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Immunological responses of turbot (*Psetta maxima*) to nodavirus infection or polyriboinosinic polyribocytidylic acid (pIC) stimulation, using expressed sequence tags (ESTs) analysis and cDNA microarrays

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Abstract To investigate the immunological responses of turbot to nodavirus infection or pIC stimulation, we constructed cDNA libraries from liver, kidney and gill tissues of nodavirus-infected fish and examined the differential gene expression within turbot kidney in response to nodavirus infection or pIC stimulation using a turbot cDNA microarray. Turbot were experimentally infected with nodavirus and samples of each tissue were collected at selected time points post-infection. Using equal amount of total RNA at each sampling time, we made three tissue-specific cDNA libraries. After sequencing 3230 clones we obtained 3173 (98.2%) high quality sequences from our liver, kidney and gill libraries. Of these 2568 (80.9%) were identified as known genes and 605 (19.1%) as unknown genes. A total of 768 unique genes were identified.

The two largest groups resulting from the classification of ESTs according to function were the cell/organism defense genes (71 uni-genes) and apoptosis-related process (23 uni-genes). Using these clones, a 1920 element cDNA microarray was constructed and used to investigate the differential gene expression within turbot in response to experimental nodavirus infection

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or pIC stimulation. Kidney tissue was collected at selected times post-infection (HPI) or stimulation (HPS), and total RNA was isolated for microarray analysis. Of the 1920 genes studied on the microarray, we identified a total of 121 differentially expressed genes in the kidney: 94 genes from nodavirus-infected animals and 79 genes from those stimulated with pIC. Within the nodavirus-infected fish we observed the highest number of differentially expressed genes at 24 HPI. Our results indicate that certain genes in turbot have important roles in immune responses to nodavirus infection and dsRNA stimulation.

Introduction

The turbot (*Psetta maxima*) is a very valuable commercial flatfish with well-established markets in the eastern North Atlantic and Mediterranean regions, and also in some Asian countries such as China and Korea. Due to its high market value, turbot has been one of the most promising aquaculture candidates in Europe [1]. As with other aquaculture species, intensive aquaculture of turbot often faces disease problems especially in early life stages; this can limit stable production of the species. The continued success of this industry in the highly competitive world market requires additional research and development activities aimed at improving health management techniques.

To efficiently manage disease problems in turbot aquaculture, information on how they respond to pathogen exposure and antigen stimulation is needed. At present our understanding of the immune response of turbot is very limited. Furthermore there are few genomic tools to study the immune system of this species. At the time of writing, there were only 176 nucleotide sequences available for turbot in public databases and of these only 32 genes are components of the immune system.

Expressed sequence tag (EST) analysis has been widely used to identify genes involved in the immune systems of commercially important fish species: Atlantic halibut (*Hippoglossus hippoglossus*) [2], Japanese flounder (*Paralichthys olivaceus*) [3–7], winter flounder (*Pleuronectes americanus*) [8], channel catfish (*Ictalurus punctatus*) [9], Japanese eel (*Anguilla japonica*) [10], rainbow trout (*Oncorhynchus mykiss*) [11], Atlantic salmon (*Salmo salar*) [12], common carp (*Cyprinus carpio*) [13,14], yellowtail (*Seriola quinqueradiata*) [15] and sea bream (*Sparus aurata*) [16]. More recently, cDNA microarray studies combined with EST analysis have been shown to be a powerful method for studying fish immune response against pathogens or immunostimulants. Such studies have been conducted for Atlantic salmon [17], Japanese flounder [18,19] and sea bream [16]. Among the flatfish, the Japanese flounder has been the most intensively studied species with respect to immune function. Studies have investigated this species responses at early time points (1 h and 6 h post-stimulation) to infection with Hirame rhabdovirus, and to stimulation with the immunostimulants Concanavalin A (Con A), phorbol myristate acetate (PMA), and lipopolysaccharide (LPS)[18,19].

Polyriboinosinic polyribocytidylic acid (pIC) is a synthetic dsRNA widely used in the study of the immune response to virus. It is a potent inducer of type I interferon genes in fish and is known to induce some, but not all, of the stimulatory effects of viral dsRNA in higher vertebrates [20,21]. Nodaviruses that infect fishes belong to the genus *Betanodavirus*, family Nodaviridae [22]. They are small (25–30 nm) single-stranded positive-sense RNA viruses that are responsible for a disease known as viral encephalopathy and retinopathy (VER), viral nervous necrosis (VNN), or fish encephalitis [22,23]. Disease outbreaks caused by nodavirus have been reported in turbot culture since the early 1990s and they are considered to be a major threat to profitable commercial culture [24,25]. For turbot, information on their immune response to viral-like antigens or virus is limited to two studies. One described the molecular cloning of myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (Mx) and its *in vivo* and *in vitro* expression in turbot tissues following pIC stimulation [26]. The other described the molecular cloning of interferon regulatory factor 1 (IRF1) and its expression in a variety of tissues at 24 h post-stimulation with pIC [27].

To improve our understanding of the immune system of turbot and the availability of genomic tools for this species we generated ESTs and a cDNA microarray. These tools were used to identify and characterize genes important in the response to stimulation with pIC and nodavirus infection. This is the first report on large-scale immune gene discovery in turbot and the first large-scale examination of the transcriptional response to pIC stimulation and nodavirus infection.

Materials and methods

EST analysis

Tissue preparation and RNA isolation

Thirty-two turbot (average weight 40 g) were injected intra-muscularly (IM) with 50 μl of nodavirus suspension (10^7 $TCID_{50}$ ml^{-1}) in the epaxial muscles at each side of the vertebrate column (10^6 $TCID_{50}$/fish). The virus (strain AH95-NorA) was originally isolated from Atlantic halibut and propagated on the SSN-1 cell line. Prior to and after infection, the fish were held in 16 °C flowing seawater at the Institute for Marine Research, Bergen, Norway. In order to obtain RNA representative of both the innate and adaptive immune systems, liver, head kidney and gill tissues were removed from 4 individuals at each of the following time points: 0 h, 6 h, 12 h, 24 h, 3 days, 8 days, 15 days, 23 days post-infection. During the infection period, no mortality occurred.

Total RNA was isolated from each sample using FastRNA Pro Green Kit (BIO 101 Systems) according to the manufacturer's instructions. For each sample, equal amounts of total RNA from the same organ of each of the four fish were