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Differentially expressed genes in *Penaeus monodon* hemocytes following infection with yellow head virus

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A cDNA microarray composed of 2,028 different ESTs from two shrimp species, *Penaeus monodon* and *Masupenaeus japonicus*, was employed to identify yellow head virus (YHV)-responsive genes in hemocytes of *P. monodon*. A total of 105 differentially expressed genes were identified and grouped into five different clusters according to their expression patterns. One of these clusters, which comprised five genes including cathepsin L-like cysteine peptidase, hypothetical proteins and unknown genes, was of particular interest because the transcripts increased rapidly (≤ 0.25 hours) and reached high expression levels in response to YHV injection. Microarray data were validated by real-time RT-PCR analyses of selected differentially expressed transcripts. In addition, comparative analysis of the hemocyte transcription levels of three of these genes between surviving and non-surviving shrimp revealed significantly higher expression levels in surviving shrimp. [BMB reports 2008; 41(9): 670-677]

INTRODUCTION

Throughout the world and especially in Asia, shrimp cultivation is often affected by outbreaks of deadly infectious diseases caused mainly by viruses (1). In the maintenance of substantial production of farmed shrimp, an understanding of the shrimp immune system would allow for the development of management strategies to control virulent or problematic pathogens encountered on shrimp farms. Shrimp immunity can be studied on one level through molecular characterization of immune effectors and analysis of gene expression in response to a given pathogen. Viral responsive genes, including those encoding structural proteins, defense and homeostasis proteins, energy metabolism enzymes, transcription or translation proteins and proteins of unknown function, have been identified in white spot syndrome virus (WSSV)- challenged shrimp and WSSV-resistant shrimp (2-4). In contrast, the responses of shrimp to YHV infection at the molecular level are currently poorly described. WSSV is an enveloped, ellipsoid and large double- stranded DNA virus (5), while YHV is an enveloped, rod-shaped and single-stranded RNA virus (6). As YHV contains a single- stranded RNA genome, the molecular basis for shrimp response to YHV might be somewhat different than that to the DNA virus WSSV.

Microarray technology has been used to identify a number of important immune modulators that are critical for the host response in marine species. In this study, a shrimp cDNA microarray chip comprising 2,028 cDNA from EST collections of two penaeid species, *Penaeus monodon* and *Masupenaeus japonicus*, was used to study the expression profile of *P. monodon* genes that are differentially expressed in response to YHV infection. In addition, the transcript abundance of selected genes in surviving and non-surviving *P. monodon* after acute YHV infection was analyzed. This study should provide useful information on the molecular mechanism of shrimp anti-YHV response, as well as more general antiviral responses.

RESULTS AND DISCUSSION

Expression profiling of genes responsive to yellow head virus (YHV) infection

Gene expression profiles were determined using a shrimp cDNA microarray that contained 2,028 unique EST clones from *P. monodon* (1,269) and *M. japonicus* (759). For analysis, the ratio of hybridization of fluorescent cDNA probes prepared from hemocyte RNA of YHV-infected *P. monodon* was compared with that of the control (lobster hemolymph medium-injected *P. monodon*). Cross-hybridization between *P. monodon* cDNA probes and *M. japonicus* targets was observed at about 85%. Of the 2,028 analyzed genes, a total of 105 YHV-responsive genes were identified as those whose expression changed more than 2-fold in at least 1 time point after injection. Approximately 47% (50 of 105) of the genes with altered expression were unknown genes. The changes in the transcript levels of these 105 genes were catalogued at four time points from 0.25 to 48 h post-injection, defined as very early (0.25 h),

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Table 1. List of genes in each cluster and fold changes at different time points

