs) and lymphoid tissue in teleost fish [39–41], the enhanced productions of chemokines and cytokines may suggest a positively feedback regulation of the proliferation and differentiation of lymphocytes in the transgenic spleen, and resulting in an overall enhancement of immune response in the transgenic rainbow trout. In the current study, the enhancement of JAK-STAT signaling effect was confirmed in all three families of the transgenic fish. Except for *ccr8*, all genes (*ccr9, cxcr3, cxcr4, cxcl11* and *cxcl12*) involved in receiving signal of chemokines, and genes (*il6st* and *il21r*) related to cytokine signaling were consistently up-regulated in the transgenic spleen. The cross-platform variation was found in the expression of *ccr8* because of the down regulation of *ccr8* in F231 (insignificant altered in F509 and F695) is inconsistent to the results of RNA-seq (up-regulated in both F073 and F180). Levels of mRNA of the down-stream transcription factors (*stat1* and *stat6*) and their target genes (*irf7* and *c4*) were also significantly altered. As reviewed by Sudhagar et al. [42], several different groups had reported genomic evidence to support this notion that cytokine and chemokine related immune pathways are greatly altered their expression profiles on different fish species. For instance, Mu et al. [43] demonstrated that interleukins (*il-1β, 2, 4, 6* and *8*), chemokine ligand 4 (*ccl4*) and chemokine receptor 1 (*ccr1*) were up-regulated in various degree to the spleen of *Aeromonas hydrophila* infected large yellow croaker (*Pseudosciaena crocea*). Transcriptomic analysis conducted by Dettleff et al. [44] on the spleen of viral (infectious salmon anemia virus) challenged Atlantic salmon (*Salmo salar*) could serve as another good example. The cytokine signaling of this viral infected salmon was perturbed in the spleen via up-regulated transcripts of interleukins and receptors (*il10*, *il10rb* and *il13ra2*) and interferon gamma and its receptor (*ifng* and *ifngr1n*); down-regulated transcripts of class II histocompatibility antigen (*mhc2*) and leucocyte antigens (*cd37* and *cd97*) [44].

The Toll-like receptor (TLR) signaling is known to contribute to defense against invading microbial pathogens [45], and very interestingly, we observed an overall up-regulation of genes in the TLR signaling pathway in the RNA-seq analysis [28]. Here, a constant increase of expression levels of Toll-like receptors (*tlr2*, *tlr3* and *tlr7*) and their adaptor protein (Myd88) were observed in RT-qPCR array analysis. The down-stream transcription factor, *irf3*, and its target gene *cxcl11* were up-regulated while the transcription factor (*irf7*) and IRF3 target gene (*tnfrsf5/cd40*) were suppressed. Since the *irf7* and *tnfrsf5* (*cd40*) are known to be cross-regulated by other signaling pathways, i.e. retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) for *irf7* [46] and Il-4/Tgf-β for *cd40* [47], the TLR signaling pathways were still considered stimulated in all of the three families of cecropin P1 transgenic rainbow trout. Some cross-species studies suggest highly altered expression profiles of TLR signaling pathways occurred in pathogenic infected fish. For example, Wang et al. [48] demonstrated *Vibrio alginolyticus* stimulates the Myd88 mediated TLR5 signaling via the bacterial flagellum in the spleen of giant grouper (*Epinephelus lanceolatus*). Additionally, Mu et al. [43], also discovered significantly perturbed TLR pathway in the spleen of *A. hydrophila* infected *Pseudosciaena crocea* at mRNA level, including up-regulations of *tlr1* and *tlr3*; down-regulations of *tlr2*. Very interestingly, Mu et al. [43] reported the stimulations of down-stream target genes of TLR pathways, such as *stat1*, *ap-1*, *il1β*, *il8* and *tnfα*, which greatly agrees to our current finding described in the previous section.

In the early studies conducted by Sakai et al. [49] in salmonid, complement components mediated opsonization and phagocytosis were found to be essential in fish immune response. Later, complement activities, which were implicated in the inactivation of lethal toxicities in rainbow trout, was reported by the same group [50]. Additionally, Johnson et al. [51], observed that complement receptor mediated phagocytic engulfing of complement components (C3b/C3bi) coated beads, *in vitro*, into salmon macrophages. Since complement cascades play important roles in fish immunity, especially in mediating phagocytosis, it agrees with our results of RT-qPCR array that consistent up-regulation of binding ligand (*mbl2*) and its down-stream complement components (*c4*, *c6* and *c7*) were observed in the spleen of cecropin P1 transgenic rainbow trout.

