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Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, *Crassostrea gigas*, challenged with Ostreid herpesvirus 1

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Abstract :

Virus-induced genes were identified using suppression subtractive hybridisation (SSH) from Pacific cupped oyster, *Crassostrea gigas*, haemocytes challenged by OsHV-1. A total of 304 clones from SSH forward library were sequenced. Among these sequences, some homologues corresponded to (i) immune related genes (macrophage express protein, IK cytokine, interferon-induced protein 44 or multicopper oxidase), (ii) apoptosis related genes (Bcl-2) and (iii) cell signalling and virus receptor genes (glypican). Molecular characterization and phylogenic analysis of 3 immune-related genes (macrophage expressed protein, multicopper oxidase and immunoglobulin domain cell adhesion molecule) were performed. Finally, quantitative PCR revealed significant changes in the expression of immune related genes (multicopper oxidase, macrophage expressed protein, myeloid differentiation factor 88 and interferon-induced protein 44) in oysters experimentally challenged with OsHV-1.

These findings provide a first basis for studying the role of innate immunity in response to viruses in bivalves and identified genes may serve as markers of interest in breeding programs in order to obtain selected oysters presenting OsHV-1 resistance.

Keywords : Pacific cupped oyster; *Crassostrea gigas* ; OsHV-1 ; Immunity ; Gene expression ; Multicopper oxidase ; Macrophage expressed protein ; Ig cell adhesion molecule ; Interferon-induced protein 44 ; Virus RNA

1. Introduction

Meanwhile the development of mollusc aquaculture worldwide (FAO, 2008), infectious diseases due to an extended variety of pathogens including viruses remain a major concern. A wide host range is reported for herpes and herpes-like viruses among bivalves including oysters, clams and scallops (Renault and Novoa, 2004). A virus infecting the Pacific cupped oyster, *Crassostrea gigas*, in France has been purified from naturally infected larvae (Le Deuff and Renault, 1999) and its genome entirely sequenced (Davison et al., 2005). This virus was classified as the unique member of the *Malacoherpesviridae* under the name *Ostreid herpesvirus 1* (OsHV-1) (Davison et al., 2005; Davison et al., 2009). OsHV-1 is a large DNA enveloped virus that infects several bivalve species. Although two OsHV-1 genotypes were previously reported in France (OsHV-1 reference and OsHV-1 var) (Arzul et al., 2001b), the presence of a third genotype, termed OsHV-1 µVar, was reported in France since 2008 in association with massive mortality outbreaks among French *C. gigas* (Segarra et al., 2010).

Despite the impact that herpes virus infections may have on shellfish, no information is, however, available on the immune responses of oysters to these viruses. The main objective of the present study was to provide knowledge of anti-viral innate immunity of the Pacific cupped oyster, *Crassostrea gigas*. For this purpose, virus-induced genes were first searched in Pacific cupped oyster haemocytes challenged by OsHV-1 using Suppression Subtractive Hybridisation (SSH). Comparing the transcriptome changes was expected to identify cellular pathways and genes that are important to OsHV-1 resistance. Molecular characterization and phylogenic analysis of 3 selected immune-related genes were then performed and the expression of candidate genes was monitored by real time PCR in oysters experimentally challended with the virus. Finally, results obtained in the present study were discussed in relation to existing knowledge on the role of innate immunity against viruses in vertebrates and invertebrates.

2. Materials and Methods

2. 1. Challenge of oyster haemocytes with OsHV-1

Pacific oysters, *Crassostrea gigas*, 2 year-old, 8-10 cm in shell length were purchased from a shellfish farm located in Marennes-Oleron Bay (France). The haemolymph was withdrawn from the pericardial cavity with a sterile syringue of 1 mL equipped with a needle (0.9 x 25 mm). Haemolymphs collected from 40 individuals were pooled. Oyster haemocytes were divided into 4 tissue culture flasks (25 cm^2, Nunc) adjusted to 7 x 10^6 viable cells each one. The culture flasks were incubated at 15º C for 2 h for cell settlement (Fig. 1a). The haemolymph (the acellular counterpart) was then removed from the flasks and kept on chilled ice. Two flasks were inoculated with the filtered tissue homogenate prepared from experimentally infected axenic larvae. Infected axenic larvae were obtained using a previously described protocol (Le Deuff *et al.*, 1994). The two other flasks received non-infected axenic larval homogenate. After a 1 h 30 contact at 22°C, filtered larval homogenates were removed from flasks and the haemolymph kept on ice was added again on cells after filtration through 0.22 µm filter. The culture flasks were then incubated 12 hours at 15°C and were used for total RNA extraction (Fig. 1b).

2. 2. Suppression Subtractive Hybridisation (SSH)

After washing twice with cold PBS, 780 µL of the Trizol® (Invitrogen) reagent were added on cells in culture flasks and RNA extracted. Briefly, cDNA was obtained from 1µg of hemocyte RNA (control and challenged haemocytes) using the SMART PCR cDNA Synthesis Kit (Clontech), which allowed the amplification of cDNA from mRNA transcripts. The SSH assay was then performed using the PCR-Select cDNA Subtraction Kit (Clontech) following manufacturer's instructions. The cDNA from the tester and from the driver were digested with *Rsa* I, and the tester cDNA was then ligated to either two different cDNA adaptors.

PCR mixtures of forward substraction were ligated using TA cloning kit (Invitrogen). PCR products from selected clones were spotted onto duplicate membranes which were screened for virus-induced genes using the PCR Select Differential Screening Kit (Clontech). Clones that hybridised to the forward-subtracted probe and to both subtracted probes when the difference of signal intensity was higher than 2 were interpreted as differentially expressed, their expression being induced in oyster haemocytes in contact with OsHV-1 compared to control haemocytes.

Selected clones were sequenced using a BigDye terminator Cycle Sequencing Ready Reaction Kit and a 3100 Avant Genetic analyzer ABI Prism (Applied Biosystem, Hitachi). Sequences were compared against the GenBank database using BLAST programs on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/).

2. 3. 5'/3' RACE PCR and sequence analysis

The full-length cDNAs were obtained by 5' and 3' RACE PCR from the sequences identified by SSH. SMART RACE cDNA amplification Kit was used according to the manufacturer's instructions. 5' and 3' primers were designed using primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and synthesized by Eurogentec (Table I). After transformation in Top 10 competent bacteria (Invitrogen), clones were sequenced from both ends with TA forward and reverse primers as described above. Sequences were blasted using NCBI-BLAST software (http://www.ncbi.nlm.nih.gov/blast/). Open reading frames and protein conserved domains were predicted (ORF finder, Search conserved Domain NCBI, SMART, ScanProsite). Multiple sequence alignments (Clustal W) and phylogenic analysis (Neighbour-Joining algorithm) were carried out using MEGA4 software.

2. 4. Oyster challenges with OsHV-1

Two experiments were carried out. For the first experiment, two year-old Pacific oysters (adults) were purchased from an oyster farm in La Tremblade (Charente Maritime, France). For the second experiment, 14 month-old Pacific oysters (juveniles) were obtained from the same farm. For each experiment, 90 healthy individuals were randomly distributed in 6 tanks supplied with 12 L of filtered (1 µm) seawater and acclimatizated at 22°C during a period of one week. Oysters were then placed out of water for 24 h at 22 C and then anesthetized (4 h at 22°C) in a solution of seawater (1 v) / distilled water (4 v) containing 7% (w/v) of magnesium chloride ($MgCl_2$, 50 g L^{-1}).

Fifty µL of a 0.22 µm filtered larval homogenate were injected into the pericardial cavity of 45 animals. OsHV-1 infected larvae (*C. gigas*) kept frozen (-20°C) served as the virus source. The 45 remaining oysters received an injection of 50 µL of sterile seawater and served as negative controls. Inoculated oysters were then placed for 48 h at 22°C in 12 L tanks supplied in filtered (1 µm) seawater without food supply or seawater change.

In order to evaluate gene expression, 3 oysters from each tank were sacrificed at each collecting time. Hemolymphs were collected 0 h, 24 h and 48 h post-injection for the first experiment and 0 h, 12 h, 24 h and 48 h post-injection for the second experiment. Haemolymphs were pooled for each tank (3 oysters per tank) at each collecting time and then haemocytes were pelleted by centrifugation (1500 *g* for 10 min, 4°C). Haemocyte RNAs were extracted from collected hemocytes as reported above.

2. 5. Gene expression by real-time PCR

In the first experiment, the expression of 6 immune relevant oyster genes identified by SSH was analysed. The 6 immune genes were: (i) immunoglobulin domain cell adhesion molecule, (ii) macrophage expressed protein, (iii) multicopper oxidase (laccase), (iv) glypican, (v) IK Cytokine, and (vi) myeloid differentiation factor 88 (MyD88) genes. In the second expriment, the six previous selected genes were again monitored and the expression of an additional gene was also quantified: the interferon-induced protein 44 (IFI44) gene. Elongation factor I (EF I) gene expression was used to normalize gene expression by real-time PCR. The EF I gene didn't revealed expression variation for both samples extracted from OsHV-1-challenged haemocytes and control haemocytes (data not shown).

Virus gene expression was also monitored by real time RT PCR targeting the OsHV-1 ORF5 (C9/C10 primers; Barbosa-Solomieu et al., 2004) in both experiments.

