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Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge

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Robalino J, Almeida JS, McKillen D, Colglazier J, Trent HF III, Chen YA, Peck ME, Browdy CL, Chapman RW, Warr GW, Gross PS. Insights into the immune transcriptome of the shrimp *Litopenaeus vannamei*: tissue-specific expression profiles and transcriptomic responses to immune challenge. *Physiol Genomics* 29: 44–56, 2007. First published December 5, 2006; doi:10.1152/physiolgenomics.00165.2006.—Infectious disease constitutes a major obstacle to the sustainability of shrimp aquaculture worldwide and a significant threat to natural populations of shrimp and other crustacea. The study of the shrimp immune system, including the response to viral infection, has been hampered by a relative lack of molecular genetic information and of tools suitable for high-throughput assessment of gene expression. In this report, the generation of a cDNA microarray encompassing 2,469 putative unigenes expressed in gills, circulating hemocytes, and hepatopancreas of *Litopenaeus vannamei* is described. The unigenes printed on the microarray were derived from the analyses of 7,021 expressed sequence tags obtained from standard cDNA libraries as well as from libraries generated by suppression subtractive hybridization, after challenging shrimp with a variety of immune stimuli. The general utility of the cDNA microarray was demonstrated by interrogating the array with labeled RNA from four different shrimp tissues (gills, hemocytes, hepatopancreas, and muscle) and by analyzing the transcriptomic response of shrimp to a lethal challenge with white spot syndrome virus. Our results indicate that white spot syndrome virus infection upregulates (in the hepatopancreas) genes encoding known and potential antimicrobial effectors, while some genes involved in protection from oxidative stress were found to be downregulated by the virus.

cDNA microarray; immune response; subtractive hybridization; expressed sequence tags; white spot syndrome virus

THE PACIFIC WHITELEG SHRIMP *Litopenaeus vannamei* is a major aquacultured crustacean species, and as a result is becoming an increasingly important subject for study, particularly its genetics, physiology, and immunity. The response of shrimp to infectious diseases is of particular interest, as disease outbreaks cause large losses to aquaculture and may also threaten wild crustacean populations (10, 11, 30, 37). Shrimp are susceptible

to infection by a wide range of pathogens, including parasites, fungi, bacteria, and viruses, but of these agents it is the viruses that pose the greatest threat to shrimp health under farming conditions (33). Substantial insights have been gained in recent years into important aspects of the crustacean immune system, including the role of phagocytic cells, blood clotting, the prophenoloxidase/melanization system, and antimicrobial peptides. While some of these well-conserved immune effector pathways (such as melanization and antimicrobial peptide production) are reasonably well understood at the biochemical level (5, 9), the molecular events that underlie the majority of crustacean immune reactions remain unknown. Some of the greatest gaps in our knowledge of crustacean immunity concern the molecular basis for antiviral response, which are only beginning to be addressed and recent work points to the role of the conserved RNA interference (RNAi) pathway as a component of the shrimp antiviral immune system (45, 54, 58).

Functional genomics offers an attractive route to gain rapid insight into the molecular basis of immune reactions in species (such as shrimp) for which little information and few tools (including cell lines, bacterial artificial chromosome libraries, and monoclonal antibody reagents) are available. In shrimp, several studies have collected expressed sequence tags (ESTs) from normal as well as pathogen-infected animals (13, 21, 31, 47, 52, 53). These studies have largely converged on a few common conclusions: 1) genes with similarity to known immune function genes from other organisms (such as protease inhibitors) can respond to immune stimulation in shrimp (47), 2) a high proportion of ESTs (~50% on average) obtained from shrimp share no significant similarity to any known sequences (47, 53), and 3) large-scale EST and genomic analyses, as well as high-throughput gene expression studies will increase the likelihood that sound hypotheses can be formulated regarding the roles of candidate immune function genes in shrimp.

Here we present the description of a cDNA microarray for the study of immune function in *L. vannamei*, including the identification of candidate immune function genes from EST mining, particularly from libraries generated by suppression subtractive hybridization (SSH). We describe the use of the microarray to interrogate the transcriptome of four tissues in the shrimp and characterize changes in hepatopancreas gene

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expression in response to challenge with white spot syndrome virus (WSSV).

MATERIALS AND METHODS

Shrimp and Isolation of Total RNA

L. vannamei from a specific pathogen-free (SPF) line (Kona, Oceanic Institute, HI) were used for all experiments. The shrimp (1–2 g) used for expression profiling were kept in individual flasks with ~100 ml of seawater as previously described (44). The shrimp used for construction of SSH libraries were kept, as groups, in tanks with recirculating artificial seawater. All tissues (except hemocytes) that were harvested for RNA preparation were collected in RNAlater reagent (Ambion, Austin, TX) and stored frozen until use. Hemocytes were isolated from plasma by centrifugation, lysed in RLT buffer (RNeasy kit; Qiagen, Valencia, CA), and frozen at -80°C until used for RNA isolation. Total RNA was extracted using RNeasy columns (Qiagen) as described by the manufacturer.