The abilities of MHC-I/II complex mediated ER-associated antigen processing and presentation are known to be critical in fish immunity [52,53]. In the preliminary cDNA microarray studies, Lo et al. [27] reported the up-regulation of antigen processing and presentation pathway in the cecropin P1 transgenic rainbow trout. In the current study, the results of the RT-qPCR array analysis further confirmed this finding because of the consistently enhancement of the expression of *calr*, *cd4*, *b2m* and two heat shock proteins (*hspa4* and *hspa5*) genes, and this finding indicated a promoted antigen processing and presentation process in the spleen of cecropin P1 transgenic fish. Although the major histocompatibility complex I and II were found suppressed in three families of transgenic fish, this suppression effect may be compensated by the stimulation of ER-chaperons (Calr, Hspa4 and Hspa5), and the overall antigen processing and presentation capacity is still considered up-regulated in the transgenic spleen. In addition, genes of lysosomal cysteine proteases cathepsins (*ctsd* and *ctsl*) and lysosomal membrane protein (*lamp2*) were constantly stimulated in the three fish families. These results may infer that an enhanced capacity of phagocytosis to engulf pathogenic antigen and increase the ability of lysosomal proteolytic digestion both taken placed in the transgenic spleen; then, the resulting small peptides might be exported via enhanced ER-mediated transportation machinery to the cell surface to be presented to recipients, e.g. T helper cells, by MHC complex. These findings are supported by the transcriptomic profiling, conducted by Zhu et al. [54], on the bacteria-infected Nile tilapia (*Oreochromis niloticus*). To the spleen of tilapia challenged by *Streptococcus iniae*, the expression levels of heat shock proteins (i.e. *hspa4*, *hspa5*, *hsp90α*, *hsp30β* and *hsp10*) were up-regulated, as well as the major histocompatibility complex (*mhc1* and *mch2*) [54]. Finally, the leukocyte trans-endothelial migration pathway was evaluated in the three fish families, and the results showed that genes of three important integrins (*itgb1*, *itgb7* and *itga4*), and the adhesion ligand (*cxcl12*) were significantly promoted, as well as the

critical collagenase (*mmp9*). This finding confirms the result of RNA-seq reported by Han et al. [28], suggesting that leukocytes might have better recognition and adhesion capacities targeting to epitheliums in the transgenic fish. In short, results the current RT-qPCR array study are consistent with those of Lo et al. [27] and Han et al. [28].

4.2. Kidney: the major hematopoiesis tissue

Because of lacking bone marrow, hematopoiesis in teleost fish is primarily carried out by the anterior kidney [55] including B cells, plasma cells and plasmablasts [56]. Recently, Lo et al. [27] and Han et al. [28] reported independently of two major functional perturbations: (1) leukocyte trans-endothelial migration and (2) ECM organization in the kidney of the transgenic cecropin P1 fish. By analyzing the expression patterns of integrin and binding ligand genes by RT-qPCR array analysis, we confirmed the down-regulation of *itgb1*, *itgb7*, *itga4* and *cxcl12*, and the matrix metallopeptidase (*mmp9*), and suggested a consistent inhibition of leukocyte trans-endothelial migration in in all transgenic fish families. Furthermore, the organization of extracellular matrix is important in modulating immunity, especially, in regulating the mobilizing and homing of hematopoietic stem cells (HSCs) in tetrapod bone marrow [57]. In the transgenic kidney, down-regulation of docking integrin, adhesive binding protein (fibronectin), basement membrane component protein (Laminin) and matrix metallopeptidase genes were observed in the current study. This notion is confirmed by the transcriptomic analysis of bacteria-infected Japanese sea bass (*Lateolabrax japonicus*). As reported by Xiang et al. [58], pattern recognition proteins (such as C-type Lectin 12, mannose receptor 1 and NOD protein 2) and docking protein (Integrin α-3) were functional perturbed in the head kidney and spleen of the *Vibrio harveyi* challenged Japanese sea bass. In the preliminary cDNA microarray analysis, Lo et al. [27] discovered the suppression of global translational machinery in the kidney of transgenic rainbow trout. By combining it to our RT-qPCR array result, an overall inhibited ECM organization capacity may be suggested by altering the micro-environment and reducing population of blood cell at early hematopoietic lineage in transgenic kidney.

