1 Characterization and expression analysis of chitinase genes (CHIT1,
2 CHIT2 and CHIT3) in turbot (*Scophthalmus maximus* L.) following
3 bacterial challenge

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Abstract: Chitinases are hydrolytic enzymes which have been employed to breakdown chitin coats of pathogenic microorganisms, thereby weaken the defense system of several pathogens and insects. In this regard, we identified the chitinase genes of turbot and characterized their expression patterns in mucosal tissues following *Vibrio anguillarum* and *Streptococcus iniae* challenge. In present study, transcripts of three chitinase genes (CHIT1, CHIT2 and CHIT3) were captured, as well as their protein structures and expression patterns following different bacterial infection were characterized. The chitinases were widely expressed in all tested tissues with the highest expression level of CHIT1 and CHIT2 in intestine, and CHIT3 in skin. Finally, these three genes showed different expression patterns following bacterial challenge. The significant quick induction of chitinases in mucosal surfaces against infection indicated their key roles to prevent pathogen attachment and entry in mucosal immunity. Functional studies should further characterize the chitinases and avail utilization of their function to increase the disease resistance in maintaining the integrity of the mucosal barriers against infection and facilitating the disease resistant family/strain selection in turbot.

Keywords: Chitinase, Mucosal surfaces, turbot, *Vibrio anguillarum*, *Streptococcus iniae*

1. Introduction

Chitinases are hydrolytic enzymes which break down the glycoside bonds in chitin [1], and were first detected in the secretions of mid intestinal gland of snail (*Helix pomatia*) [2]. Chitin is a β -1, 4-linked polymer of N-acetylglucosamine and one of the most abundant biopolymers in nature [3]. Chitin makes up the exoskeleton of arthropods, including crustaceans, insects, molluscs, nematodes and worms [4], also contributes to nitrogen and carbon generation of the ecosystem [5]. Chitinases have received special attention due to their roles in the bio-control of fungal pathogens and harmful insects [6]. A variety of pathogenic microorganisms contain chitin coats which provide protection against external factors. Chitinases have been employed to breakdown these protective coats and weaken the defense system of several pathogenic microorganisms and insects [7].

Chitinase have broad spectrum of distribution in nature including bacteria, fungi, plants, insects, protozoa, human and animal [8]. The roles of chitinases in these organisms are diverse. For the organisms encrusted by a tough layer of chitin, and the organisms that consume other chitin-containing organisms as a source of food, the strong activities of chitinases are essential for their life. For instance, in bacteria, chitinases are usually involved in mineralization of chitin nutrition and parasitism [8]. Except the above scenarios, the discovered chitinases in other organisms might contribute to degrade the protective shield of the invading chitin-coated microorganisms. In mammals, chitinases were shown to play important roles in the pathogenesis of T(H)2 inflammation, as well as the host anti-parasite responses [9]. In fish species, many studies have explored their critical roles in several biological processes, especially the localization, distribution, purification, identification and

functional characterization in different biological tissues, including silver croaker (*Pennahia argentatus*), chub mackerel (*Scomber japonicas*), red sea bream (*Pagrus major*), channel catfish (*Ictalurus punctatus*), Nile tilapia (*Oreochromis niloticus*), Japanese flounder (*Paralichthys olivaceus*), and turbot (*Scophthalmus maximus*) [10-16] respectively. But only few studies have characterized its detailed immune roles against pathogen infection, for example, a significant increase of chitinase expression was observed in lamprey (*Lampetra japonica*) following stimulation by bacteria or fungi [17]. It is clear that chitinase genes play vital roles for fish innate immune responses. In addition, different from their terrestrial counterparts, fish species depend more heavily on mucosal barriers as living in the pathogen-rich aquatic environment, while their mucosal surfaces (skin, gill and intestine) are continuously interacting with a broad range of commensals and opportunistic and primary pathogens [18]. However, our knowledge of the roles of chitinase genes in fish mucosal immunity against infection is still limited.

Turbot (*S. maximus*) is one of the extensively maricultured species in China, suffering from widespread disease outbreaks due to several pathogens, including *Vibrio anguillarum*, *Streptococcus iniae* and *Edwardsiella tarda*. In order to develop disease control and prevention measures, much efforts have been made to identify the immune genes in turbot, as well as their associated immune activities following infection [19-23]. The mucosal surfaces constitute the first immune barrier of fish host defense, a better understanding of mucosal barriers is urgent for aquaculture research to improve vaccine delivery via immersion or feeding [24]. In this regard, we sought here to characterize chitinase genes in turbot, as well as their expression patterns in mucosal barriers following different bacterial infection.