For microarray analysis of immune-challenged shrimp, we used RNA from animals that had been injected with 20 μl of a standard tissue homogenate containing WSSV [diluted 1×10^{-6} wt/vol, a dose sufficient to cause 100% mortality in 5–7 days (44, 45)], followed by an injection of sterile saline 24 h postinfection. Controls were treated identically, except that they received tissue homogenate from SPF shrimp.

Standard cDNA Libraries

Some of the ESTs used in this study have been previously reported (21), and were obtained from cDNA libraries prepared from hemocytes and hepatopancreas of adult *L. vannamei* using the SMART cDNA library construction kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. An additional new library was prepared for the present study from gills pooled from multiple individuals, using similar methods. A total of 1,552 sequences from these three libraries were used to select probes for microarray construction.

Depletion of Highly Redundant Sequences From cDNA Libraries

To maximize the rate of gene discovery by EST collection, certain libraries from hemocytes were prepared in which the most highly redundant sequences were depleted by affinity chromatography. For this, a collection of 80 PCR products was generated, each representing a gene that had been found to be highly redundant in unmodified cDNA libraries (21). These PCR products were amplified using universal 5'-biotinylated primers and mixed in approximately equimolar amounts, to use as a depletion probe. Two libraries from hemocytes were prepared by two slightly different approaches. For the first library, cDNA was prepared from hemocyte total RNA using the long-distance PCR (LD-PCR) protocol from the SMART cDNA library construction kit (BD Biosciences), as recommended by the manufacturer. Double-stranded cDNA (~3 μg) was mixed with the biotinylated probe (~0.1 μg), 4.5 μl of water, and 2.5 μl of hybridization buffer (Clone Capture kit, BD Biosciences). The cDNA-probe mix was heated at 98°C for 5 min and allowed to hybridize at 68°C overnight. The hybridization mixture was then diluted to 30 μl with water, and biotin-containing complexes were removed by binding twice to magnetic streptavidin-coated beads (Clone Capture kit, BD Biosciences). The unbound materials (~60 μl) were diluted to 200 μl with water, and ethanol precipitated. The precipitated DNA was reconstituted in 79 μl of water and incubated in PCR reaction buffer (including Taq polymerase, Advantage PCR kit, BD Biosciences) at 98°C for 30 s and 68°C for 8 min, in the presence of LD-PCR primers (SMART cDNA library kit, BD Biosciences). This depleted cDNA was used to construct a library using the SMART cDNA library construction kit (BD Biosciences), following the instructions from the

manufacturer. A total of 1,152 clones from this library were propagated, sequenced, and deposited in <http://www.marinegenomics.org>; sequences of sufficient quality were also deposited in GenBank. The second depleted hemocyte library was derived from a previously constructed unmodified SMART cDNA library (21). In brief, the entire phage library was amplified on solid medium, and 4×10^6 clones were converted, in vivo, to circular plasmid DNA. The plasmid library was then titered, and a total of 3×10^6 clones were plated, and the colonies were pooled and used to isolate plasmid DNA by chromatography (Nucleobond Megaprep, BD Biosciences) followed by density gradient fractionation on CsCl (49). The plasmid library (~1 μg) was mixed with the biotinylated probe (~0.1 μg) in the presence of RecA and RecA reaction mix (Clone Capture kit, BD Biosciences), and biotinylated complexes were removed by binding the mixture twice to streptavidin-coated magnetic beads, following the manufacturer's instructions. The unbound material was extracted with phenol and chloroform and precipitated with ethanol following standard procedures (49). The depleted plasmid library was transformed into *Escherichia coli* and a total of 480 clones were propagated, sequenced, and deposited in <http://www.marinegenomics.org>; sequences of sufficient quality were also deposited in GenBank.

SSH

SSH libraries were constructed from three tissues of shrimp: hepatopancreas, hemocytes, and gills, using the PCR Select cDNA subtraction kit (BD Biosciences) according to the manufacturer's instructions. In every case, RNA from multiple individuals was pooled to generate samples for subtraction. Poly(A)-selected mRNA was used to construct hepatopancreas and gill SSH libraries, while total RNA was used for hemocyte SSH libraries due to the low yield of RNA typically obtained from these cells. The subtracted cDNA pools obtained from the PCR Select protocol were cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) to generate SSH libraries. A summary describing these SSH libraries is shown in Table S1 (the online version of this article contains supplementary materials). Essentially, four conditions were explored in each of the three tissues: infection with WSSV, hyperthermia in WSSV-infected shrimp, stimulation with heat-killed microbes, and injection of double-stranded RNA (dsRNA) [an inducer of antiviral immunity in shrimp, (46)]. A total of 5,760 clones isolated by SSH from the three tissues of interest were sequenced, and sequences of adequate quality were deposited in GenBank.