Gene ID	Highest homology[a]	Accession no.[b]	*E* value	Closest species	Signal intensity ratios (infected shrimp/control shrimp)			
					0.25 h	6 h	24 h	48 h
Cluster I								
N145	Unknown	DW042574	–	–	1.16	1.29	0.72	3.14
HPAN0214	Ferritin	EB409360	0.00	*Litopenaeus vannamei*	1.65	0.98	0.86	3.38
MJ_H_3_230	Unknown	AB374095	–	–	1.30	1.15	1.25	4.22
IF160	Arginase	DW042869	1.00E-42	*Xenopus tropicalis*	1.18	0.83	1.33	3.30
SH2008	CG16742-PA isoform A	DW042750	9.00E-07	*Tribolium castaneum*	1.23	1.02	1.49	3.73
N60	Hypothetical protein YP_758557	DW042528	3.00E-05	*Plutella xylostella*	1.29	0.87	1.60	4.15
PJV703R	Ribosomal protein S19	AU176244	7.00E-15	*Bombyx mori*	0.92	0.96	1.15	2.37
HPAN0099	Unknown	FC996011	–	–	1.00	1.20	1.17	2.13
PJA136R	Ribosomal protein L35	AU175612	3.00E-37	*Xenopsylla cheopis*	1.02	0.98	1.20	2.02
SH719	Ribosomal protein L19	DW042564	1.00E-49	*Drosophila melanogaster*	0.87	1.03	0.93	2.62
SH811	Ribosomal protein L30	CO408866	7.00E-44	*Argopecten irradians*	1.06	1.02	0.97	2.45
IF117	Ribosomal protein LP1	CO576787	3.00E-19	*Argas monolakensis*	0.86	0.85	1.20	2.93
IF468	Unknown	DW042957	–	–	0.92	0.86	1.44	2.23
SH971	Ribosomal protein LP1	ES609103	3.00E-21	*Argas monolakensis*	0.97	0.86	1.32	2.00
PJH489R	Unknown	AU175410	–	–	1.04	0.74	1.15	2.33
LPV0611	Unknown	EE662402	–	–	1.17	0.78	1.37	2.36
PJV603F	Theileria-specific sub-telomeric protein	AU176210	1.00E-07	*Theileria annulata* strain Ankara	0.99	0.80	1.21	2.55
PJH518F	Unknown	AU175426	–	–	1.05	0.84	1.35	2.53
MJ_H_1_242	Eukaryotic translation initiation factor	AB374092	6.00E-18	*Nasonia vitripennis*	1.27	1.47	1.49	2.17
MJ_H_3_255	Unknown	AB374097	–	–	1.45	1.14	1.10	2.18
LPN0282	Unknown	FC996012	–	–	1.13	1.26	1.13	2.45
SH932	CG12012-PA	DW042658	1.00E-20	*Drosophila melanogaster*	1.07	1.25	1.11	2.27
LPV0400	Zinc finger protein 416	CO777321	4.00E-32	*Macaca mulatta*	1.08	1.22	1.20	2.24
MJ_H_3_172	Unknown	AB374094	–	–	1.02	1.08	1.02	2.04
MJ_H_3_239	Unknown	AB374096	–	–	1.17	1.11	1.09	2.12
HPAN0305	Ribosomal protein S21	CO777168	5.00E-34	*Eurythoe complanata*	1.15	1.13	0.94	2.01
SH2043	Nascent polypeptide-associated complex alpha chain	DW042732	4.00E-17	*Homo sapiens*	1.14	1.03	0.94	2.10
PJH798R	Carboxylesterase-6	AU175528	2.00E-51	*Bombyx mori*	1.18	1.09	1.26	2.54
MJ_H_3_135	Unknown	AB374093	–	–	1.28	1.11	0.98	2.60
Cluster II								
N70	Unknown	DW042534	–	–	1.89	1.54	2.09	3.10
LPN0336	Unknown	EE661916	–	–	2.59	1.61	1.59	1.87
LPN0106	Unknown	ES609114	–	–	2.04	1.78	2.03	0.96
LPV0202	Unknown	EE662577	–	–	1.86	2.92	1.88	1.35
LPV0547	Unknown	EE662352	–	–	0.86	3.28	1.16	1.17
Cluster III								
N124	Pherophorin-dz1 protein	DW042567	3.00E-63	*Volvox carteri*	0.64	1.07	2.55	0.05
SH970	Crustin-like peptide type 3	BI018072	1.00E-57	*Marsupenaeus japonicus*	0.73	0.84	1.33	0.05
SH834	Penaeidin	DW042613	9.00E-35	*Penaeus monodon*	0.72	0.81	1.91	0.03
MJ_H_2_078	Unknown	AB308271	–	–	0.86	0.80	2.03	0.59
N59	Hypothetical protein	DW042527	2.00E-12	*Ornithorhynchus anatinus*	0.69	0.46	3.30	0.24
IF309	Sperm mitochondria-associated cysteine-rich protein	DW042910	5.00E-08	*Mus musculus*	0.77	0.58	2.50	0.12
Cluster IV								
LPV0060	Unknown	EE662532	–	–	2.08	0.82	0.79	1.17
SH920	Selenoprotein W1	DW042654	1.00E-22	*Danio rerio*	1.48	0.63	0.33	0.78
SH962	Unknown	EE332501	–	–	0.81	1.38	0.87	0.12
SH922	Hemocyte kazal-type proteinase inhibitor	DW042655	5.00E-15	*Penaeus monodon*	0.79	1.51	1.03	0.19
LPV0020	Unknown	ES609117	–	–	0.75	1.34	0.73	0.19
N167	Transglutaminase	ES609106	9.00E-42	*Penaeus monodon*	0.92	0.62	0.91	0.20
SH71	Antilipopolysaccharide factor	ES609107	2.00E-53	*Penaeus monodon*	1.43	0.57	0.66	0.26
LPN0064	Unknown	EE662062	–	–	0.79	1.02	0.61	0.19
IF485	Hemocyte kazal-type proteinase inhibitor	ES609109	0.00	*Penaeus monodon*	0.99	0.90	0.78	0.12
LPV0559	Unknown	EE662364	–	–	0.86	1.06	0.72	0.11
LPV0054	Antilipopolysaccharide factor	ES609116	2.00E-10	*Penaeus monodon*	1.13	0.65	0.49	0.13