2. Materials and methods

2.1. Sequence identification and analysis

Chitinase genes from other species including human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), frog (*Xenopus laevis*), fugu (*Takifugu rubripes*), zebrafish (*Danio rerio*), channel catfish (*I. punctatus*), Japanese flounder (*P. olivaceus*) were used as queries to BLAST against our transcriptome database with a cutoff E-value of 1e-10 [25]. The retrieved initial pool of sequences was aligned to delete the repeated entries to generate a set of unique sequences for further analysis. Following identification, the candidate sequences were translated using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The predicted ORF sequences were further verified by BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against NCBI non-redundant protein sequence database. The identification, annotation and signal peptides of the protein domains were identified by the simple modular architecture research tool (SMART; <http://smart.embl-heidelberg.de/>). Signal peptide sequences were further analyzed using the web-based SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) with the settings ‘eukaryotes’, default D-cutoff value and ‘SignalP-TM’ as method. ExPASy server was used to analyze the theoretical pI, molecular mass and N-glycosylation sites (<http://web.expasy.org/protparam/>). The percentages of similarity and identity were calculated by MatGAT program [26].

2.2. Primary structure analysis

ExPASy's Prot Param server [27] was used to characterize the physiochemical characters of chitinase genes and designed fusion constructs such as theoretical isoelectric point (pI), molecular weight, molecular formula, total number of positive and negative residues, instability index, aliphatic index and grand average hydropathicity (GRAVY). The instability index provides an estimate of a protein's stability in vitro. Proteins with instability index smaller than 40 are predicted as stable. A value above 40 indicates that the protein may be unstable. The aliphatic index of a protein is regarded as a positive factor for the increase of thermostability of globular proteins and is mainly defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine and leucine). The GRAVY score is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence.

2.3. Phylogenetic analysis

The phylogenetic tree was constructed by selecting the amino acid sequences of chitinase genes of turbot and the other organisms, including human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), green sea turtle (*Chelonia mydas*), frog (*X. laevis*), fugu (*T. rubripes*), zebrafish (*D. rerio*), tilapia (*O. niloticus*), channel catfish (*I. punctatus*), Japanese flounder (*P. olivaceus*), Japanese mackerel (*Scomber japonicus*), three wire angeles perch (*Parapristipoma trilineatum*), large yellow croaker (*Larimichthys crocea*) and Atlantic salmon (*Salmo salar*). The multiple protein sequence alignment was conducted in ClustalW2 program [28]. The neighbor-joining method within the Molecular Evolutionary Genetics Analysis (MEGA 6) package was utilized to perform the phylogenetic and molecular

evolutionary analyses [29]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the neighbor-joining trees was evaluated by 1,000 bootstrapping replications.

2.4. Bacteria challenge and sample collection

In order to investigate the immune roles of chitinase genes in the host defense against bacterial infection, the Gram-negative bacteria *V. anguillarum* and the Gram-positive bacteria *S. iniae* were selected to conduct the bath challenge. Turbot fingerlings were obtained from turbot hatchery (Haiyang, Shandong, China) with average weight and length at 15.6 g and 5.5 cm, respectively. Prior to experimental challenge, the fish were acclimated in the laboratory in a flow-through system for at least one week. The bacterial isolate of *V. anguillarum* and *S. iniae* were provided by the disease lab in Qingdao Agricultural University. After a pre-challenge, the bacteria were re-isolated from symptomatic fish and biochemically confirmed before cultured. During challenge, symptomatic fish were confirmed to be infected with *V. anguillarum* and *S. iniae*, respectively. During the experiments, the fish were immersed in the bacteria solution for 2 h and then samples were collected at certain time point after treatment under tricaine methanesulfonate (MS-222) at 200 mg/L (buffered with sodium bicarbonate).