DNase treated RNA aliquots of 500 ng were reverse transcribed using SuperScript™ III reverse transcriptase (Invitrogen). The relative levels of gene transcripts in haemocytes from control and challenged oysters were monitored by real-time PCR using a MX3000P apparatus (Stratagene). Generated cDNAs were diluted at 1/30 with sterile water before use. Real-time PCR was carried out in triplicates in a total volume of 25 µL containing diluted cDNA (5 µL), Full Velocity® SYBR® Green QPCR Master Mix (12.5 µL, Stratagene®), sterile water (2.5 µL, Promega) and each specific primer pair (2.5 µL, Eurogentec) (Table I). PCR conditions consisted of DNA denaturation (10 min, 95 °C) followed by 40 amplification cycles (10 s at 95°C and 30 s at 60°C). A melting curve of PCR products (60 to 95°C) was performed to ensure the detection of a single specific product. Amplification of a specific cDNA (Elongation factor I) was carried out to confirm the steady-state expression of a housekeeping gene. Each microplate included negative control (total RNA treated with DNase) and blank control (sterile water). PCR efficiency (E) was determined for each primer pair by constructing a standard curve from serial dilutions. The relative expression ratio for a considered gene is based on the PCR efficiency (E) and the Ct of the challenged oyster samples versus the control samples expressed in comparison to the reference gene (EF I) (Pfaffl, 2001).

2. 6. Statistical analysis

Results for gene expression were expressed as means ± standard error. Statistical tests were carried out using XLSTAT 2009. Since the low number of data, the Mann-Whitney test was used to analyse OsHV-1 effects at each sampling date. U values equal to 0 and P values lower than 0.05 were used to identify significant differences.

3. Results

3. 1. Identification of OsHV-1-induced genes by SSH

From the forward-subtracted library, a total of 304 EST sequences was obtained and analysed. Putative homologues were identified on the basis of systematic comparison with sequence databases. Only matches with E-values smaller than 10^{-3} were retained as significant. A preliminary classification was thus proposed distinguishing between (i) sequences identified as homologues of genes (E-values $< 10^{-3}$) encoding proteins with a known function (93, 30.6%), (ii) sequences corresponding to unknown genes (hypothetical proteins) (32, 10.5 %) and (iii) sequences which yielded no hits or had poor similarity (E–values $\geq 10^{-3}$) (179, 58.9%).

The 93 sequences that matched known genes coalesced into 18 clusters and 54 singletons, suggesting that the redundancy of the library was around 20% and most of these sequences fall in gene categories related to cell structure, DNA replication, transcription, repair, cell cycle control or different metabolism processes (Table II). Other interesting putative homologues corresponded to (i) immune related genes including macrophage express protein, myeloid differenciation factor 88, IK cytokine, interferon-induced protein 44 or multicopper oxidase, (ii) apoptosis related genes such as Bcl2 and Acheron-like and (iii) cell signalling and virus receptors such as glypican (Table II). The genes identified were clustered into 7 categories. EST sequences were submitted to GenBank with the following accession numbers (HS513778 to HS513832) (Table II).

3. 2. Molecular characterization and phylogenic analysis of immune-related genes

The complete sequence of 11 genes was obtained by 5'/3' RACE PCR and deposited in the GenBank (Table III). Three of these genes interpreted as immune effectors, i.e. multicopper oxidase (laccase), macrophage expressed protein and immunoglobulin domain cell adhesion molecule genes, were more extensively characterized.

The complete sequence of *Crassostrea gigas* multicopper oxidase (laccase) cDNA (2255 bp) consisted of a 5'-untranslated region (UTR) of 87 bp, an open reading frame (ORF) of 2007 bp that encoded a 668 amino acid protein, and a 3'-UTR of 161 bp including a 27 bp poly (A) tail. The *Crassostrea gigas* multicopper oxidase cDNA sequence was deposited in the GenBank under accession no. EU678320 (Table III). The estimated molecular mass of the 668 amino acid protein was 74.2 kDa with a theoritical isoelectric point at 6.26. Three amino acid regions from 30 to 149, 183 to 323 and 400 to 578 shared a high homology with conserved Cu-oxidase domains. *Crassostrea gigas* multicopper oxidase showed significant similarity with proteins identified in hydrozoans, nematodes, echinoderms, insects, branchiostoms, plants and fungi (Fig. 2).

The complete sequence of *Crassostrea gigas* macrophage expressed protein 1 like protein (CgMpeg1) cDNA (2847 bp) consisted of a 5'-UTR of 164 bp, an ORF of 1866 bp encoding a 621 amino acid protein, and a 3'-UTR of 817 bp including a 22 bp poly (A) tail. The CgMpeg1 sequence was deposited in the GenBank under accession no. EF627979 (Table III). Analysis of sequences based on BLAST searches demonstrated that CgMpge1 nucleotide sequence showed a significant degree of identity with nucleotide sequences from *Crassostrea virginica*, *Haliotis corrugata*, *H. rufescens* and *H. diversicolor supertexta*. Ninety percent of 383 bp, 72% of 2595 bp, 72% of 2977 bp and 69% of 2781 bp were identical to macrophage expressed protein mRNA from *C. virginica* (GenBank accession no. EU437745), *H. corrugata* (GenBank accession no. AY485640), *H. rufescens* (GenBank accession no. AY485641) and *H. diversicolor supertexta* (GenBank accession no. EF529460), respectively.

The estimated molecular mass of the 621 amino acid protein was 69.2 kDa and the theoritical isoelectric point was 7.49. SignalP 3.0 analysis didn't allow identification of a putative signal sequence and no transmembrane domain was also detected using the TMHMM tool. SMART and ScanProsite analyses revealed that the amino acid region from 89 to 312 shared a high homology (E.value 9.46e-10) with the conserved membrane-attack complex/perforin (MACPF) domain. CgMpge1 shared significant similarity with proteins identified in both invertebrates and vertebrates. For example, CgMpeg1 showed 49% identity and 66% similarity with *H. diversicolor supertexta* Mpeg1 (GenBank accession no. ABP96718), 45% identities and 61% similarity with a hypothetical protein from *Nematostella vectensis* (GenBank accession no. EDO37879), and 41% identities and 58% similarity with Mpeg1 from *Homo sapiens* (GenBank accession no. AAI12231). Comparison of various proteins using MEGA4 generated tree of phylogenetic relationship (Fig. 3).

The complete sequence of *Crassostrea gigas* immunoglobulin domain cell adhesion molecule subfamily protein cDNA (573 bp) consisted of a 5'-UTR of 52 bp, an ORF of 246 bp encoding a 81 amino acid protein, and a 3'-UTR of 275 bp including a 25 bp poly (A) tail. The *Crassostrea gigas* immunoglobulin domain cell adhesion molecule cDNA sequence was deposited in the GenBank under accession no. EU678312 (Table III). The estimated molecular mass of the 81 amino acid protein was 9.15 kDa and the theoritical isoelectric point was 6.02. SMART and ScanProsite analyses showed that the amino acid region from 1 to 62 shared a homology with the conserved immunoglobulin domain cell adhesion motif (IgCAM, Ig-like domain). *Crassostrea gigas* immunoglobulin domain cell adhesion molecule shared significant similarity with proteins identified in invertebrates including L1 CAM adhesion molecule (lad-2) from *Caenorhabditis elegans* (GenBank accession no. ABQ57414, 37% identities and 53% similarity).

3. 3. Gene expression in OsHV-1 challenged oysters

To confirm the induction of transcripts of identified genes related to immunity, RT-PCR assays with RNA from challenged oysters (OsHV-1 contact) and from control oysters were carried out using specific sets of primers (Table I).

In both experiments, analysis of viral gene expression showed the detection of viral RNA in OsHV-1 challenged oysters 48 h post-injection (data not shown).

In the first experiment, a significant increase in mRNA levels was reported for 4 genes MyD88, macrophage expressed protein, multicopper oxidase and immunoglobulin domain cell adhesion molecule) in challenged oysters 48 h post injection in comparison to non-challenged animals (controls) (Fig. 4). A significant increase in mRNA levels was also observed for two genes (macrophage expressed protein and immunoglobuline domain cell adhesion molecule) in challenged oysters 24 h post injection in comparison to control animals (Fig. 4). No significant differences were observed for other genes, i.e. glypican and IK cytokine (data not shown).

In the second experiment, the analysis of immune-relevant gene expression demonstrated a significant up-regulation of 4 genes (MyD88, macrophage expressed protein, multicopper oxidase and IFN-induced protein 44) 48 h post-injection in OsHV-1 challenged oysters in comparison to non-challenged oysters (Fig. 5). Moreover, two of these genes (multicopper oxidase and IFN-induced protein 44) presented a significant increase in transcript levels 24 h post OsHV-1 injection in comparison to controls (Fig. 5). Finally, MyD88 gene expression was also up-regulated 12 h post injection (Fig. 5).

4. Discussion

4. 1. Developing a study model

The suppression substractive hybridisation technique (SSH) was used to characterize Pacific cupped oyster (*Crassostrea gigas* L.) genes induced by OsHV-1 (Ostreid herpesvirus 1). The most adapted solution to carry out SSH appeared to use haemocytes maintained in vitro in contact with the virus (Fig. 1). As no cell lines allow OsHV-1 replication in vitro, the experimental infection of Pacific cupped oyster larvae was selected as the most suitable way to produce infectious virus particles (Arzul *et al.*, 2001; Le Deuff *et al.*, 1994; Le Deuff *et al.*, 1996).