Table 1. Continued

Gene ID	Highest homology[a]	Accession no.[b]	*E* value	Closest species	Signal intensity ratios (infected shrimp/control shrimp)			
					0.25 h	6 h	24 h	48 h
IF174	Unknown	DW042874	—	—	1.14	0.80	0.58	0.12
IF459	Unknown	ES609110	—	—	0.83	0.79	0.56	0.13
IF438	Insulin-like growth factor binding protein 7 precursor	EE332504	2.00E-09	*Bos taurus*	0.74	0.83	0.45	0.13
SH889	WAP four-disulfide core domain 5	DW042639	4.00E-07	*Bos taurus*	1.07	1.16	0.44	0.26
SH881	C-type lectin	DW042635	1.00E-104	*Penaeus monodon*	1.13	0.95	0.66	0.36
PJA815R	Unknown	AU175958	—	—	1.03	0.89	0.56	0.40
LPN0248	Unknown	ES609113	—	—	1.14	1.51	0.34	0.44
SH2065	Unknown	DW042743	—	—	1.24	1.28	0.43	0.52
PJI216R	Unknown	AU176329	—	—	1.22	1.51	0.69	0.34
N194	Histone H1-beta	ES609105	1.00E-17	*Strongylocentrotus purpuratus*	1.18	1.31	0.99	0.49
SH750	Beta-tubulin	DW042578	1.00E-139	*Mythimna separata*	1.20	1.38	0.92	0.49
SH2052	Moesin-ezrin-radixin	ES609102	2.00E-45	*Culex pipiens quinquefasciatus*	0.92	1.38	0.92	0.38
IF348	Unknown	DW042923	—	—	1.08	1.29	0.82	0.33
SH602	Beta-tubulin isotype 2	DW042503	1.00E-122	*Cyathostomum catinatum*	1.11	1.37	0.81	0.34
LPV0510	Unknown	EE662326	—	—	1.23	1.22	0.98	0.28
PJI304F	Unknown	AU175958	—	—	1.25	1.22	0.90	0.33
N225	Unknown	DW042623	—	—	0.99	1.24	1.14	0.25
N237	Secreted protein SST3	DW042615	2.00E-13	*Mus musculus*	0.95	1.30	0.82	0.26
N292	Unknown	ES609104	—	—	0.84	1.23	0.71	0.26
PJA356R	Unknown	AU175301	—	—	1.59	0.97	0.86	0.49
PJH463F	Unknown	AU175382	—	—	1.20	0.85	1.17	0.47
SH2007	Actin E	DW042749	1.00E-105	*Litopenaeus vannamei*	1.32	0.83	1.08	0.49
SH2063	Ribosomal protein S3a	CO408886	3.00E-96	*Tribolium castaneum*	1.10	0.98	1.05	0.47
LPN0056	Cofilin	ES609115	4.00E-22	*Bombyx mori*	1.05	1.02	0.00	0.45
N242	Unknown	DW042618	—	—	0.91	0.94	1.05	0.46
MJ_H_1_191	Unknown	AB308274	—	—	0.91	0.96	1.04	0.50
LPN0185	Unknown	EE661813	—	—	0.97	0.97	1.02	0.48
SH656	Skeletal alpha-actin	DW042525	2.00E-44	*Sparus aurata*	1.10	0.82	0.95	0.36