In *V. anguillarum* challenge, bacteria were inoculated in LB broth and incubated in a shaker (180 rpm) at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 1 ml of 10-fold serial dilutions onto LA agar plates. During challenge, the treatment groups were immersed for 2 h at a final concentration of 5×10^7 CFU/mL. Meanwhile, control fish were

immersed in sterilized media alone. Aquaria were randomly assigned for 2 h, 6 h, 12 h and 24 h post-treatment and 0 h control respectively, with thirty fish in each aquarium. At each time point after treatment, skin, gill and intestine tissue from 15 fish were collected (three pools of 5 fish per pool).

In *S. iniae* challenge, the bacteria were isolated from a single colony and inoculated in LB medium in a shaker incubator at 28 °C overnight. For the challenge, fish were immersed for 2 h at a final concentration of 5×10^6 CFU/mL, while control fish were immersed in sterilized media alone. At each time point following challenge, namely, 2 h, 4 h, 8 h and 12 h treatment, intestine, skin and gill tissues were collected from 15 fish (three pools of 5 fish per pool). All samples from both experiments were flash-frozen in liquid nitrogen and then stored in a -80 °C ultra-low freezer until preparation of RNA.

2.5. Total RNA extraction

Prior to RNA extraction, samples were homogenized using mortar and pestle under liquid nitrogen. Total RNA was extracted using Trizol® Reagent (Invitrogen, USA) according to the supplied protocol. RNA degradation and contamination was monitored on 1% agarose gels. The quality and quantity of RNA of each sample was measured on a Nanodrop 2000 (Thermo Electron North America LLC, FL). All extracted samples had an A260/280 ratio greater than 1.8.

2.6. Real-time PCR analysis

The expression patterns of chitinase genes in different tissues following different

bacterial challenge were examined by real-time PCR (qPCR). Gene specific primers were designed using Primer3 software based on the turbot chitinase gene sequences (Supplementary Table 1)[30]. And turbot 18S rRNA gene was used as an internal control for normalization of the expression levels. First strand cDNA (500 ng RNA per 10 µl reaction) was synthesized by PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to manufacturer's protocol. qPCR was performed on a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. The thermal cycling profile was performed as follows: the PCR reaction mixture was denatured at 95 °C for 30 s and then subjected to 40 cycles of 95 °C for 5 s, 58 °C for 5 s and followed by dissociation curve analysis, 5 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s increment, to verify the specificity of the amplicons. Triple RNA samples at each time-point were analyzed for gene expression. The fold differences were calculated by the relative quantification method using the Relative Expression Software Tool (REST) to capture significance at the level of $P < 0.05$ [31]. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. A no-template control was run on all plates.

3. Results and discussion

3.1. Identification of turbot chitinase genes

Three cDNA and putative amino acid sequences (CHIT1, CHIT2 and CHIT3) were identified, and they were all validated by the sequencing. In detail, CHIT1 (GenBank Accession: KY421058) was 1,440 bp containing a 1437 bp open reading

frame (ORF) that encoded 479 amino acids. The deduced CHIT1 protein was predicted to have a molecular mass of 52.09 kDa and a theoretical pI of 7.03, with seven protein kinase C phosphorylation sites, four casein kinase II phosphorylation sites, but none N-glycosylation site (Table 1). Results of a sequence homology search of entire open reading frame showed 91.8% identity to Japanese flounder ortholog, and 90.7% to large yellow croaker, 78-80% to other piscine and 60.9% to human orthologs (Table 2). Turbot CHIT1 was most identical to Japanese flounder with 93.2% identity, and most similar to large yellow croaker with 97.1% similarity (Table 2). The amino acid sequence identities and similarities between Glyco_18 domains were approximately 3~14% higher than the entire ORF identities and similarities, with the highest identity (95.6%) between turbot and Japanese flounder and similarity (98.8%) between turbot and large yellow croaker (Table 2).

Moreover, the full-length cDNA of the turbot CHIT2 (GenBank Accession: KY421059) was 1,660 bp containing a 1,458 bp ORF that encoded 486 amino acids. The deduced CHIT2 protein was predicted to have a molecular mass of 53.01 kDa and a theoretical pI of 5.01, with five protein kinase C phosphorylation sites, two casein kinase II phosphorylation sites, and only one N-glycosylation site (Table 1). Similar to CHIT1, results of sequence homology search of entire open reading frame also showed most identical with 77% identity to Japanese flounder ortholog, followed by large yellow croaker and other piscine (Table 3). Similar, turbot CHIT2 was also most identical and similar to Japanese flounder, with 90.8% identity and 96.1% similarity (Table 3). The amino acid sequence identities and similarities between Glyco_18 domains were still higher than the entire ORF identities and similarities, with the highest identity (92.2%) and similarity (97.4%) between turbot and Japanese flounder, also between turbot and large yellow croaker (Table 3).