Virus gene expression was monitored through real time RT PCR in both OsHV-1-challenged oyster experiments in order to demonstrate effective virus infection. It is the first report of the detection of virus RNA for OsHV-1. C9/C10 primers were selected as they have been successfully used to detect virus DNA by real time PCR (Pepin et al., 2008). This primer pair targets the ORF5 encoding a unknown protein. Although this approach needs to be improved in selecting carefully virus genes, OsHV-1 RNA quantification appears as a suitable tool to differentiate different status of infection (i.e. effective virus replication versus latent infection).

4. 2. Identification of OsHV-1-induced genes

Although previous studies reported the discovery of genes in different bivalve species in response to bacteria or protozoan parasites through SSH (Gestal et al., 2007; Tanguy et al., 2004; Wang et al., 2009), this approach was used for the first time in the present study in order to identify genes in a marine bivalve after a virus challenge.

Only clones derived from the forward-subtracted library were sequenced and analysed as the main objective of the present study was to search and identify OsHV-1-induced genes. However, it is well known that herpesviruses induce also a shut-down of cell protein expression (Roizman and Batterson, 1985). Clones identified as up-regulated in the presence of OsHV-1 were sequenced and compared to sequences available in databases. Clone sequences were identified as (i) sequences corresponding to genes with known function and (ii) sequences corresponding to unknown genes encoding proteins with unknown functions. The latter are certainly the most promising ones. However, the present study focused on oyster genes encoding proteins presenting homologies with proteins possessing known functions.

Several ESTs were selected because homology search demonstrated that they could be involved in virus/host cell interactions and in anti-viral immunity. It is well known that expression of host cell genes in response to viral infections constitutes a major step in the initiation of the host defence system (O'Shea, 1997; Welsh and Sen, 1997). Thus, the complete sequence of 11 oyster genes was obtained using RACE PCR (Table III).

We characterized for the first time a multicopper oxidase (laccase) gene from a marine mollusc (Table III). Multicopper oxidases consist of 2, 3 or 6 homologous Cu-oxidase domains and include laccases, 3 Cu-oxidase domain enzymes. Multicopper oxidases are oxidoreductive enzymes that catalyze a one-electron oxidation of broad range of polyphenols and aromatic substrates (Claus, 2004). Multicopper oxidases particularly phenoloxidases are shown to be important for immune defence in invertebrates (Soderhall and Cerenius, 1998) including marine molluscs (Aladaileh et al., 2007). Peter and Raftos (2002) showed that *Marteilia sydeneyi* infection (QX infection) lowered the level of phenoloxidase activity in the haemolymph of the Sydney rock oyster, *Saccostrea glomerata*. Moreover, *S. glomerata* bred

for QX resistance demonstrated higher phenoloxidase activity than unselected oysters (Butt and Raftos, 2008).

The *Crassostrea gigas* immunoglobulin domain cell adhesion molecule (Table III) is a small protein (81 aa) containing a single Ig-like domain that is normally found in several copies in large transmembrane proteins involved in cellular adhesion and cell-to-cell communication (*i.e.*, five C2-like immunoglobulin domains). It is the first identification of such a small molecule in marine bivalves. While a classical Ig domain contains about 100 residues, smaller ones (74-90 residues) have been observed in several Ig-related molecules.

Macrophage-expressed proteins have already been reported in marine molluscs (Mah et al., 2004; Roberts et al., 2009; Wang et al., 2008). These proteins possess a conserved membrane-attack complex/perforin (MACPF) domain. Perforin-like proteins are present in NK cells in vertebrates and are key actors in anti-viral defence (Young et al., 1986). SignalP 3.0 analysis didn't allow identification of a putative signal sequence for the macrophage-expressed protein from the Pacific cupped oyster. Although a signal sequence has been reported in proteins from abalone and vertebrates, ranging from 703 to 730 amino residues, no signal has been identified for the protein from anthozoans (*Nematosella vectensis*) presenting less amino acids (583 aa) such as CgMPeg1 (621 aa).

IFI44 was first reported in the liver of chimpanzees infected with hepatitis C (Kitamura et al., 1994). The IFI44 gene is an inducible interferon-specific gene (ISG). Its expression is up-regulated in the presence of interferon in cell cultures and it may be involved in anti-hepatitis C virus activity (Liu et al., 2007). However, its function remains unclear. As proposed by Hallen et al. (2007), IFI44 may induce a cellular GTP depletion that abolishes extracellular signal-regulated kinase signalling and results in cell cycle arrest. Green et al. (2008) reported a homologous gene in the Sydney rock oyster, *Saccostrea glomerata*. The expression of the Sydney rock oyster gene was significantly up-regulated in animals presenting neoplasia. Interestingly, these authors suggested that the aetiological agent of neoplasia among *Saccostrea glomerata* could be a virus (Green et al., 2008).

A gene encoding a protein presenting significant homologies with Bcl-2 was also identified in *Crassostrea gigas* in the present study and its complete sequence defined (Table III). During viral infections of multicellular organisms, induction of apoptosis is often observed and can be regarded as a primitive anti-viral mechanism (Everett and Mc Fadden, 1999; Koyama et al., 2000; O'Brien 1998). Upon viral infection, cells that die by apoptosis limit the ability of the virus to replicate and spread. There is consequently a selective advantage for viruses that subvert apoptotic processes. Several viruses carry genes that interfere with the host's apoptotic machinery. Such genes have been found in mammalian herpesviruses (Henderson et al., 1993; Zhu et al., 1995) and insect baculoviruses (Huang et al., 2000). Herpesviruses infecting bivalves have also been associated with apoptosis (Renault et al., 2000; Renault et al., 2001). Moreover, genes encoding proteins significantly related to inhibitor of apoptosis proteins (IAPs) in mammalian and insect cells have been described in OsHV-1 genome (Davison et al., 2005).

Kinases and phosphatases (phosphoinositide 4-kinase, serine/threonine protein kinase 38 and protein tyrosine phosphate) were also identified by SSH from OsHV-1-challenged haemocytes. Such enzymes are involved in cell signalling pathways and signal transduction through phosphorylation/dephosphorylation of target proteins. It has been demonstrated that viruses including herpesviruses are able to modulate host cell signalling pathways. Hale and Randall reported that binding of NS1 protein of influenza A virus induced an activation of phosphoinoside 3-kinase in virus-infected cells (Hale and Randall, 2007).

IK cytokine has been identified as a factor down regulating class II major histocompatibility complex antigens (class II MHC) (Krief et al., 1994). Genes presenting homologies with IK

cytokine have been also identified in different invertebrates including *Nematostella vectensis* (Putnam et al., 2007) and *Branchiostoma floridae* (Putnam et al., 2008). Although class II MHC antigens and their down regulation play a key role in anti-viral-immunity through specific recognition mechanism in vertebrates, it remains unclear how IK cytokine may be involved in invertebrate immunity.

Finally, a transmembrane receptor presenting high homologies with a melatonin receptor was identified by SSH in OsHV-1 challenged haemocytes. It has been demonstrated that melatonin has a role in the immune system modulating different functions such as cytotoxicity of natural killer cells (Maestroni et al., 1986). Due to its capability to diminish deleterious effects of different viruses including herpesviruses, melatonin has been considered as a therapeutic alternative to fight viral diseases (Bonilla et al., 2004; da Silva Nunes and Souza Pereira, 2008).

4. 3. Expression of immune-related genes in OsHV-1 challenged Pacific oysters

A main objective of the present study was to investigate the expression of candidate genes in Pacific cupped oysters after a virus challenge (OsHV-1 challenged oysters and non-challenged oysters) using real time PCR. Seven immune-related genes were selected to be monitored on the basis of sequence similarity and putative functions.

Three genes (MyD88, macrophage-expressed protein and multicopper oxidase) showed a significant up-regulatation of transcripts in both experiments suggesting that the products of these genes are involved in defence mechanisms against OsHV-1 and/or in disease development. Moreover, higher transcript levels were observed in the second experiment for these genes in comparaison to the first experiment. Finally, an up-regulation of *Crassostrea gigas* immunoglobulin domain cell adhesion molecule gene and IFI44 gene was reported in the first experiment and in the second experiment, respectively. Differences between both experiments may be explained in part by the age of oysters (14 month-old oysters versus 24 month-old animals). It has been reported that young oysters appear more susceptible to OsHV-1 infection than adults (Renault, 2008).

MyD88 is a Toll/IL1 receptor (TIR) domain containing cytoplasmic protein and it plays a role in signalling by the TLRs (McGetick and O'Neill, 2005). The signalling pathway via MyD88 leads to activaton of NF-κB transcription factors, c-Jun NH_2 terminal kinase (Jnk) and mitogen-activated protein kinases (MAPKs). MyD88-deficient mice ($MyD88^{-/-}$) presented increased susceptibility to pathogens including herpesviruses. Mansour et al. (2005) demonstrated that $MyD88^{-/-}$ mice were highly susceptible to HSV-1 infection suggesting that innate resistance to HSV-1 is mediated by MyD88. The role of MyD88 in immunity against genital HSV-2 infection in mice was also investigated by Tengvall et Harandi (2008). $MyD88^{-/-}$ mice showed more rapid disease progression, earlier death and higher HSV-2 titers compared to control mice (Tengvall and Harandi, 2007). Upon Murine Cytomegalovirus (MCMV) infection, $MyD88^{-/-}$ mice were more susceptible and presented increased viral loads compared to control mice (Delale et al., 2005). Moreover, NK cells from MCMV-infected $MyD88^{-/-}$ mice showed an impaired IFNγ production (Delale et al., 2005).

The protein encoded by *C. gigas* macrophage-expressed protein gene has sequence similarity to perforin and, therefore, may be involved in direct killing of infected cells and/or in inactivating OsHV-1 through virus envelop degradation. Moreover, the macrophage-expressed protein was identified as an executing molecule of a MyD88-dependent signalling pathway in the sponge *Suberites domuncula* (Wienst et al., 2005).