4.3. Liver: the major energy metabolism tissue

The mammalian liver is considered as the most important immune relevant organ, including the pathogen clearance, leukocyte recruitment and antigen presentation to lymphocytes [40,59]. Very much alike to mammals, the liver in fish also plays a critical role in the host immunity although the function of the fish liver is slightly different from that of mammals [60]. In fish, the liver functions to synthesize and degrade metabolic protein as well as is known to biosynthesize fatty acids (FA) [60]. Lo et al. [27] and Han et al. [28] have observed significant functional perturbations in the energy metabolism related pathways (e.g. PPAR, arachidonic acid and phospholipid metabolism processes) in the liver of cecropin P1 transgenic fish, and these pathways were considered as indirectly impacted the immune responses. In the current study, we found up-regulation of genes of Acetyl-CoA synthase (*slc27a2*) and phospholipid transporter (*pltp*) in the transgenic liver. In addition, the signaling receptor complex genes (*ppar-α* and *rxr-γ*) and the down-stream target gene (*cyp27a1*) were consistently suppressed among the three transgenic fish families. In the expression profiling of Poly I:C (polyriboinosinic polyribocytidylic acid, mimicry of viral infection) stimulated liver of yellow catfish (*Pelteobagrus fulvidraco*), similar results were demonstrated. As reported by Liu et al. [61], enrichment analysis, conducted via the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, revealed that multiple energy metabolism related pathways, including retinol metabolism, PPAR signaling, glyoxylate/dicarboxylate metabolism and butanoate metabolism, were significantly altered in the liver of Poly I:C challenged yellow catfish. In addition, we observed that two arachidonic acid metabolism pathway related genes, phospholipase (*pla2g12b*) and cytochrome P450 (*cyp2j2*), were down-regulated, and the down-regulation of these genes indicated a consistent alteration of the FA metabolism in transgenic rainbow trout. Although these results are slightly inconsistent to those of DNA microarray and RNA-seq analyses, the findings still support the notion that processes of lipid metabolism in the transgenic liver were highly impacted. Therefore, the immunomodulatory effect of cecropin P1 transgene in trout liver is confirmed since the FA composition and metabolism have been shown indirectly modulating immunity in salmonids [62,63].

Another functional perturbation reported in the preliminary cDNA microarray and mRNA-seq analyses was the obvious suppression of lysosomal phagocytosis in the transgenic liver. As reviewed by Jenne et al. [59], scavenging phagocytes is also one of the crucial function in mammalian liver. In the current study, we found that the expression of two essential lysosomal protease genes, cathepsin D and S (*ctsd* and *ctss*), was constantly down-regulated, while the lysosome-membrane bound glycoprotein gene, *lamp2*, was shown partially enhanced in fish families, F231 and F695, but suppressed in F509. As shown in Fig. 3A and C, up-regulated *fabp1* was discovered in two (F509 and F695) of the three families, and up-regulated *lamp2* was found in two (F231 and F695) of the three. Since the up-regulations of *fabp1* and *lamp2* were consistent to the finding of RNA-seq, and, thus, the inconsistency among families may be resulted from insufficient sample repeats. Although this slightly inconsistency was observed, the global suppression of the expression of genes in lysosomal phagocytosis pathway in the transgenic liver is inferred.

5. Conclusion

In this study, by employing the assay of RT-qPCR array, we have confirmed the results of Lo et al. [27] and Han et al. [28] that the tissue-specific expression profiles of immune relevant genes in the spleen, kidney and liver among all families of cecropin P1 transgenic rainbow trout are consistently up-or down-regulated. The profiles of the differential expression of immune relevant genes account well for the phenotype of enhanced resistant to infection by microbial pathogens in the cecropin P1 transgenic fish. The data presented in this paper supports the notion that cecropin P1 transgene product not only eliminates pathogens directly but also modulates host immune functions, and the combined effects boost the disease resistance characteristic of transgenic rainbow trout. With the advantages of high sensitivity and reliability of the assay method, the developed RT-qPCR array technology promises a fast, effective and inexpensive screening method for selecting and breeding disease resistant fish strains in aquaculture. This study may also encourage further explorations of related scientific research topics such as tissue distributions of, microbial infectious effects of, and proteomic studies of AMPs in marine species.

Declarations

Ethics approval and consent to participate

Both transgenic and non-transgenic rainbow trout (one year old) were maintained in the Salmon Disease Laboratory at Oregon State University by following the OSU protocol # 4282. Prior to tissue collection, fish were euthanized by treatment with MS222, Tricaine mesylate, following the specification in OSU protocol #4282.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific research.

Author's contributions

YCH prepared RNA samples, performed RT-qPCR assays,

bioinformatics analysis, and wrote the manuscript; TTC provided the research plan and edited the manuscript.

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Abbreviations

mRNA-seq	mRNA deep sequencing
DEG	Differentially expressed gene
RT-qPCR	Quantitative reverse transcription real time PCR
ECM	Extracellular matrix
PPAR	Peroxisome proliferator-activated receptor
AMP	Antimicrobial peptide
IHNV	Infectious hematopoietic necrosis virus
VHSV	Viral hemorrhagic septicemia virus
SHRV	Snakehead rhabdovirus
IPNV	Infectious pancreatic necrosis virus
IL-1β	Interleukin 1 beta
COX2	Cycloxygenase 2
TLR	Toll-like receptor
Fc ε RI	Fc region of immunoglobulin epsilon (IgE) receptor
Fc γ RI	Fc region of immunoglobulin gamma (IgG) receptor
NTC	Non-template control
NRC	Non-reverse transcription control
Ct	Threshold cycle
GOI	Gene of interest
HKG	Housekeeping gene
JAK	Janus kinase
STAT	Signal transducer and activators of transcription
MHC	Major histocompatibility complex
IDR	Innate defense regulator
MMCs	Melanomacrophage centers
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
MMP	Matrix metallopeptidase
HSC	Hematopoietic stem cell
FA	Fatty acid
KEGG	Kyoto Encyclopedia of Genes and Genomes

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.027.

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