CHIT3 (GenBank Accession: KY421060) was 1,227 bp containing a 408 amino acids ORF. The deduced CHIT3 protein was predicted to have a molecular mass of 45.69 kDa and a theoretical pI of 6.26, with six protein kinase C phosphorylation sites, six casein kinase II phosphorylation sites, and none N-glycosylation site (Table 1). Similarity, turbot CHIT3 showed high percentages of ORF identity to that of Japanese flounder with 59.5%, and amino acid sequence identity to that of fugu rubripes with 48.6%. In addition, the amino acid sequence identities and similarities between Glyco_18 domains also showed the highest identity (53.1%) between turbot and Japanese flounder (Table 4).

In addition, multiple protein sequence alignment found the conserved structural motifs such as Tyrosine (Y), Tryptophan (W), Aspartic acid (D), Alanine (A) and Methionine (M), and several conserved functional domains including Glyco_18 domain and ChtBD2 domain in three turbot chitinases (Fig. 1). Although these three proteins have similar domains, however, variations in the starting and ending positions exist in three proteins (Fig. 2). Previously studies have revealed that the ChBD might help localizing the enzyme on insoluble substrates to increase the efficiency of chitin cleavage [32]. The immune protective role of chitinases is mediated through the degradation of the key structural component of pathogens-chitin. The high affinity binding ability of chitinase proteins to pathogen-associated molecular patterns encoded in chitin, which is highly depend on the structural characteristics of chitinase, is crucial for the pathogen recognition. Further studies need to be conducted in teleost to characterize the relationship between the structural factors and the immune activities of chitinases.

3.2. Phylogenetic analyses

In order to further validate the gene identification, as well as the phylogenetic relationship of turbot chitinase genes, a neighbor-joining phylogenetic tree was constructed using MEGA6 with the chitinase genes from other species (Fig. 3). As shown in Fig. 3, the turbot CHIT1 gene showed the closest relationship to *P. olivaceus*, followed by *L. crocea* and *S. salar*. And the turbot CHIT2 gene also showed the closest relationship to *P. olivaceus*, and then clustered with *L. crocea* and *P. trilineatum*, and then fell into the clade with *I. punctatus* and *S. japonicas*. Different in turbot CHIT3, the CHIT3 gene from zebrafish and *I. punctatus* was clustered with *O. niloticus*, *P. olivaceus* and *T. rubripes* and then next to turbot CHIT3 gene (Fig.3). The other clade was consisted with human and mouse, and then clustered with turtle and frog. Overall, the phylogenetic tree analysis was consistent with their phylogenetic relationships, and all branching nodes were supported by high bootstrap values. Collectively, the phylogenetic analysis confirmed the identification of turbot chitinase genes and showed the strong orthology to their counterparts identified in other species.

3.3. Basal tissue expression of turbot chitinase genes

In order to characterize the tissue distribution patterns of chitinase genes in turbot, the expression analysis was conducted in eight healthy turbot tissues, including blood, liver, spleen, gill, skin, intestine, head kidney and brain by real-time PCR. Overall, all three chitinase genes were widely expressed in all the examined tissues, but with distinct expression patterns (Fig.4). In detail, CHIT1 showed the lowest expression level in head kidney, and the strongest expression level in intestine, followed by liver

and gill, and had the modest expression level in spleen and brain (Fig.4A). Similar to CHIT1, CHIT2 also showed the strongest expression level in intestine, but with the lowest expression level in liver, and the modest expression level in brain and gill (Fig.4B). Similar to CHIT1, the lowest expression level of CHIT3 was also detected in head kidney, with the strongest expression level in skin, followed by spleen and intestine (Fig.4C). Previously, chitinase genes also showed relatively high expression level in intestine in *Sardinops melanostictus* [33]. In mammals, the expression level of chitinase was observed much higher than two housekeeping genes in gastrointestinal tissues [34]. Especially, intestine is the largest vulnerable surface that faces the external environment, the high expression level of chitinase might indicating its protective roles against invasive pathogens. Additionally, the modest expression level in traditional immune tissues (spleen and liver) has also been detected. Some previous studies have implied that chitinases may play a crucial role in the defense against microbial pathogens through nonspecific immune response in *L. japonica* and *Crassostrea gigas* [17, 35]. Collectively, the detected high expression levels in mucosal tissues (skin, gill and intestine) suggested the involvement of chitinases in turbot mucosal immunity. Indeed, further studies are needed to characterize the detailed roles of chitinase genes in mucosal immune response against infection in teleost.