Phenoloxidase (PO) functions as an important immune response against many pathogens including viruses in invertebrates and is routinely used as a measure of immune

competence. PO has been detected in the haemolymph of *C. gigas* (Hellio et al., 2007). Xing et al. (2008) reported an increase of PO detection in the haemocytes of scallop *Chlamys farreri* after a challenge with the acute virus necrobiotic virus (AVNV).

The *Crassostrea gigas* immunoglobulin domain cell adhesion molecule may act as a recognition protein through its single Ig-like domain. It may promote binding between hemocytes and foreign bodies including virus particles, and subsequent phagocytosis acting as an opsonin in the oyster internal defense response. A link between hemolin, the most strongly induced immune gene known in *Lepidoptera*, and the prophenoloxidase system has been demonstrated by Terenius et al. (2007). These authors suggested that hemolin is a pattern recognition protein containing several Ig-like domains with the ability to bind pathogens and that it acts through the prophenoloxidase activating pathway. It can be speculated that such a link exists in bivalves and an increase of multicopper gene expression may enable a protection against OsHV-1.

5. Conclusions

Present results suggest that OsHV-1 recognition through a protein containing a single Ig-like domain, the *Crassostrea gigas* immunoglobuline domain cell adhesion molecule, may induce cell activation through an opsonin activity. A signal transduction involving MyD88 and protein kinase recruitment may result in an elevated expression of macrophage-expressed protein and multicopper oxidase acting as immune effectors. These findings provide a first basis for studying the role of the innate immune system to viruses. In turn, this will be of benefit to the identification of new targets for preventative actions in different cultured aquatic species.

However, repeated experiments are needed to confirm results and to study oyster gene expression mainly after 48 hours. Other oyster genes such as α2 macroglobuline or phosphatidylinositol 4-kinase can be also selected for gene expression analysis by real time PCR. Production of recombinant proteins represents the necessary following step in order to demonstrate biological activities of oyster proteins against OsHV-1 and their involvment in anti-virus immunity. The genes identified during the course of the present study may serve as markers of interest in breeding programs in order to obtain selected oysters presenting OsHV-1 resistance or tolerance (Sauvage et al., 2009). Finally, it would be very inetersting to compare the gene modulation using different OsHV-1 genotypes including OsHV-1 µVar.

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Figures

Figure 1. Challenge of Pacific cupped oyster haemocytes with OsHV-1. Fig 1A: oyster haemocyte collection and settlement in culture flasks. Fig 1B: oyster haemocyte challenge with OsHV-1 (contact with filtered larval homogenate).

Figure 1A

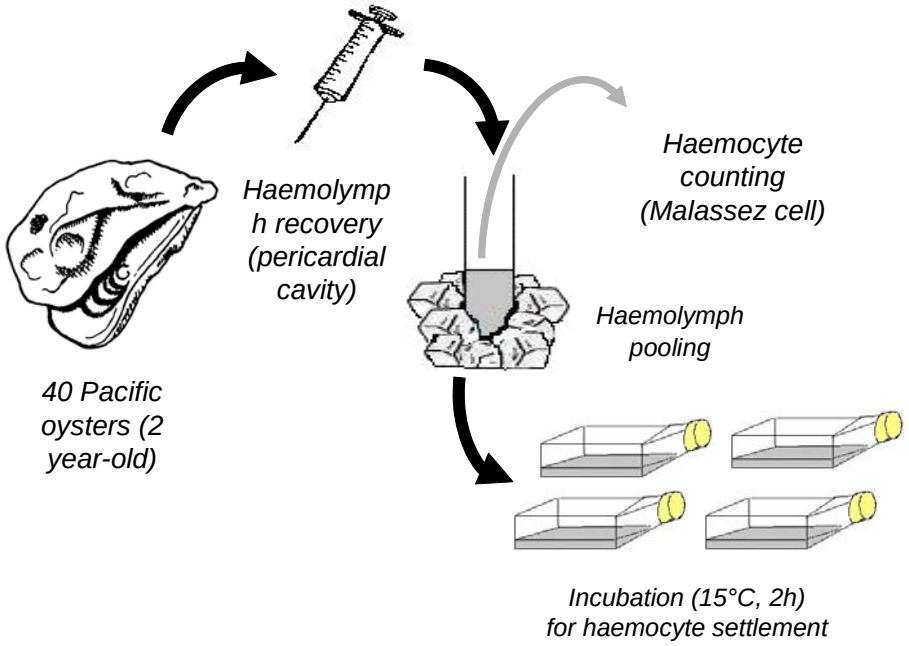


Figure 1B

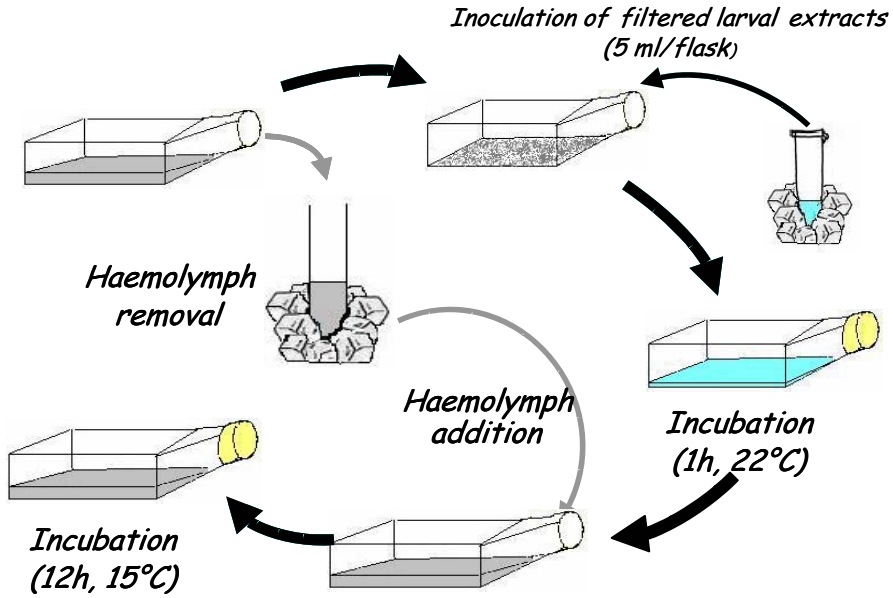


Figure 2. Neighbour-joining (NJ) tree showing phylogenic analysis of multicopper oxidase amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch (0.1 subsitution/site) is shown below the tree.

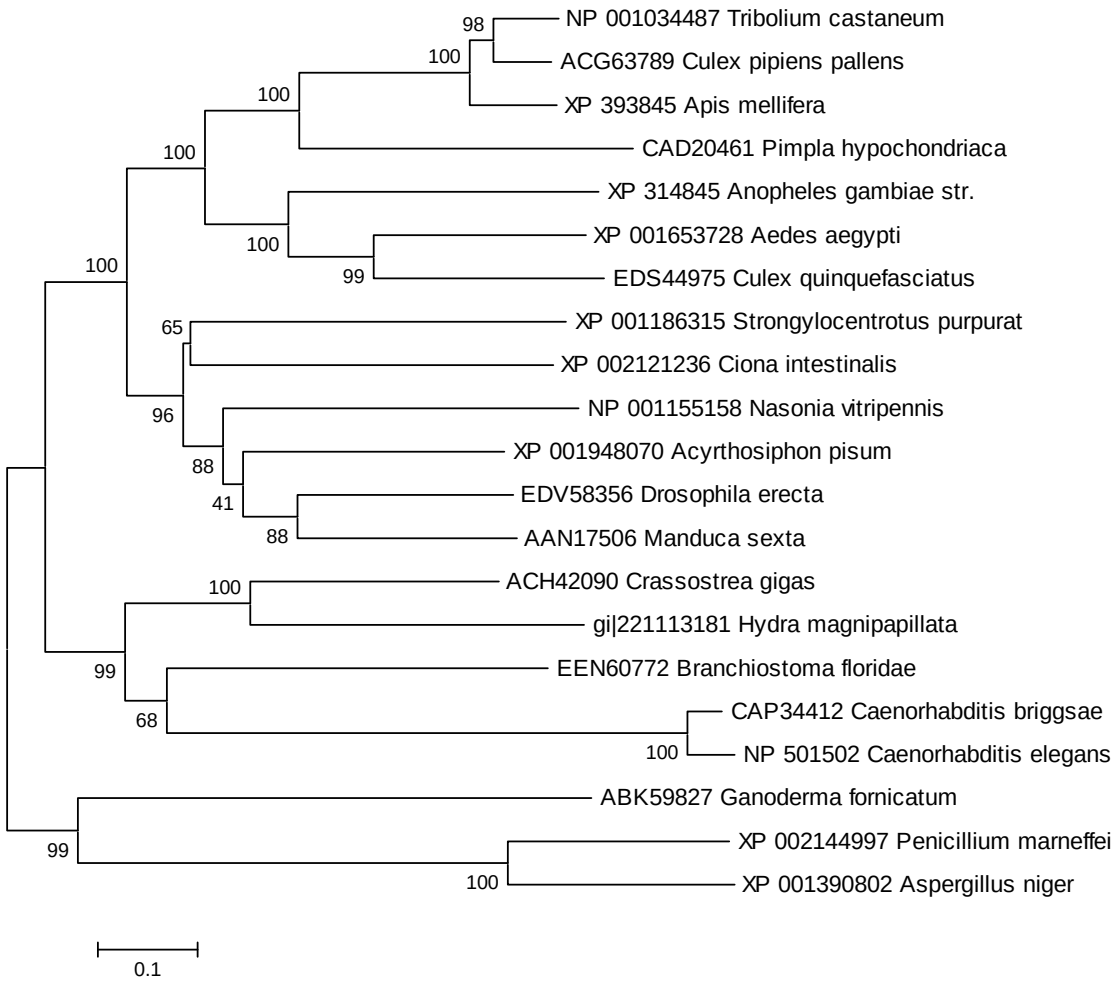


Figure 3. Neighbour-joining (NJ) tree showing phylogenic analysis of macrophage expressed protein 1 like protein (Mpeg1) amino acid sequences from various species. The tree is based on 1000 bootstrap replications. The scale for branch (0.2 subsitution/site) is shown below the tree.