IF279	Unknown	DW042904	—	—	0.99	0.90	0.99	0.38
PJH466R2	CG10440-PA	AU175384	2.00E-56	*Tribolium castaneum*	1.14	0.88	0.92	0.48
KS-2-6_H07	DDBDRAFT_0216556	AU175363	5.00E-18	*Dictyostelium discoideum* AX4	1.12	0.85	0.87	0.41
IF76	Crustin-like protein fc-2	DW042843	3.00E-12	*Fenneropenaeus chinensis*	0.57	1.55	0.88	0.43
IF127	Unknown	DW042857	—	—	0.88	1.29	0.64	0.46
N239	Small ubiquitin-like modifier SUMO	DW042617	1.00E-37	*Apis mellifera*	0.93	1.27	0.85	0.48
LPV0131	Unknown	EE662516	—	—	1.16	1.03	0.78	0.48
LPV0508	Unknown	EE662324	—	—	0.91	1.07	0.75	0.45
MJ_H_1_039	CG7698-PA	AB308273	4.00E-16	*Tribolium castaneum*	1.03	1.10	0.72	0.47
SH615	Gamma-interferon inducible lysosomal thiol reductase-like protein	DW042506	4.00E-15	*Aedes aegypti*	0.85	0.99	0.96	0.30
N439	Unknown	DW042710	—	—	0.75	0.99	0.82	0.40
IF39	Allergen Pen m 2 (arginine kinase)	EE332503	2.00E-38	*Penaeus monodon*	0.93	0.84	0.82	0.35
SH685	Prophenoloxidase	BI018090	7.00E-52	*Penaeus monodon*	0.83	0.93	0.72	0.35
KS-2-5_C09	MAP kinase	AB308272	3.00E-85	*Bombyx mori*	0.86	1.01	0.96	0.48
IF284	14-3-3-like protein	ES609111	0.00	*Penaeus monodon*	0.83	1.01	0.92	0.49
IF436	Unknown	DW042947	—	—	0.83	1.01	1.21	0.44
N202	Unknown	DW042601	—	—	0.76	0.93	1.15	0.48
SH1070	Actin related protein	DW042717	6.00E-22	*Mus Musculus*	0.87	0.85	0.81	0.45
IF501	Sodium/potassium-transporting ATPase subunit beta	ES609108	1.00E-90	*Penaeus monodon*	0.86	0.89	0.82	0.48
LPN0148	Unknown	EE661801	—	—	0.88	0.90	0.94	0.41
LPN0213	Unknown	EE661833	—	—	0.96	0.90	0.91	0.47
Cluster V								
IF415	ENSANGP00000023397	DW042937	3.00E-09	*Anopheles gambiae str. PEST*	7.69	13.24	13.57	6.44
LPN0428	Y43E12A.2	EE661984	6.00E-21	*Tribolium castaneum*	5.70	4.25	5.80	1.52
LPN0255	Cathepsin L-like cysteine peptidase	ES609112	2.00E-18	*Penaeus monodon*	2.52	3.64	2.63	1.60
LPN0252	Unknown	EE661861	—	—	2.55	3.83	2.85	2.05
LPV0254	Unknown	EE662563	—	—	3.32	3.64	4.09	1.39

