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Molecular characterisation of TNF, AIF, dermatopontin and VAMP genes of the flat oyster *Ostrea edulis* and analysis of their modulation by diseases

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ABSTRACT

Bonamiosis and disseminated neoplasia (DN) are the most important diseases affecting cultured flat oysters (*Ostrea edulis*) in Galicia (NW Spain). Previous research of the response of *O. edulis* against bonamiosis by suppression subtractive hybridisation yielded a partial expressed sequence tag of tumour necrosis factor (TNF) and allograft inflammatory factor (AIF), as well as the whole open reading frame for dermatopontin and vesicle-associated membrane (VAMP). Herein, the complete open reading frames of TNF and AIF genes were determined by the rapid amplification of cDNA, and the deduced amino acid sequences of the four genes were characterised. Phylogenetic relationships for each gene were studied using maximum likelihood parameters. Quantitative-PCR assays were also performed in order to analyse the modulation of the expression of these genes by bonamiosis and disseminated neoplasia. Gene expression profiles were studied in haemolymph cells and in various organs (gill, gonad, mantle and digestive gland) of oysters affected by bonamiosis, DN, and both diseases with regard to non-affected oysters (control). TNF expression in haemolymph cells was up-regulated at heavy stage of bonamiosis but its expression was not affected by DN. AIF expression was up-regulated at heavy stage of bonamiosis in haemolymph cells and mantle, which is associated with heavy inflammatory response, and in haemolymph cells of oysters affected by DN. AIF expression was, however, down-regulated in other organs as gills and gonads. Dermatopontin expression was down-regulated in haemolymph cells and digestive gland of oysters affected by bonamiosis, but DN had no significant effect on its expression. Gills and gonads showed up-regulation of dermatopontin expression associated with bonamiosis. There were significant differences in the expression of TNF and VAMP depending on the bonamiosis intensity stage whereas no significant differences were detected between light and heavy severity degrees of DN for the studied genes. VAMP expression showed also differences among haemolymph cells and the organs studied. The occurrence of both diseases in oysters involved haemolymph cell gene expression patterns different from those associated to each disease separately: no significant effect was observed in TNF expression, dermatopontin was up-regulated and marked up-regulation of AIF and VAMP was recorded, which suggests a multiplier effect of the combination of both diseases for the latter two genes.

1. Introduction

Flat oyster (*Ostrea edulis*) populations in Galicia (NW Spain) have declined significantly since the 19th century, due to overexploitation and diseases. Since the 1980's, infections by protozoans of the genus *Bonamia* have caused mass mortalities of oyster populations hampering the population recovery (Montes et al., 1991). Bonamiosis is an infection caused by parasites of the genus *Bonamia*, haplosporidian protozoans, which are able to survive and proliferate within oyster's haemocytes. These parasites evoke a heavy inflammatory reaction associated with necrosis at the late stage of infection and frequently lead to the oyster's death (Balouet et al., 1983; Bucke, 1988; da Silva et al., 2008). The parasites induce changes in the oyster haemocytes to neutralise microbicidal mechanisms (Comesaña et al., 2012; Morga et al., 2009). Another important pathological condition affecting *O. edulis* populations is disseminated neoplasia (DN) (Alderman et al., 1977; da Silva et al., 2005, 2011). DN is an oyster disease resembling leukaemia that is characterised by a quick proliferation of abnormal circulating cells of undetermined origin, which infiltrate into the connective tissue

Abbreviations: AIF, Allograft inflammatory factor; cDNA, Complementary deoxyribonucleic acid; CDD, Conserved domain; CDR, Cysteine rich domain; DG, Digestive glands; DN, Disseminated neoplasia; EST, Expressed sequence tag; TRAF6, TNF-receptor associated factor 6; Hsc, Heat shock cognate; Hsp, Heat shock protein; ORF, Open reading frame; PI, Isoelectrical point; Q-PCR, Quantitative polymerase chain reaction; RACE, Rapid amplification of cDNA ends; RNA, Ribonucleic acid; SSH, Suppressive subtractive hybridisation; SUMO, Small ubiquitin-like modifier; THD, TNF homology domain; TIMP, Tissue inhibitor of metalloproteinase; TNF, Tumour necrosis factor; UTR, Untranslated region.

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of most organs resulting in most cases in the death of the affected individuals (Barber, 2004). Neoplastic cells show impaired immune activities with regard to normal circulating cells (haemocytes), as well as a lack of adhesion, spreading and phagocytic ability (Beckmann et al., 1992; Diaz et al., 2011).