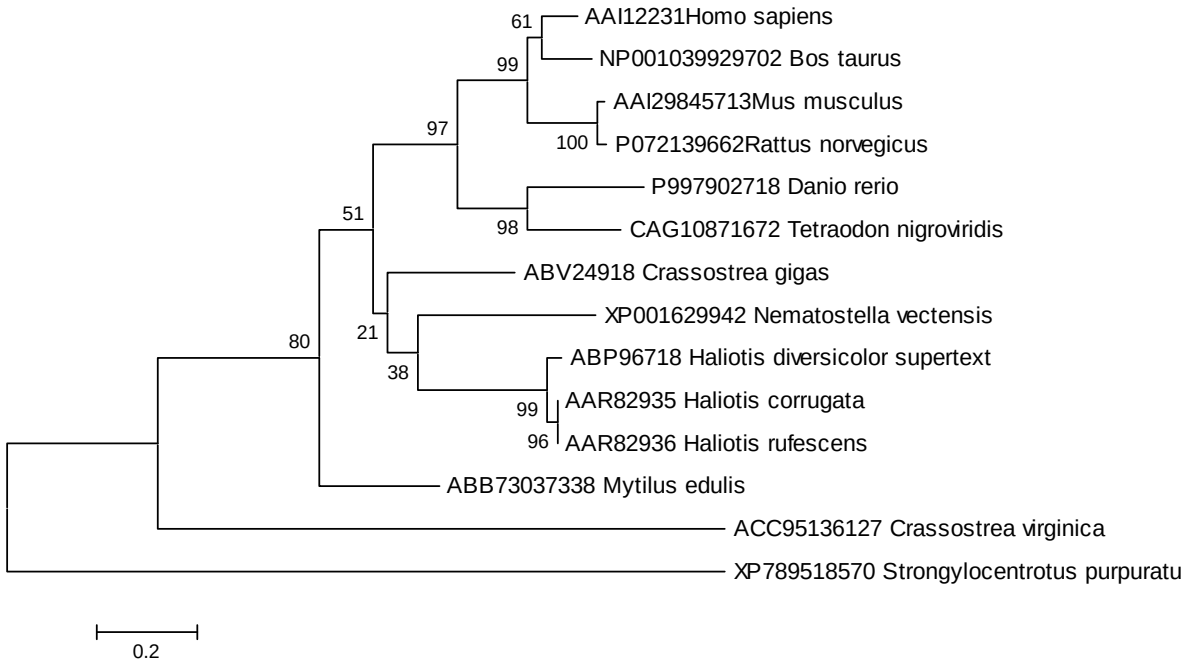


Figure 4. First experiment - Relative expression by real time PCR of 6 selected genes (immunoglobulin domain cell adhesion molecule, macrophage expressed protein, multicopper oxidase, glypican, IK Cytokine, and myeloid differentiation factor 88) 0h, 24 h and 48 h post infection. Expression levels were normalized to EF I and presented as relative expression to controls (mean ± SD, n = 9). Controls are arbitrarily assigned to a value of 1. * indicates a significant difference of gene expression compared to controls.