3.4. Expression profiles of chitinase genes following bacterial challenge

In order to reveal the roles of chitinases for turbot mucosal immunity, the expression patterns of three chitinases (CHIT1, CHIT2 and CHIT3) were characterized in mucosal tissues following bacterial infection. As we focused on

mucosal immunity, the mucosal tissues (gill, skin and intestine), which are the first places for host-bacteria interactions were selected. The tissues were collected at the early time points which are critical time for pathogen attachment and entry, following one Gram-negative bacteria *V. anguillarum*, and one Gram-positive bacteria *S. iniae* challenge. This study represents the first spatial expression analysis of chitinases following bacterial immersion challenge in teleost species in mucosal immunity.

Following *V. anguillarum* challenge, CHIT1 showed different expression patterns in different tissues (Fig.5). In detail, CHIT1 was induced significantly in the intestine as early as 2 h with 3.71 fold, but was quickly suppressed to -55.46 fold at 6 h, and continued to be down-regulated with -39.03 fold at 24 h (Fig.5A). Different to intestine, in gill, it was down-regulated as early as 2 h with -3.64 fold, and was down-regulated dramatically for -48.39 fold at 6 h and -10.56 fold at 24 h (Fig.5A). In contrast, no significant changes of CHIT1 expression were detected in skin following challenge. Different from CHIT1, CHIT2 was down-regulated in all the mucosal tissues. Especially, it showed the significant down-regulation at all time-points in gill (Fig.5B). while the most down-regulation was detected with -20.68 fold as early as 2 h, and then significantly down-regulated with -11.29 fold at 6 h, -8 fold at 12 h and -11.82 fold at 24 h. Moreover, in skin, CHIT2 was significantly down-regulated for -3.88 fold at 6 h and -4.51 fold at 12 h. While in intestine, the CHIT2 was only suppressed at 6 h with -4.62 fold (Fig.5B). However, CHIT3 was only induced for 3.07 fold at 12 h in intestine, and for 3.33 fold at 24 h in skin. No significant changes of CHIT3 expression were detected in gill following challenge (Fig.5C).

The distinct expression patterns were observed after *S. iniae* infection, and the significant different expression patterns were also detected among the three chitinases. In detail, CHIT1 was only down-regulated at 12 h for -2.31 fold in intestine, and no

significant changes were detected in skin and gill following challenge (Fig.6A). Different from CHIT1, CHIT2 showed an early response at 2 h with 3.17 fold in intestine, but was quickly returned to basal level at 4 h and 8 h, and was up-regulated again at 12 h with 5.24 fold (Fig.6B). While in skin, similar to that in *V. anguillarum* challenge, CHIT2 was down-regulated significantly for -10.88 fold at 4 h and -13.58 fold at 8 h respectively (Fig.6B). Obviously, a clear trend of up-regulation was observed for CHIT2 in gill, with 7.23 fold at 4 h, 9.34 fold at 8 h, and 37.36 fold at 12 h (Fig.6B). Notably, CHIT3 was significantly changed at all the timepoints in all the mucosal tissues (Fig.6C). A clear trend of up-regulation was observed in intestine, with 3.11 fold at 2 h, 3.18 fold at 4 h, 2.15 fold at 8 h and 50.33 fold at 12 h. In contrast, a clear trend of down-regulation was observed in skin, with -37.53 fold at 2 h, -25.93 fold at 4 h, -34.22 fold at 8 h and -3.22 fold at 12 h. Finally, in gill, it was suppressed with -6.45 fold as early as 2 h, but then up-regulated with 4.31 fold at 6 h, and reached the peak value at 12 h with 14.16 fold (Fig.6C).