[a]Sequence of clones obtained by universal primer was subjected to homology search against NCBI database. Homology is based on expected values of BlastX and BlastN matches less than E-4. [b]GenBank accession number. All cDNA sequences were submitted to the GenBank database.

early (6 h), mid (24 h) and late (48 h) stages of infection. Expression of most genes was altered at the mid and late infection stages, and only a minor proportion of the transcripts showed up-regulation at the very early or early infection stages (Table 1). Using a hierarchical average linkage clustering program to group the 105 responsive genes revealed at least five different patterns of transcript regulation (Fig. 1). These five clusters and the fold changes of each differentially expressed gene are listed in Table 1.

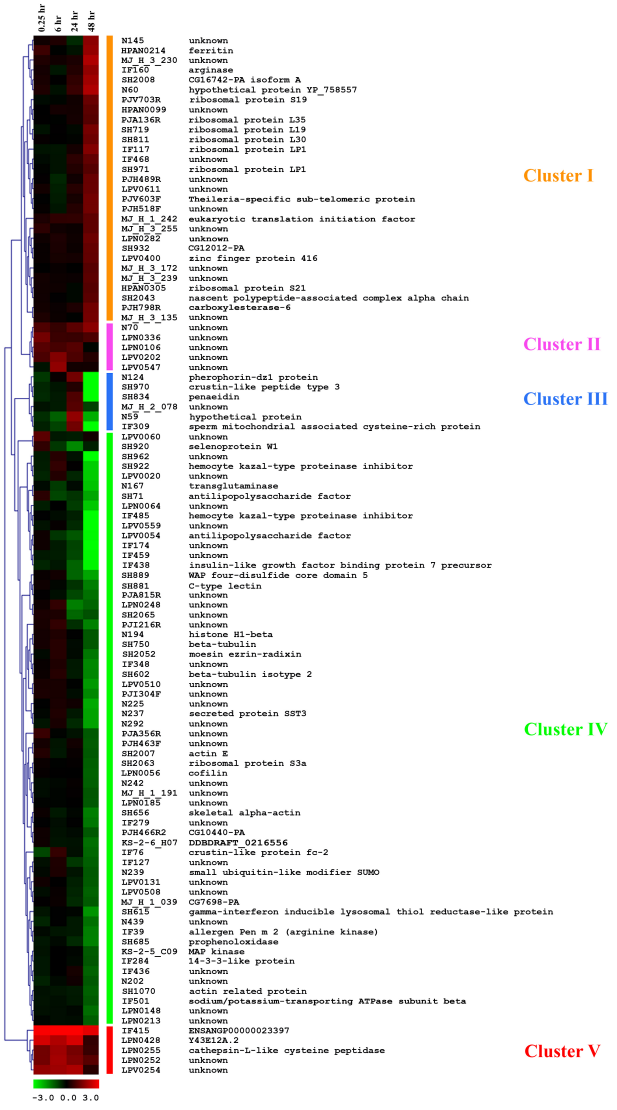

Fig. 1. Hierarchical clustering of 105 YHV-responsive genes in *P. monodon* hemocytes. Cluster analysis was performed with the Manhattan distance metric and the average linkage. Each column represents a time point of YHV infection, and each row represents the expression profile of each gene over 4 time points. The color saturation reflects the magnitude of the log-2 expression ratio of infected and control shrimp (Cy5/Cy3) for each transcript.

As shown in Fig. 1, genes in cluster I (29 genes) were up-regulated after 48 h with YHV. Genes encoding defense-related proteins (ferritin, carboxylesterase-6), amino acid metabolism (arginase), transcription or translation proteins (zinc finger protein 416, nascent polypeptide-associated complex alpha chain, eukaryotic translation initiation factor), ribosomal proteins, as well as hypothetical proteins and unknown genes, were found in this cluster. Ferritin and carboxylesterase are known to play an important role in detoxification. Ferritin was also up-regulated in WSSV-resistant shrimp and in WSSV-challenged shrimp (2, 7). Carboxylesterase was also up-regulated at the protein level in taura syndrome virus-challenged shrimp (8). In the present study, induction of arginase and ribosomal protein mRNAs was also observed in this cluster during viral infection. The role of arginase in the specific antiviral responses of crustaceans is not known. Interestingly, mRNAs of ribosomal proteins were up-regulated only in this cluster, but were previously found to be increased in hemocyte cDNA libraries of WSSV-infected shrimp (9, 10). The up-regulation of genes in this cluster suggests that they may be involved in shrimp antiviral responses at the late stage of infection. However, since *P. monodon* shrimp at 48 h post-injection are moribund, up-regulation only during this stage may be independent of immunity and reflect either viral changes to host gene expression or general host cell stress responses. For example, the specific late infection stage up-regulation of ribosomal protein genes seen in this study is more likely reflective of the viral use of the host cell machinery to replicate and produce new virions.

In contrast to the late-stage regulation of genes in cluster I, those in clusters II and V (five genes each) were up-regulated from the very early or early infection stages. Several genes in cluster II, however, were up-regulated by less than 2-fold at some time points, and all were annotated with, and are homologous with genes annotated with, unknown function. In contrast, all five genes in cluster V were all up-regulated to high levels at the very early infection stage and maintained elevated expression until the late infection stage. These five genes are consequently of some interest and were comprised of cathepsin L-like cysteine peptidase, two hypothetical proteins (ENSANGP00000023397 and Y43E12A.2 homologs) and two unknown genes. Indeed, one of these genes, IF415, was the most significantly up-regulated gene found in this study (up to 13-fold) by microarray hybridization and was strongly up-regulated throughout the infection process from 0.25 to 48 h. Its predicted amino acid sequence showed closest identity to ENSANGP00000023397 from *Anopheles gambiae* (36% identity), but remains of unknown function. The second most significantly up-regulated gene, LPN0428, had a 5-fold increase in expression, and was also found in category V; this gene encoded a homolog (54% identity) of *Tribolium castaneum* Y43E12A.2, which is also annotated without function. However, despite the lack of known functionality, the expression profiles imply that these genes are intimately involved