Figure 4A

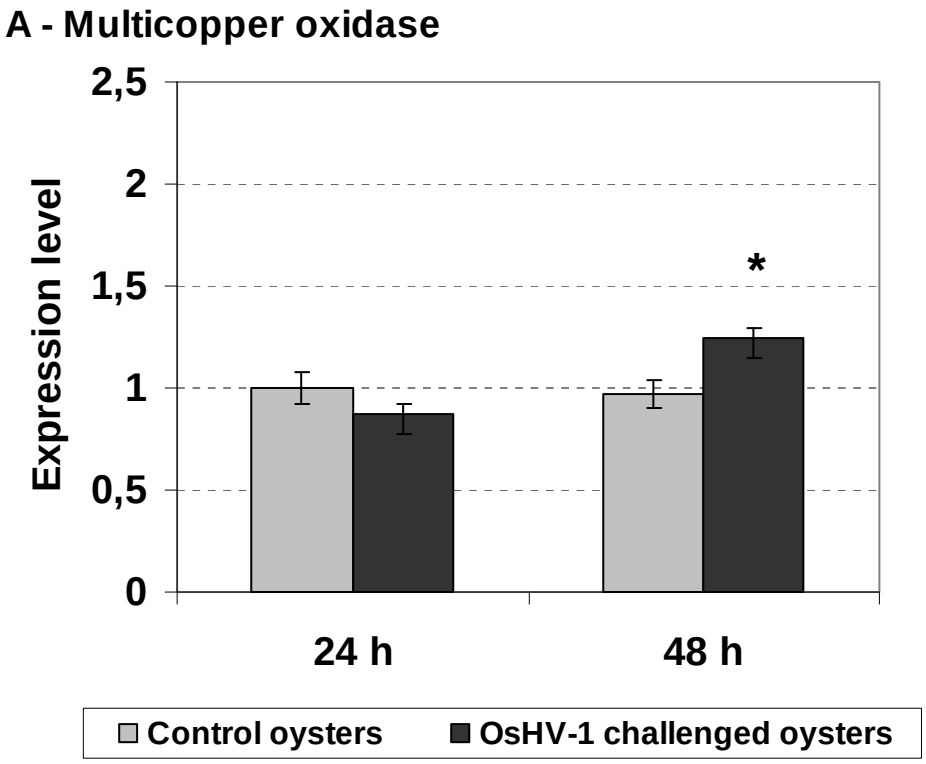


Figure 4B

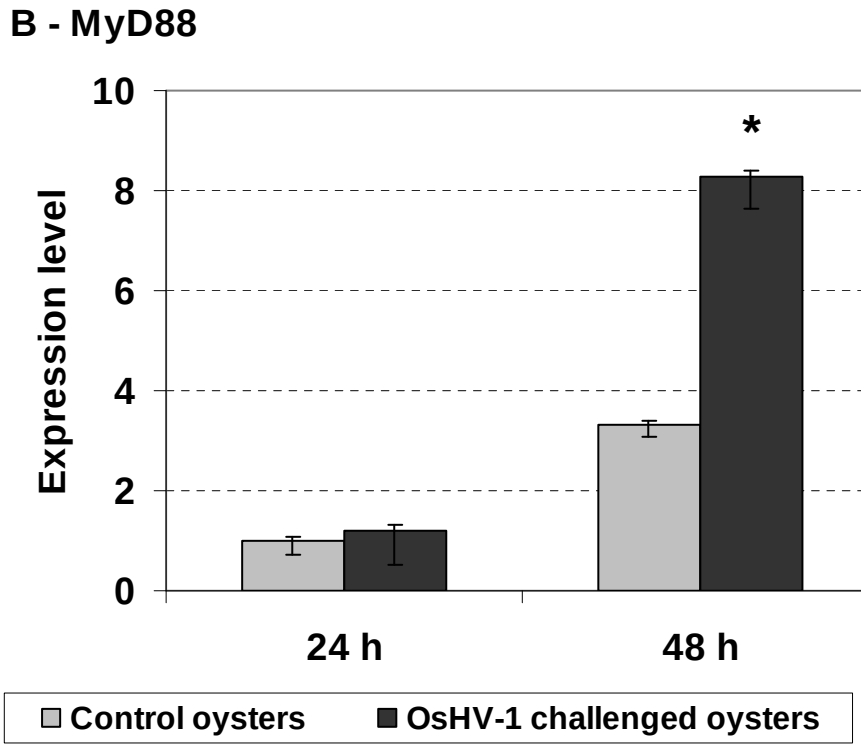


Figure 4C

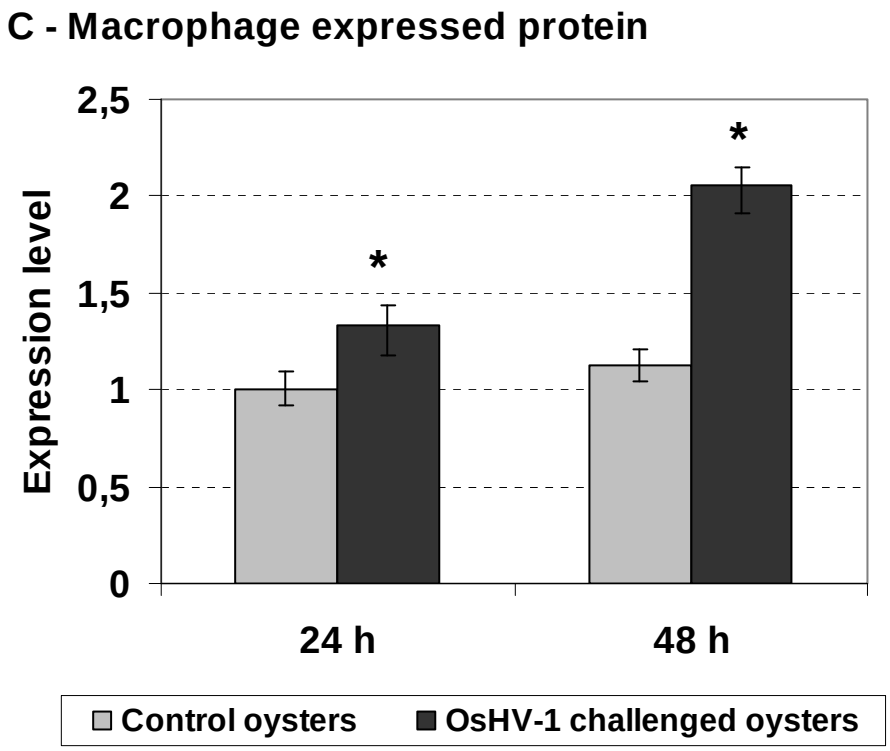


Figure 4D

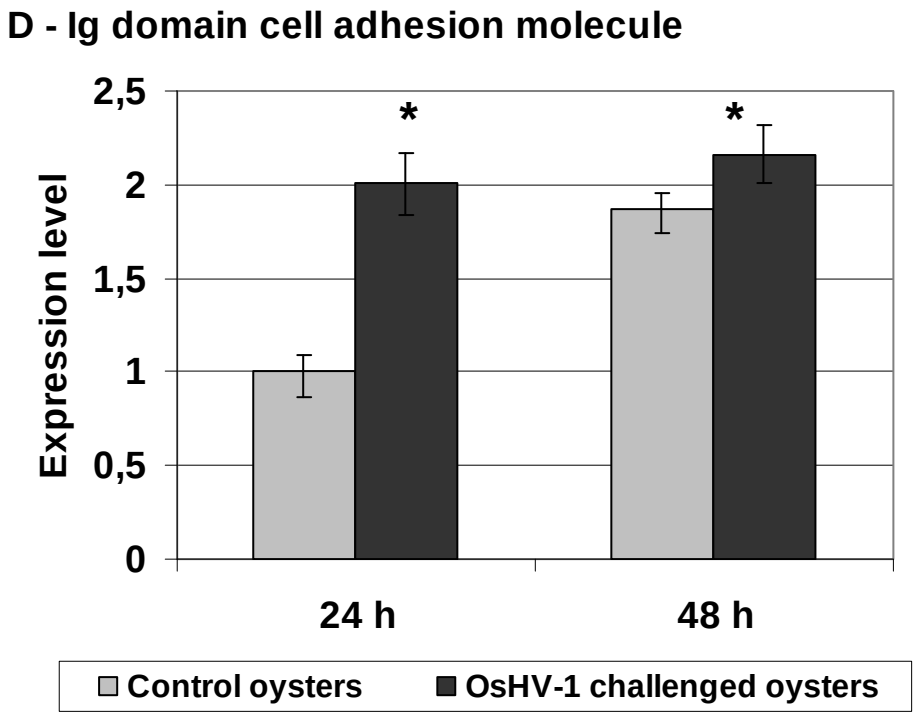


Figure 5. Second experiment - Relative expression by real time PCR of 7 selected genes (immunoglobulin domain cell adhesion molecule, macrophage expressed protein, multicopper oxidase, glypican, IK Cytokine, myeloid differentiation factor and interferon-induced protein 44) 0 h, 12h, 24 h and 48 h post infection . Expression levels were normalized to EF I and presented as relative expression to controls (mean ± SD, n = 9). Controls are arbitrarily assigned to a value of 1. * indicates a significant difference of gene expression compared to controls.