As described above, the significant chitinases expression level that detected in mucosal tissues at early time points following infection suggested their putative roles in mucosal immunity. Chitinases consist of two primary domains, including Glyco_18 domain and ChtBD2 domain. Glyco_18 is a widespread group of hydrolytic enzymes that hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. Chitinases exhibited strong hydrolytic ability to break the integrity of the bacteria chitin coats, resulting in cell lysis in the hypoosmotic environment. But there are large differences of the bacteria chitin coats structures between the Gram-negative and Gram-positive bacteria, as the Gram-negative bacteria is additionally covered by a lipopolysaccharide-containing outer membrane, which is not directly accessible for chitinases. Therefore, the

chitinases need to utilize different strategies to fight against the different bacterial infection, so here we selected one Gram-negative and one Gram-positive bacteria to characterize and compare the expression profiles of chitinases during infection.

Indeed, we observed different responses of chitinase genes against different infection. Interestingly, almost all the three chitinase genes were rapidly up-regulated as early as 2 h in intestine. In mammals, chitinases have been shown to be up-regulated in intestine following bacterial infection, and required for the activations of NF- κ B- and MAPK-pathways [36]. And it was also demonstrated that the chitinase knockout (KO) mice showed suppressed Th2-immune responses, with lower tissue inflammation and higher immune cell apoptosis [37]. Moreover, previous studies have suggested that intestine might serve as the primary portal of entry for *V. anguillarum*. In turbot, following administration of *V. anguillarum* by anal or intragastric intubation, the bacteria could be detected in spleen as early as 2 h, indicating the ability of quickly pass through the intestine mucosal barrier [38]. Following bath vaccination with live attenuated *V. anguillarum* vaccine in zebrafish, the bacteria proliferated rapidly in 3 h and maintained at a high level in the intestine. Besides, bacteria persisted in the intestine for a longer time whereas decreased rapidly in the skin and gills [39]. In sea bass, after exposure to *V. anguillarum*, intact bacteria in the gut lumen were observed in close contact with the apical brush border [40]. Here, the quick up-regulation of chitinases in intestine might indicate the rapid invasion and proliferation of bacteria in intestine. And the most dramatic down-regulation in both challenge was found in CHIT1 in intestine, which was quickly suppressed to -55.46 at 6 h following the up-regulation in 2 h, which suggested the massive invasive bacteria in the intestine seeking to penetrate the intestinal barrier. In shrimp, chitinase was quickly up-regulated in gill following both *S. agalactiae* and *V. harveyi* challenge [41].

In our results, CHIT2 also showed the maximum expression level in gill following *S. iniae* infection. However, CHIT2 and CHIT3 were both down-regulated in skin following *S. iniae* infection. A considerable conjecture about the portal of entry of streptococcal pathogens have been reported. Especially, in *Lates calcarifer*, bath exposure of *S. iniae* could resulted in higher level of mortality within the first 48 h, which is much more significant than oral exposure [42]. In addition, the pathogenic characteristic of *S. iniae* infection was marked petechial hemorrhages of the skin [43]. Here, the expression changes of chitinases were much more significant in skin and gill than that in intestine following *S. iniae* infection, suggested the primary portal of entry for *S. iniae* is rather gill and skin than intestine. Currently, the expression analysis of chitinases in mucosal tissues following *S. iniae* infection is very limited. Further studies are needed to characterize the exact roles of different chitinases against infection and thus to identify the key player for disease resistance selection.

As a multifunction protein, chitinase has been identified in insects, yeasts, and crustaceans, plants and mammals. In the species that could synthesize or metabolize chitin, many studies have revealed its ability in anti-parasite/pathogen invasions, particularly the chitin-containing pathogens. In the species that could not synthesize or metabolize, it was shown to be constitutively expressed in epithelial cells to provide the first line of defense against infection by mediating the inflammation and immune processes, such as involved in lung diseases, bowel and airway inflammation [44-46]. However, the identification and functional characterization of chitinase during immune responses are still lacking in fish species, compared to its well documented immune roles in other species. Previously, fish chitinases were reported to have high activity in the stomach, exhibited markedly high activity toward chitin nanofibers, as fish efficiently degrade chitin ingested as food [47-49]. The chitinase