in the regulation of shrimp defense responses against YHV. Cathepsin L-like cysteine peptidase was the only gene of known (annotated) function that was up-regulated at the very early infection stage. Cathepsins are thought to be involved in apoptosis. A number of genes encoding apoptotic-related proteins were expressed in high proportions in a WSSV-infected library of *M. japonicus* (9) and in viral-resistant shrimp (2), suggesting up-regulation upon infection. The up-regulated transcript expression levels of cathepsin L following YHV infection described here may be the result of host defense mechanisms to eliminate virus-infected cells by apoptosis, although this requires experimental verification. Because of their rapid induction and high level of expression in response to YHV infection, the other two unknown genes in cluster V are of particular interest despite their lack of annotated function. The innate immune system is regarded as the first line of defense in protecting shrimp in the first few hours to days after infection, with the initial distinct phase of the shrimp immune response occurring within the first 12 h after challenge (11). The rapid response of the genes in cluster V is, therefore, consistent with (but not conclusive of) an important role in the anti-YHV, and possibly general antiviral, defenses of *P. monodon* shrimp.

The common feature of the six genes in cluster III was their up-regulation at mid-stage and down-regulation at late infection stage. The known antimicrobial molecules crustin-like peptide type 3 and penaeidin were found in this cluster. Cluster III also contained genes encoding pherophorin-dz1 protein, sperm mitochondrial-associated cysteine-rich protein, a hypothetical protein and an unknown gene. The levels of up-regulation of genes in this cluster were low with a maximum of up to 3-fold.

Cluster IV was the largest cluster and comprised 60 genes. The common feature of the genes in this cluster (except for LPV0060) was a significant down-regulation in expression with a peak at mid or late infection stages. Several genes in this cluster grouped into defense and homeostasis categories, including antimicrobial molecules (antilipopolysaccharide factor, C-type lectin, crustin-like protein fc-2, gamma-interferon inducible lysosomal thiol reductase-like protein, WAP four-disulfide core domain 5), antioxidant enzyme (selenoprotein W1), proteinase inhibitor (kazal-type proteinase inhibitor) and ProPO system (prophenoloxidase, transglutaminase). Recently, antilipopolysaccharide factor (ALF) was found to provide protection against WSSV infection in the crayfish, *Pacifastacus leniusculus*, since knockdown of ALF by RNAi specifically resulted in higher rates of viral propagation (12). In this study, the expression ratios of the two genes encoding ALF were 1.4 and 1.1 at 0.25 h post-infection and decreased thereafter with values less than 0.5 at 48 h post infection. However, that the expression of ALFs was not appreciably induced does not *per se* negate a potential role for ALF proteins in YHV responses. C-type lectin is an invertebrate non-self innate immune system, and its gene expression was induced in response to WSSV infection (13). However, the other two C-type lectin genes did not change significantly after WSSV infection when comparing EST expression levels from healthy and WSSV-infected shrimp (14). Both ALF and C-type lectin seem to have antiviral activity, but YHV infection did not alter their gene expression profiles in *P. monodon* hemocytes in our study. It is of note that previous studies have focused on the response of shrimp genes to WSSV, not to the unrelated YHV, which may evoke somewhat different immunological responses and/or molecular manipulations of host genes by the virus, especially since YHV is a ssRNA virus rather than a DNA virus. Crustacean transglutaminase is an enzyme that cross-links hemolymph clots to prevent excess blood loss from a wound and invasion of micro-organisms into the wound (15); its down-regulation at a late stage of YHV infection in our study was in accordance with that reported in WSSV-infected Chinese shrimp (16). Using proteomic approaches, Bourchookarn *et al.* (6) found down-regulation of hemolymph clottable protein and up-regulation of transglutaminase upon YHV infection.

In addition to genes related to defense and homeostasis, cluster IV also contained genes that encode structural and cytoskeleton proteins (moesin/ezrin/radixin, β-tubulin, actins, actin-related protein, histone H1-β, cofilin), energy metabolism enzymes (arginine kinase, Na^+/K^+-ATPase β subunit), signaling/communication proteins (14-3-3-like protein, MAP kinase), insulin-like growth factor binding protein 7 precursor, ubiquitin-like modifier SUMO and secreted protein SST3. Down-regulation of several cell structure proteins and energy metabolism enzymes such as arginine kinase, actin, ATP synthase β-subunit and ATPases have been observed in WSSV-infected shrimp (3, 17). In addition, reduction of the carbohydrate metabolic enzyme, 6-phosphogluconate dehydrogenase, was identified from comparative proteomic analysis of normal and YHV-infected shrimp. However, the proteomic data revealed an increase of ATP synthase β-subunit (6). β-Tubulin homologues were down-regulated in YHV infection, but were up-regulated in WSSV infection. Tubulin has long been known to be involved in the assembly and transport of virus particles (3, 4). However, the response of β-tubulin to YHV infection and its functions in viral pathogenesis of WSSV and YHV are currently unknown. Regardless, the down-regulation of many genes at the mid and especially at the late stages of infection, including most genes related to defense and homeostasis, may well reflect intensive viral propagation at the late stage of infection that restrains the expression of the vast majority of host genes.