The internal defence system of molluscs comprises humoral and cellular elements that operate in a coordinated way during the recognition and destruction of invading organisms. Cellular response is carried out by haemocytes, being a key protection mechanism through phagocytosis and various cytotoxic reactions, such as the release of antimicrobial peptides and lysosomal enzymes. They are also involved in the respiratory burst which controls the production of microbicidal oxygen metabolites (Chu, 2000). In bivalves, prior studies on the immune response have been conducted mainly by traditional approaches, such as functional assays and histopathological studies. In recent years, the use of molecular approaches has allowed the identification and characterisation of various immune-regulatory genes and has facilitated the study of gene expression during disease processes in clams and oysters (Barreau-Roumiguière et al., 2003; Fan et al., 2007; Green and Barnes, 2009; Gueguen et al., 2006; Itoh et al., 2010; Lelong et al., 2007; Montagnani et al., 2004, 2007, 2008; Roberts et al., 2008; Yu et al., 2007). Knowledge on the molecular bases of defence mechanisms in flat oyster *O. edulis* is scarce. Boutet et al. (2003) cloned the heat shock cognate 70 (*hsc70*) and heat shock protein 70 (*hsp70*) genes. Recently, Morga et al. (2010, 2011) studied in vitro the molecular response of *O. edulis* haemocytes to *Bonamia ostreae*. To further understand the molecular basis of the immune response of the flat oyster against bonamiosis and DN, Martín-Gómez et al. (2013) combined suppression subtractive hybridisation and quantitative PCR approaches, identifying numerous genes involved in the development of the diseases. Gene ontology analysis of subtracted libraries previously sequenced by Martín-Gómez et al. (2012), revealed two partial ESTs with high homology to TNF (tumour necrosis factor) and AIF protein (allograft inflammatory factor), both included in the up-regulated library, and two ESTs comprising the whole open reading frame (ORF) for the transcripts of VAMP (vesicle-associated membrane) (up-regulated library) and dermatopontin (down-regulated library) proteins. These genes were chosen for further study among the entire identified oyster ESTs due to their very likely involvement in immune response and/or cancer process, according to previous information on their roles in mammals, as well as to analyse the differential expression patterns by quantitative PCR. TNF is a cytokine that plays an important role in diverse host responses such as septic shock, induction of other cytokines, cell proliferation, differentiation, necrosis and apoptosis (Rahman and McFadden, 2006). AIF is a molecule involved in inflammatory responses, allograft rejection and in the activation and role of macrophages. Both act as potent proinflammatory cytokines playing also roles in the neoplasia process. Dermatopontin is a widely distributed small molecular weight protein in the extracellular matrix, with cell binding properties, that play important roles in cell–matrix interactions and matrix assembly (Forbes et al., 1994). VAMP has a main role in the homotypic fusion of both early and late cell endosomes (Wade et al., 2001). Both dermatopontin and VAMP could be of interest in the bonamiosis because of their involvement in the interaction of host cells with the parasite and the phagocytosis process.

2. Materials and methods

2.1. Rapid amplification of cDNA ends (RACE)

Suppressive subtractive hybridisation (SSH) libraries previously constructed in our laboratory yielded highly expressed transcripts in haemocytes of *O. edulis* infected by *B. ostreae* and *Bonamia exitiosa*. One EST identified in the down-regulated library was annotated as dermatopontin, and three ESTs obtained from the up-regulated library were annotated as allograft inflammatory factor (AIF), tumour necrosis factor (TNF) (ligand) superfamily and vessicle-associated protein (VAMP). Transcripts of dermatopontin and VAMP comprised the whole open reading frame (ORF). In order to obtain the whole ORF of TNF and AIF genes, 5′ and 3′ RACE-PCRs were carried out with the RNA pool of haemolymph cells from oysters with heavy *Bonamia* spp. infections (infection intensity level 3, according to da Silva and Villalba (2004), and showing co-infection with *B. ostreae* and *B. exitiosa*). BD SMART™ RACE cDNA amplification kit (BD biosciences, Clontech, California, USA) was used according to the manufacturer's instructions. Specific primers used in RACE reactions were designed with the programme Primer-3 (Rozen and Skaletsky, 2000); they are shown in Table 1. The PCR profile consisted of an initial denaturation for 2 min at 94 °C, 25 cycles of 30 s at 94 °C, 30 s annealing at 65 °C (TNF) or 63 °C (AIF) and 3 min elongation at 72 °C, followed by a final elongation of 7 min at 72 °C. PCR products were seen on a 2% agarose gel and subsequently ligated into cloning vector pCR2.1 and transformed into *Escherichia coli* one shot top 10F′ chemically competent cells (Invitrogen life technologies). Transformed cells were screened by PCR using the vector's primers M13 (Table 1) and the PCR profile consisted of an initial denaturation for 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s annealing at 55 °C (M13 primers) or 68 °C (nested primers) and 90 s elongation at 72 °C, final elongation 7 min at 72 °C. The positive clones were cleaned for sequencing using ExoSap-IT (USB Corporation) enzyme, as supplied by the manufacturer. PCR products were sent to Secugen Company for sequencing. Sequence chromatograms were analysed using ChromasPro version 1.41 Technelysium Pty Ltd.

Table 1
Sequences of the primers used in this study.

Primer name	Sequence 5′-3′	Purpose
M13 forward	GTA AAA CGA CGG CCA G	Vector primer
M13 reverse	CAG GAA ACA GCT ATG AC	Vector primer
Nested PCR 1	TCG AGC GGC CGC CCG GGC AGG	Vector primer
Nested PCR 2	AGC GTG GTC GCG GCC GAG GT	Vector primer
TNF-GSP1-RACE	GATCAAAGTCCGTCACTCTGACCAT	To amplify the 5′ end of TNF cDNA and qPCR
TNF-GSP2-RACE	CCTATTTCCCGAACACTGGGAGATT	To amplify the 3′end of TNF cDNA
AIF-GSP1-RACE	TTCTGGATCTCCAGGTGAGTCTTAG	To amplify the 5′ end of AIF cDNA and qPCR
AIF-GSP2-RACE	GACCGACACTACATACTCGGAAGTT	To amplify the 3′ end of AIF cDNA and qPCR
TNF-Rev	GACCTAGCGTCCTCTTGGTCCAT	TNF reverse primer, qPCR
Derm-F	TCACACAGGCTCTGGATCTG	Dermatopontin forward primer, qPCR
Derm-R	TAGCCTGAACTTGGAGCACA	Dermatopontin reverse primer, qPCR
VAMPF	ACCAGAGCAGAAGACCTGGA	VAMP forward primer, qPCR
VAMP-R	CGCCACTGTTGTTTCCTCTT	VAMP reverse primer, qPCR
Cyt forward	GAGCCAGCTGGAGTGTGAAT	Reference forward primer, qPCR
Cyt reverse	GAGGTATCCCTGCCAAACAA	Reference reverse primer, qPCR