enzyme activities have been characterized in digestive tract of the cod [50], Arctic charr [51], and Japanese Sardine [52]. Additionally, chitinase was expressed throughout the life span of *Chirostoma estor*, with maximum expression in the hepatopancreas of juvenile stage [53]. In zebrafish, chitinase was stably detectable in early embryonic development stage [54], indicating the critical roles of chitinases in innate immunity functioned early than previously assumed. Remarkably, the expression level of chitinase was specifically up-regulated in skin in the resistant pink salmon compared to susceptible fish, and identified as one of the most highly up-regulated genes in juvenile pink salmon during salmon lice infection [55]. Although the knowledge of the detailed roles of chitinases in fish mucosal immunity are still limited, the significant expression changes observed in current study suggested the protective roles of chinitases in fish mucosal surfaces. Further functional studies should be performed to better characterize chitinases family members, and to identify the roles in different mucosal tissues in protecting teleost fish from the pathogenically hostile environment.

In conclusion, the chitinase genes, including CHIT1, CHIT2 and CHIT3, were identified from turbot, and their expression patterns were first examined following different bacteria challenge in mucosal tissues. They are the orthologs of other fishes and higher vertebrate. The gene structures, the amino acid sequences and predicted structural domains are conserved across species. In present study, the significant mucosal expression signatures of chitinase genes indicates their key roles to prevent pathogen attachment and entry in the first line of host defense. Expanding the knowledge of chitinases and mucosal immune response would definitely avail the utilization of chitinases to increase the disease resistance in the mucosal surfaces and facilitating the disease resistant family selection.

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657 **Table.1** Primary structural analysis. Properties of CHIT1, CHIT2 and CHIT3
658 determined by ProtParam.

Protein	CHIT1	CHIT2	CHIT3
No. of amino acids	479	486	408
Molecular weight	52088.45	53009.45	45693.42
Theoretical pI	7.03	5.01	6.26
Total No. of negatively charged residues (Asp + Glu)	35	45	44
Total No. of positively charged residues (Arg + Lys)	35	35	41
Formula	C ₂₃₄₀ H ₃₄₈₆ N ₆₁₆ O ₆₉₆ S ₂₂	C ₂₃₇₅ H ₃₅₄₆ N ₆₁₂ O ₇₂₁ S ₂₅	C ₂₀₅₀ H ₃₀₉₈ N ₅₄₀ O ₆₁₅ S ¹⁷
Instability index	28.37	34.76	23.99
Aliphatic index	63.38	65.31	70.51
Grand average of hydropathicity (GRAVY)	-0.296	-0.272	-0.388

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678 **Table 2.** Comparison of turbot CHIT1 amino acid sequences with those of other
679 teleost and human.

	Turbot	Atlantic salmon	Large yellow croaker	Japanese flounder	Channel catfish	Human
Accession no.	KY421058	XP_013987245.1	XP_010745222.1	BAD15059.1	XP_017306451.1	NP_003456.1
ORF (bp)	1440	1479	1452	1455	1446	1401
Amino acid residues	479	492	483	484	481	466
Entire ORF^a	-	79.4(79.4)	90.7(90.8)	91.8(92.0)	78.4(78.8)	60.9(61.7)
Entire amino acids^a	-	83.7(90.7)	92.6(97.1)	93.2(95.7)	82.2(89.4)	52.2(68.9)
Glyco_18 domain^{a, b}	-	86.7(93.0)	94.8(98.8)	95.6(97.7)	84.6(91.6)	56.7(74.3)

680 ^aThe percentages of sequence identity and similarity were calculated by the MatGAT program and
681 the values expressed as identity (similarity); ^b Predicted Glyco_18 domains based on the results
682 from the SMART program.

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702 **Table 3.** Comparison of turbot CHIT2 amino acid sequences with those of other
703 teleost.

	Turbot	Atlantic salmon	Large yellow croaker	Japanese flounder	Channel catfish
Accession no.	KY421059	XP_013991842.1	XP_010736405.1	BAD15060.1	XP_017341907.1
ORF (bp)	1660	1458	1488	1464	1494
Amino acid residues	486	485	495	487	497
Entire ORF ^a	-	54.7(55.3)	75.5(76.7)	77.0(77.1)	65.4(66.7)
Entire amino acids ^a	-	51.3(70.2)	88.5(94.3)	90.8(96.1)	78.3(88.9)
Glyco_18 domain ^{a, b}	-	57.3(74.5)	92.2(97.4)	92.2(97.4)	81.2(92.2)

704 ^a The percentages of sequence identity and similarity were calculated by the MatGAT program and
705 the values expressed as identity (similarity); ^b Predicted Glyco_18 domains based on the results
706 from the SMART program.