Figure 5A

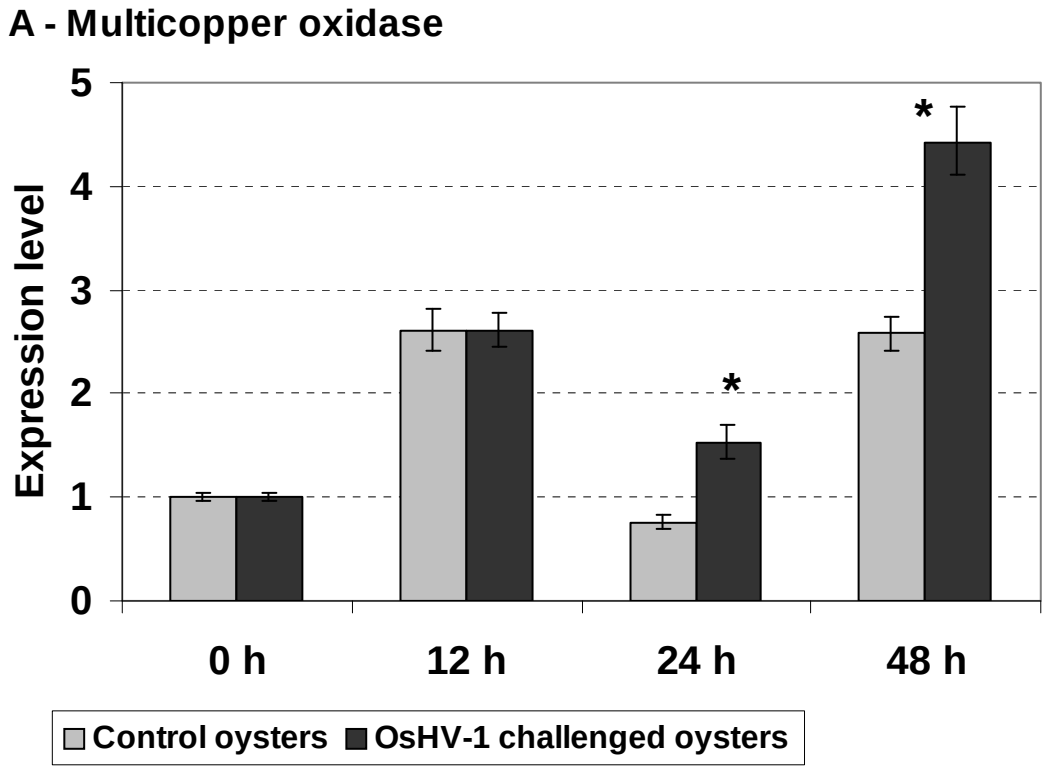


Figure 5B

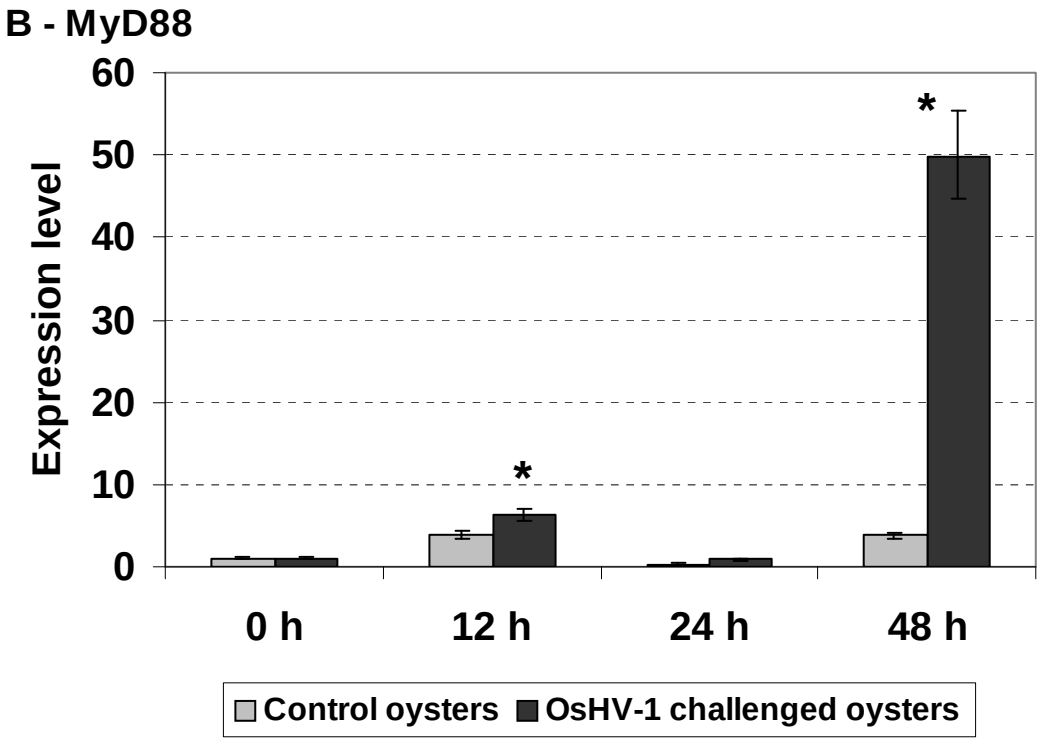


Figure 5C

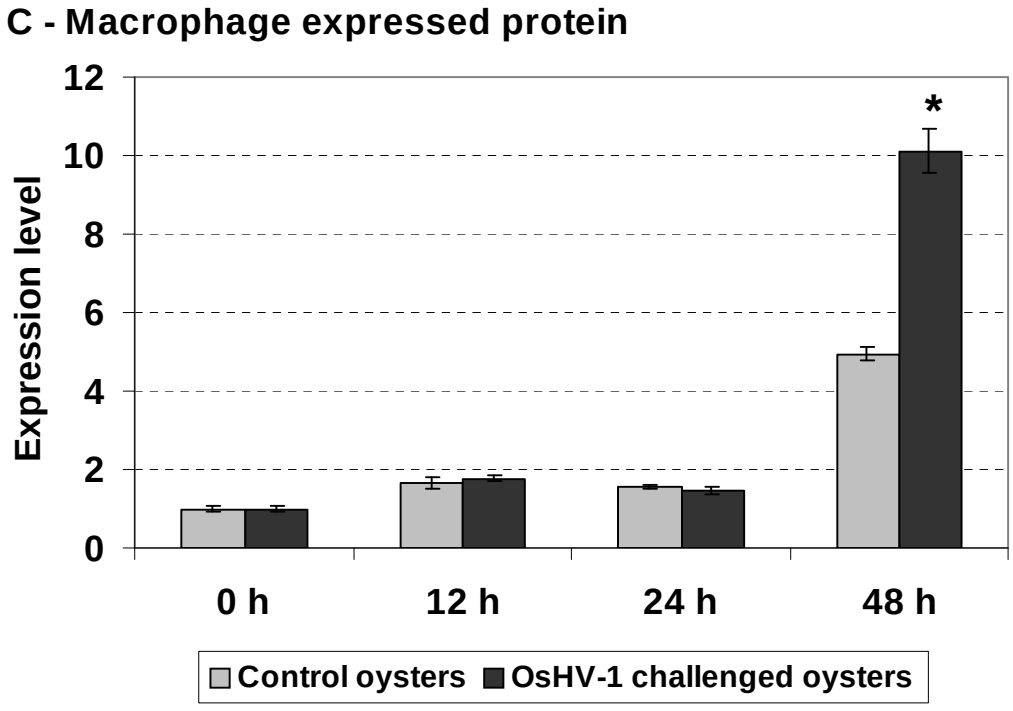
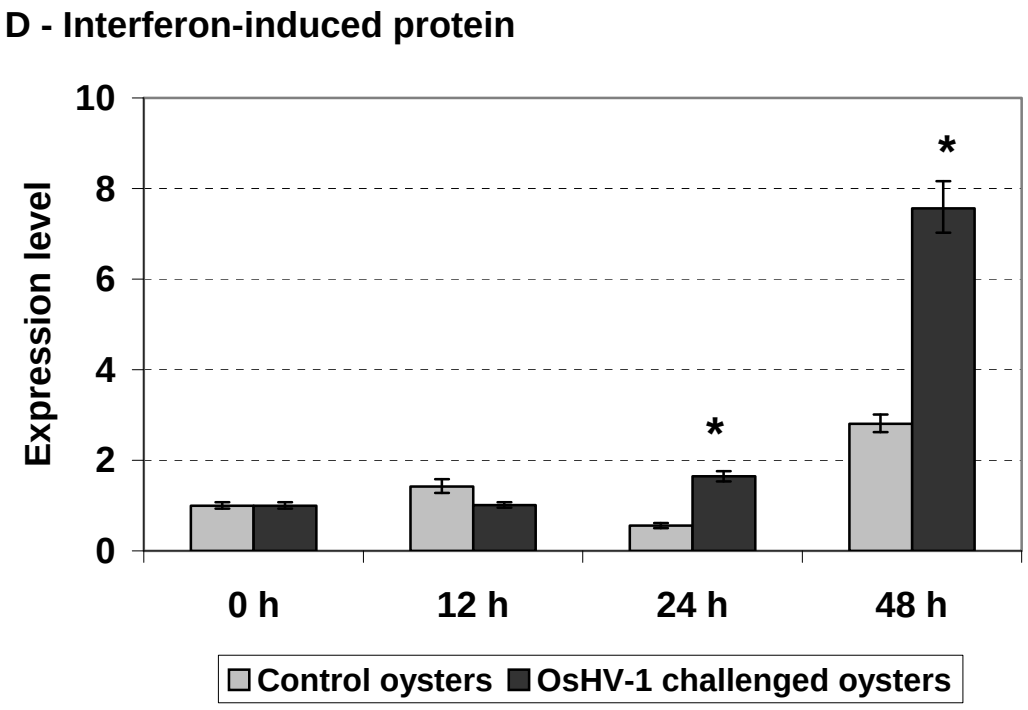


Figure 5D


D - Interferon-induced protein
Expression level
10
8
6
4
2
0
*
*
0 h
12 h
24 h
48 h
Control oysters
OsHV-1 challenged oysters

Tables

Table I - Combination of primers used in RACE PCR and/or real time PCR assays

Gene name	GenBank accession	Oligonucleotide	Sequence Race and Sequence real time PCR
Bcl-2	EU678310	Forward primer	5’-CTCACACAACGGGGGATGGGTAAGTG-3’ (a)
		Reverse primer	5’-GCACAAGCCCCTAACCATGCAAAAC-3’ (a)
Elongation Factor	AB122066	Forward primer	5’-CAAGAACGGAGATGCTGGTATGG-3’ (b)
		Reverse primer	5’-TTTCACTCTTTCCACCGGCTTT-3’ (b)
Glypican	EU678311	Forward primer	5’-CGAGTTCTCCCTGGGCAGGTACC-3’ (a)
		Reverse primer	5’-GGCTCGTTGAGTTCCGAGTGGTAGGC-3’ (a)
		Forward primer	5’-AACTACTGCCTCAACACCATGA-3’ (b)
		Reverse primer	5’-TAGGTGTCACCAACAGAACCAC-3’ (b)
IK cytokine	EF627976	Forward primer	5’-GGAGCGCGAGGAAGAGGAGATAATGG-3’ (c)
		Reverse primer	5’-ATCCGTCCCGGCAGAAACAGCTC-3’ (c)
Immunoglobulin domain cell adhesion molecule subfamily protein	EU678312	Forward primer	5’-CATTCTGCCCGGAGACAATCAGATG-3’ (c)
		Reverse primer	5’-TCCCTCCAGCGATCACTAGATCCAC-3’ (c)
Interferon-induced protein 44	FJ440108	Forward primer	5’-CTCCTGATTGGTCAGATTGGTGCGGG-3’ (c)
		Reverse primer	5’-CGGTAGACAGTGGTGAGGCTGTGCT-3’ (c)
Macrophage expressed protein 1-like protein	EF627979	Forward primer	5’-GCCACCGAAAGCCGGAGAAGATGTC-3’ (c)
		Reverse primer	5’-ACCGAGACCGAGTTTCAGGGGGTAG-3’ (c)
Multicopper oxidase	EU678320	Forward primer	5’-GATGGGTGGGAGGCGAAACGACTTG-3’ (a)
		Reverse primer	5’-CATCCATGTAAGGGGTCCCCGTCTG-3’ (a)
		Forward primer	5’-TGGTTCCTGCATTGTCACAT-3’ (b)
		Reverse primer	5’-AAGAGTATCAGCCGCGAAAA-3’ (b)
Myeloid differentiation factor 88	DQ530619	Forward primer	5’-CGTGCCATGGACGGATAACAACG-3’ (b)
		Reverse primer	5’-GGCCCAGCAGTACCTCTGTGGAATC-3’ (b)
Phosphoinositide 4-kinase beta	EU678316	Forward primer	5’-CGAGTGCTGGTCTCAGGAGGATGATG-3’ (a)
		Reverse	5’-GCTTTTGGGAGCTGTGAAGTGCTCAG-3’ (a)

		primer	
Protein tyrosine phosphatase	EU678317	Forward primer	5’-ATTGCGTCCCAAGGCCCTAAGTCAAAC-3’ (a)
		Reverse primer	5’-GTTGGCTTTGGTTTGACTTCCGGTG-3’ (a)
Serine/threonine-protein kinase 38	EU678314	Forward primer	5’-AAATTGCCCATGTACGAGCCGAGAG-3’ (a)
		Reverse primer	5’-CGTCATCATGTCGCCTCCTGGTAAAAAC-3’ (a
Transmembrane receptor (putative melatonin receptor)	HM034838	Forward primer	5’-GGCGATTTCCGTCATTGTGATCCTC-3’ (a)
		Reverse primer	5’-GCCGTCAGCCACAAGCGTTACATAC-3’ (a)
C9	AY509253	Forward primer	5’-GAGGGAAATTTGCGAGAGAA-3’ (b)
C10		Reverse primer	5’-ATCACCGGCAGACGTAGG-3’ (b)

(a): Race
(b): Real time PCR
(c) : Race and real time PCR

Table II – Up-regulated EST identified in *Crassostrea gigas* haemocytes 12 h after OsHV-1 challenge