Since WSSV contains a DNA genome whereas YHV is a ssRNA virus, their mechanisms of virulence and pathogenesis might differ somewhat. As discussed above, WSSV infection modulates the expression of various kinds of genes. Genes encoding apoptotic-related proteins were the most commonly induced genes in several previous studies of WSSV. The other commonly induced genes were calcium-dependent genes and transcription- and translation-related genes. However, in this study, YHV infection of *P. monodon* did not affect the expression level of several calcium-dependent genes including calnexin, calponin, calreticulin and calcium-sensitive chloride channel, in contrast to the reported up-regulation of calreticulin in WSSV-infected shrimp at

both the mRNA and protein levels (3, 18). In addition, no calcium-dependent protein was identified by comparative proteomic analysis between normal and YHV-infected shrimp (6). The up-regulation of several calcium-related genes in WSSV infection may be a compensating reaction of the host genes. Basically, Ca^{2+} plays a key role in many pathological processes, including viral infections (19). In summary of this and previously reported studies, different gene expression profiles are observed in response to WSSV and YHV, suggesting different host immune responses to the two pathogenic viruses.

To validate the microarray results, real-time RT-PCR was performed on seven selected genes that represented different regulation patterns on microarray analysis. The expression of these transcripts, penaeidin, cathepsin L-like, WAP four-disulfide core domain 5, ribosomal protein LP1 and hypothetical proteins (IF415, LPN0428, N59), were analyzed in control and YHV-injected shrimp at 0.25, 6, 24 and 48 h post-injection. In general, the results were all in accordance with the microarray data, although the rank order and magnitude differed between the two methods (Table S2).

Determination of transcript abundance in surviving and non-surviving *P. monodon* after acute YHV infection

The very early increased, sustained and high expression levels of cluster V genes made them attractive candidates for analysis of transcript abundance in surviving and non-surviving shrimp after acute YHV infection. From these five genes we selected the best two hypothetical proteins and one known gene: IF415, LPN0428 and cathepsin L-like, according to their expression analyses by microarray (Fig. 1 and Table 1) and real-time RT-PCR (Table S2). Using real-time PCR, we determined the relative expression of the three transcripts in non-infected hemocytes of *P. monodon* shrimp that either survived or did not survive subsequent acute YHV infection. Higher expression levels were seen for all three transcripts in the surviving shrimp than in non-surviving shrimp (Fig. 2). Of the three transcripts studied, LPN0428 showed the highest difference in transcript abundance between surviving and non-surviving shrimp, followed by cathepsin L-like and IF415. This correlation suggests that these transcripts may be essential components for the antiviral mechanism and are certainly worthy of further investigation to validate this hypothesis. For example, genes whose expression is associated with the ability of shrimp to survive pathogen infection could be used as markers to monitor the capacity of shrimp to resist pathogens.

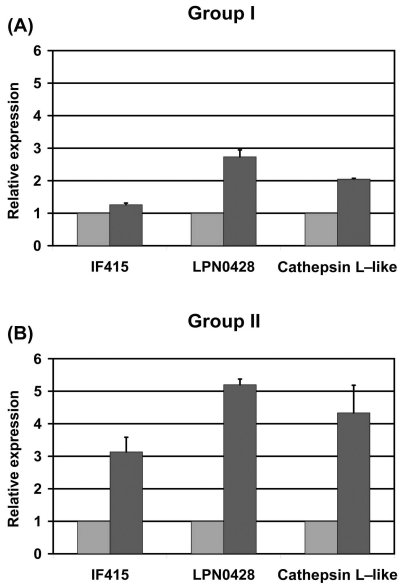

Fig. 2. Relative expression levels of IF415, LPN0428 and cathepsin L-like transcripts in hemocytes of *P. monodon* shrimp that either survive subsequent acute YHV infection (solid bars) or not (non-surviving; grey bars). Two different weight groups of *P. monodon* were used in the study: group I, 25-g shrimp (A) and group II, 16-g shrimp (B). Relative expression levels were calculated according to the $2^{-\Delta\Delta C}{}_{T}$ method (20), using β-actin as an internal reference gene and non-surviving shrimp as calibrator (relative expression = 1). Data represent the average of three independent experiments ± 1SD.