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726 **Table 4.** Comparison of turbot CHIT3 amino acid sequences with those of other
727 teleost.

	Turbot	Fugu rubripes	Large yellow croaker	Japanese flounder	Channel catfish
Accession no.	KY421060	XP_011600979.1	XP_019119111.1	BAD15061.1	XP_017352011.1
ORF (bp)	1227	1362	1497	1434	1419
Amino acid residues	408	453	498	477	472
Entire ORF ^a	-	56.8(57.6)	51.0(51.8)	59.5(59.9)	56.8(57.0)
Entire amino acids ^a	-	48.6(64.7)	37.4(54.8)	46.6(62.3)	45.4(63.3)
Glyco_18 domain ^{a, b}	-	51.9(68.2)	45.6(65.2)	53.1(69.1)	50.7(69.7)

728 ^a The percentages of sequence identity and similarity were calculated by the MatGAT program and
729 the values expressed as identity (similarity); ^b Predicted Glyco_18 domains based on the results
730 from the SMART program.

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Fig. 2 Schematic representation of the domain architecture of chitinases (CHIT1, CHIT2 and CHIT3) in turbot. Domains were determined using SMART program. The three proteins have similar domains; however, variations in the starting and ending positions exist in three proteins, e.g., Signal peptide (CHIT1: 1-21; CHIT2: 1-21; CHIT3: no signal peptide), Glyco_18 (CHIT1: 22-364; CHIT2: 22-365; CHIT3: 12-325), ChtBD2 (CHIT1: 431-479; CHIT2: 438-486; CHIT3: 354-406).

Fig. 3 Phylogenetic tree for the turbot chitinase genes. The phylogenetic tree was constructed based on the amino acid sequences of chitinases from species of fish and mammals, using the neighbor-joining method in MEGA 6. Gaps were removed by complete deletion and the phylogenetic tree was evaluated with 1,000 bootstrap replications, and the bootstrapping values were indicated by numbers at the nodes. Dark solid circles, dark filled triangle and dark filled inverted triangle indicated the newly characterized turbot CHIT1 gene, CHIT2 gene and CHIT3 gene, respectively.

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792 **Fig. 4** Gene expression analysis of the chitinases in different healthy turbot tissues.

793 Expression levels were calibrated against tissue which had the lowest expression level,

794 and 18S rRNA was used as a reference gene. HK was the abbreviation for head kidney.

795 (A) Tissue distribution of CHIT1 in turbot; (B) Tissue distribution of CHIT2 in turbot,

796 (C) Tissue distribution of CHIT3 in turbot.

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Fig. 5 Real-time qPCR analysis for chitinases expression levels following *Vibrio anguillarum* infection. The chitinases expression was measured in the mucosal tissues including skin, gill, and intestine at the time points of 2 h, 6 h, 12 h, and 24 h post-infection. Fold change was calculated by the change in expression at a given time point relative to the untreated control and normalized by change in the 18S housekeeping gene. The results were presented as mean $\pm$ SE of fold changes and * indicated statistical significance at $P < 0.05$. (A) Relative gene expression level of CHIT1 following *Vibrio anguillarum* infection in the mucosal tissues at different time points; (B) Relative gene expression level of CHIT2 following *Vibrio anguillarum* infection in the mucosal tissues at different time points; (C) Relative gene expression level of CHIT3 following *Vibrio anguillarum* infection in the mucosal tissues at different time points.

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Fig. 6 Real-time qPCR analysis for chitinases expression following *Streptococcus iniae* infection. The chitinases expression levels were measured in the mucosal tissues including skin, gill, and intestine, at the time points of 2 h, 4 h, 8 h and 12 h post-infection. Fold change was calculated by the change in expression at a given time point relative to the untreated control and normalized by changes in the 18S housekeeping gene. The results were presented as mean $\pm$ SE of fold changes and the * indicated statistical significance at $P < 0.05$. (A) Relative gene expression level of CHIT1 following *Streptococcus iniae* infection in the mucosal tissues at different time points; (B) Relative gene expression level of CHIT2 following *Streptococcus iniae* infection in the mucosal tissues at different time points; (C) Relative gene expression level of CHIT3 following *Streptococcus iniae* infection in the mucosal tissues at different time points.

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