Category and gene identity. BlastX	Insert size (bp)	Homolog species	*e*-value	Number of clone	User identification code	GenBank Accession Number
1. Immunity					**2.**	**3.**
Cathepsin B	211	*Strongylocentrotus purpuratus*	9e-27	1	CgHV-575	HS513799
Immunoglobulin domain cell adhesion molecule	573	*	-	1	-	EU678312
Alpha macroglobulin	286	*Chlamys farreri*	7e-19	1	CgHV-722	HS513827
Macrophage expressed protein 1 like protein	2825	*	-	1	-	EF627979
Macrophage stimulating 1 (hepatocyte growth factor-like)	241	*Danio rerio*	4e-12	1	CgHV-748	HS513831
Interferon-induced protein 44	624	*	-	1	-	FJ440108
IK cytokine	1010	*	-	1	-	EF627976
Multicopper oxidase	2255	*	-	2	-	EU678320
Adseverin-like protein	297	**3.1. Crassostrea gigas**	9e-35	2	CgHV-667	HS513819
3.2. Cell Signalling/Cell activation					3.3.	3.4.
Phospholipase C	323	*	-	1	-	EF627978
Calmodulin	382	*Monodelphis domestica*	2e-11	1	CgHV2-10bR	HS513780
Nicastrin	792	*	-	1	-	EU678321
Notch 3	201	*	-	2	-	EF999949
Protein tyrosine phosphatase	1795	*	-	2	-	EU678317
Transmembrane protein 184C	423	*Danio rerio*	1e-30	1	CgHV-565	HS513798
Serine/threonine-protein kinase 38	1304	*	-	1	-	EU678314
Transmembrane receptor (putative melatonin receptor)	2208	*	-	1	-	HM034838
Myeloid differentiation factor 88	405	**3.5. Crassostrea gigas**	2e-39	1	-	**
Ras-related protein Rab-1A	253	*Harpegnathos saltator*	3e-16	1	CgHV-608	HS513805
Ras-like GTP-binding protein Rho1	535	*Harpegnathos saltator*	5e-68	1	CgHV2-13cR	HS513782
RAL (Ras-related GTPase)	334	*Pediculus humanus corporis*	5e-17	1	CgHV-645	HS513813
Misexpression suppressor of ras 3	484	*Drosophila melanogaster*	2e-11	1	CgHV4-22dF	HS513787
Glypican	2991	**3.6. ***		1	-	EU678311

Syntaxin-binding protein 5	346	**3.7. Camponotus floridanus**	7e-33	1	CgHV-533	HS513794
Semaphorin 2A precursor	420	*	-	2	-	EU678318
BMP type 1b receptor	440	**3.8. Crassostrea gigas**	2e-76	1	CgHV-681	HS513821
Transmembrane sweet-taste receptor of 3 GCPR	329	**3.9. Tribolium castaneum**	1e-08	1	CgHV-708	HS513824
Munc18-1-interacting protein 1	337	**3.10. Lymnaea stagnalis**	2e-36	2	CgHV-638	HS513809
MEGF10 protein, partial	407	**3.11. Apis mellifera**	2e-19	1	CgHV-644	HS513812
Rho-GDI related protein	396	**3.12. Crassostrea gigas**	6e-58	1	CgHV-655	HS513816
Phosphoinositide 4-kinase beta	2902	*	-	1	-	EU678316
4. Apoptosis/ Cell cycle					**5.**	**6.**
Translation initiation factor	336	*Saccoglossus kowalevskii*	1e-38	2	CgHV2-13dR	HS513783
SnF7	1068	*	-	1	-	EU678319
Cyclin L1	427	*Monodelphis domestica*	1e-32	1	CgHV-590	HS513803
Bcl-2 protein	982	*	-	1	-	EU678310
Archeron-like protein	691	**6.1. ***	-	1	-	EU678313
Tumor protein p63	302	**6.2. Camponotus floridanus**	2e-12	1	CgHV-537	HS513796
7. Cell structure					**8.**	**9.**
Actin	350	*Crassostrea gigas*	8e-52	4	-	**
Myosin	254	*Mizuhopecten yessoensis*	2e-29	2	CgHV-557	HS513797
Protein unc-45 homolog A	316	**9.1. Pongo abelii**	7e-27	1	CgHV-578	HS513800
Nucleoporin	446	**9.2. Gallus gallus**	3e-12	1	CgHV4-19bF	HS513786
9.2.1. Replication/Repear/Transcription/Translation					9.2.2.	9.2.3.
PHD finger protein 10	1657	*	-	1	-	EU678315

Elongation factor 1 alpha	416	*Crassotrea gigas*	4e-56	2	-	**
Splicing factor 3B subunit 3	414	*Taeniopygia guttata*	5e-67	1	CgHV-140	HS513792
Nucleotidase 4F8	388	*Ixodes scapularis*	2e-22	1	CgHV-711	HS513826
Postreplication repair protein hRAD18p	348	*Strongylocentrotus purpuratus*	7e-19	1	CgHV-728	HS513828
Splicing factor, arginine/serine-rich 8 isoform 1	331	*Mus musculus*	8e-13	1	CgHV-741	HS513830
Transcription factor Rel 2	266	*Crassotrea gigas*	1e-30	1	CgHV-750	HS513832
Block of proliferation protein	338	*Ixodes scapularis*	3e-28	1	CgHV-582	HS513801
Zinc finger protein	267	*Strongylocentrotus purpuratus*	4e-09	1	CgHV-632	HS513807
Nuclear ribonucleoprotein	405	**9.3. Monodelphis domestica**	1e-14	1	CgHV4-659	HS513817
Heterogeneous nuclear ribonucleoprotein AB isoform a	358	*Canis familiaris*	4e-15	1	CgHV4-6aF1	HS513785
10.					**11.**	**12.**
13. Metabolic process					**14.**	**15.**
Cytochrome oxidase subunit I	387	*Crassostrea angulata*	8e-35	1	CgHV4-35cF	HS513790
Cytochrome c oxidase subunit I	507	*Crassostrea gigas*	3e-68	1	CgHV2-11aR	HS513781
Sodium/potassium-transporting ATPase alpha chain	625	*		2	-	EF627777
ATP-synthase delta subunit	408	**15.1. Crassotrea gigas**	5e-42	1	CgHV-648	HS513814
NADPH oxidase	518	*Saccoglossus kowalevskii*	1e-37	1	CgHV4-28cF	HS513788
NADPH oxidase	360	*Crassostrea angulata*	1e-14	1	CgHV-693	HS513822
Alpha/beta hydrolase, putative-like	426	*Saccoglossus kowalevskii*	2e-36	2	CgHV-637	HS513808
Carnitine O-acetyltransferase	296	*Bos taurus*	8e-23	2	CgHV-705	HS513823
Ubiquitin specific peptidase 16	350	*Oryctolagus cuniculus*	2e-07	1	CgHV-732	HS513829
Ubiquitin specific peptidase 16	364	*Strongylocentrotus purpuratus*	6e-12	1	CgHV-536	HS513795
Flavin adenine dinucleotide synthetase	316	*Ciona intestinalis*	2e-28	1	CgHV-613	HS513806
Transketolase-like 2	193	*Camponotus floridanus*	1e-20	1	CgHV-643	HS513811
Peroxisomal membrane protein	558	**15.2. Aedes aegypti**	4e-12	1	CgHV2-1bF	HS513778
Multi EGF-like domains 6	421	**15.3. Gallus gallus**	1e-07	1	CgHV4-3bR	HS513784
Acyl-CoA desaturase	331	*Salmo salar*	3e-08	1	CgHV-139	HS513791
Acetylcholinesterase/butyrylcholinesterase	224	**15.4. Ixodes scapula**	8e-07	1	CgHV-665	HS513818
Mesoderm specific transcript	322	**15.5. Takifugu rubripes**	9e-14	2	CgHV-142	HS513793

Importin beta-2	215	**15.6. Aedes aegypti**	5e-34	2	CgHV-710	HS513825
Peptide methionine sulfoxide reductase	325	**15.7. Oncorhynchus mykiss**	2e-37	1	CgHV-585	HS513802
Voltage-dependent anion-selective channel protein 3	287	**15.8. Crassostrea gigas**	2e-18	1	CgHV-600	HS513804
Translocon-associated protein, gamma subunit	382	**15.9. Ixodes scapularis**	5e-26	1	CgHV-640	HS513810
Wurst-like protein	424	*Saccoglossus kowalevskii*	9e-27	1	CgHV-654	HS513815
Translocase of outer mitochondrial membrane	260	*Apis mellifera*	3e-13	1	CgHV2-3bR	HS513779
Ribosomal protein						
Ribosomal protein L34	366	*Branchiostoma belcheri tsingtauense*	1e-05	1	CgHV4-28dF	HS513789

*: Complete cDNA sequences obtained in the present study were deposited in GenBank (see Table III).

**: These EST sequences were not deposited because they correspond to previously existing complete cDNA sequences in GenBank.

Table III - cDNA complete sequences obtained by 5'/3' RACE PCR from up-regulated EST sequences identified in *Crassostrea gigas* haemocytes after OsHV-1 challenge

Gene ontology annotation : biological process	Gene Name	GenBank accession	Gene length	Protein lenght	Homology	E-value (Blastx)
Immunity						
	Macrophage expressed protein 1-like protein	EF627979	2825	621	*Haliotis diversicolor supertexta*	1e-164
	Interferon-induced protein 44	FJ440108	624	193	*Saccoglossus kowalevskii*	2e-43
	IK cytokine	EF627976	1010	310	*Danio rerio*	5e-85
	Multicopper oxidase	EU678320	2255	668	*Hydra magnipapillata*	1e-160
	Immunoglobulin domain cell adhesion molecule	EU678312	573	81	*Lymnaea stagnalis*	7e-10
Cell signalling/Cell activation						
	Glypican	EU678311	2991	554	*Taeniopygia guttata*	2e-118
	Transmembrane receptor (melatonin receptor)	HM034838	2208	273	**15.10. Branchiostoma floridae**	8e-40
	Protein tyrosine phosphatase	EU678317	1795	487	*Pediculus humanus corporis*	1e-32
	Phosphoinositide 4-kinase beta	EU678316	2902	637	*Apis mellifera*	0.0
	Serine/threonine-protein kinase 38	EU678314	1304	245	*Ixodes scapularis*	9e-99
Apoptosis/Cell cycle						
	Bcl-2	EU678310	982	150	*Saccoglossus kowalevskii*	1e-33