MATERIALS AND METHODS

Animals and experimental infection

Juvenile *P. monodon* shrimp were checked for the absence of white spot syndrome virus (WSSV), yellow head virus (YHV) and the bacteria *Vibrio harveyi* before being used in subsequent experiments. For the microarray analysis and validation of microarray results, juvenile shrimp (approximately 20-g each) were assigned to two groups: control and experimental. Each experimental shrimp was injected with 100 µl of a 600-fold diluted hemolymph previously isolated from a moribund YHV-infected *P. monodon* shrimp; the control group received 100 µl of lobster hemolymph medium, which was used as diluent for YHV. Hemocyte samples were collected at 0.25, 6, 24 and 48 h post-injection.

For analysis of transcript abundance in surviving and non-surviving *P. monodon* shrimp after acute YHV infection, two groups of animals (50 shrimp each) with an average body weight of 25 and 16-g were used for group I and group II, respectively. After acclimatizing the shrimp for three days, 300 µl of hemolymph was collected from each individual. The shrimp were maintained in aquaria for three more days prior to experimental infection with YHV by injection with 50 µl of *P. monodon* hemolymph containing YHV particles. The shrimp from each group were categorized as surviving and non-surviving according to the survival period following this experimental infection. Shrimp that survived the first day but died at day 2 post-infection were designated as non-surviving shrimp. Those that survived for at least six (group I) or four (group II) days after injection were designated as surviving shrimp. These classifications were based upon the observations that high mortalities among the group I YHV-infected shrimp appeared from days 2

to 5, while those for group II appeared from days 2 to 3. Hemolymph samples from surviving and non-surviving shrimp taken before YHV infection were used for real-time RT-PCR analysis of gene expression levels.

Microarray analysis

A mixed species shrimp cDNA microarray that contained 2,028 unique EST clones was constructed from 1,269 (62%) unique EST clones derived from various cDNA libraries of *P. monodon* prepared from different tissues (10). The remaining 759 EST clones were from hemocyte libraries of *M. japonicus* (9). Each EST was spotted in duplicate.

cDNA transcribed from 20 μg of total RNA from 10 individual *P. monodon* was labeled with Cy3 or Cy5 using a Labelstar Array kit (QIAGEN, Germany). The arrays were hybridized with the labeled cDNA sample pairs (one with Cy3 and the other with Cy5) overnight at 42°C. After hybridization, the arrays were washed at 30°C in 5X SSC/0.1% (w/v) SDS for 10 min, then twice in 0.5X SSC for 2 min each, and finally briefly in 0.5X SSC/0.01% (v/v) Tween 20. The arrays were scanned using a GenePix 4000B, and the raw images were analyzed with GenePix Pro ver. 6.0 (Amersham Biosciences Corp., USA). Three normalization steps were performed in MIDAS (http://www.tigr.org/software): (a) a low-intensity filter in which signal intensity $<$ 10,000 was removed; (b) a Lowess (Locfit) normalization; and (c) a flip dye consistency check in which the Cy3 and Cy5 labels were swapped between control versus experimental groups. After normalization, the values of spot replicates were averaged. A gene was considered differentially expressed by YHV infection if its expression ratio increased or decreased by 2-fold or more compared to the control samples. The responsive genes were hierarchically clustered using the MultiExperiment Viewer (MeV) software (http://www.tm4.org/mev.html).

SYBR Green I real-time RT-PCR

SYBR Green I real-time RT-PCR was utilized to confirm the microarray results and to determine quantifiable differences in gene transcript abundance between *P. monodon* shrimp that survived and those that did not survive YHV infection. The hemocyte total RNA from each group of five shrimp was pooled and 1 μg of total RNA was reverse transcribed into cDNA using the ImPromp II Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. The real-time PCR was performed using the cDNA in an iCycler iQ™ Real-Time Detection system using iQ SYBR Green Supermix (Bio-Rad, USA) with the following settings: 95°C for 540 s, 40 cycles of 95°C for 20 s, annealing temperature for 25 s, 72°C for 30 s. The primers and annealing temperatures of the eight selected genes are shown in the Supplementary material (Table S1). Each PCR reaction was done in triplicate.

The relative quantification values for each gene were calculated by the $2^{-\Delta\Delta C}{}_T$ method using β-actin as an internal reference gene (20). The data are presented as the fold change in gene expression normalized to β-actin and relative to another sample to permit comparison between the two groups. Fold changes in target genes for validation of the microarray results were calculated relative to the control shrimp, whereas non-surviving shrimp were used as a calibrator to determine the differences in gene transcript abundance between surviving and non-surviving shrimp.

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