EST Analysis and Databases

The EST analysis pipeline utilized in this study is hosted at <http://www.marinegenomics.org> and has been described previously (36). The sequences of all the ESTs reported in the present study can be accessed at this site and identified by either a unique Marine Genomics ID no. (MGID) or by accession numbers assigned by the National Center for Biotechnology Information (NCBI), where the ESTs have also been deposited (dbEST). Before basic local alignment search tool (BLAST) (4) and Gene Ontology (GO) (22) analyses, the ESTs were automatically trimmed to remove vector and adaptor sequences, and uninformative sequences (e.g., short or poor quality reads) were removed from analyses. Further manual curation was performed to maximize the accuracy of the trimming and selection processes.

Microarray Generation

DNA for clones representing 2,469 predicted genes [based on CAP3, (27)] were amplified by PCR using universal primers (5'-TCGAGCGGCCGCCGGCAGGT and 5'-AGCGTGGTCGCGGCCGAGGT for SSH clones; 5'-AGTCCGAGATCTGGACGAGC and 5'-TAATACGACTACTATAGGGC or 5'-CTCGGAAGCGC-GCCATTGTG and 5'-CGAATTGGCCAAGTGAGCTCG for

SMART library clones). PCR products were purified by ion-exchange chromatography (QiaQuick, Biorobot 9600, Qiagen), quantified in a spectrophotometer (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA), dried down (SpeedVac; Thermo Savant, Waltham, MA), and dissolved in water to a concentration of 75 $\mu\text{g}/\text{ml}$. We transferred 20 μl to 384-well plates and mixed that with 10 μl of 100% dimethyl sulfoxide, and 10 μl of this mix were further transferred to 384-well spotting plates (Genetix, New Milton, UK). Glass slides (GAPSII Amino-Silane; Corning, Corning, NY) were spotted using a Q-ArrayMax (Genetix), baked for 2 h at 80°C, and stored under vacuum until used. Amplicons were spotted as neighboring duplicates, in a total of 48 subarrays of dimension 16 \times 17 (13,056 features in total). At least two pairs of duplicate features were spotted for each clone, and each pair was spotted in a different region of the array.

RNA Labeling and Microarray Hybridization

Total RNA (1 μg) from gills, hepatopancreas, or muscle obtained from individual shrimp was used in one round of linear RNA amplification using the Amino Allyl MessageAmp II aRNA kit (Ambion). For hemocyte samples, essentially all the RNA extracted from one individual shrimp was used (the amount of RNA was often below the level of reliable detection by spectrophotometry), and two rounds of amplification were applied, as instructed by the manufacturer. We used 10 μg of amino allyl-modified RNA (aRNA) for labeling with reactive Cy3 (Ambion) and for subsequent hybridizations. Microarrays were soaked in 0.2% SDS for 1 min, rinsed briefly with water, dipped for 1 min in boiling water, rinsed briefly again with water, and dipped in 70% ethanol before air drying. After this treatment, slides were prehybridized in 50% formamide, 2.5 \times Denhardt solution, 4 \times sodium chloride-sodium phosphate-EDTA (SSPE), 2.4% SDS, and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA for 1 h at 50°C. Labeled target aRNA was boiled for 1 min and prehybridized at 50°C for 1 h in the presence of 33% formamide, 2.6 \times SSPE, 1.6% SDS, 1.7 \times Denhardt, poly(dA) (22 $\mu\text{g}/\text{ml}$), and mouse cot-1 DNA (22 $\mu\text{g}/\text{ml}$). The prehybridization buffer was washed from the slide by a brief dip in water, labeled target was added to the dry slide, and a coverslip was placed on top. Hybridization was allowed to proceed overnight at 50°C in a humidified air incubator (InSlideOut 241000; Boekel, Feasterville, PA). After hybridization the slides were washed once in 2 \times SSC-0.1% SDS for 5 min, twice in 0.2 \times SSC-0.1% SDS for 5 min, twice in 0.2 \times SSC for 5 min, and once in 0.1 \times SSC for 5 min. After being rinsed in water, the slides were air dried and scanned using a ScanArray Express (Perkin Elmer, Boston, MA). Expression data were collected from images using QuantArray software (Perkin Elmer), and data were uploaded onto the microarray analysis pipeline hosted at <http://www.marinegenomics.org> for analysis.

Microarray Data Analyses

The basic approach used in this study to quantify differential expression involves data-driven modeling of the variance in microarray signals that is unrelated to experimental treatment of shrimp, by use of one or more sets of calibrator samples where the treatment does not change. We assessed the differential expression between an experimental and a reference dataset based on this data-driven model, by assigning to every gene a value of *df*, a measure of the strength of differential expression (*df* is defined below in the section *Assessment of differential expression*). The statistical significance associated with any measurement of *df* was also assessed by a sign rank Wilcoxon-type *P* value. Rank-ordered intensity data were used to reconstruct a normalized intensity value for each gene within each array, which was then used to calculate an average fold-change between signals in experimental and reference datasets. This approach allows the evaluation of differential expression based on a probabilistic indicator (*df*) that is accompanied by an index of reproducibility (*P* value), together with the more familiar ratio of differential expression. It is worth noting that even if there is a fundamental equivalence between

parametric ratios and nonparametric shifts in rank order, the latter are more reliable.

A custom-designed microarray analysis pipeline was developed to execute the analysis summarized above. The bioinformatic details of this analysis tool were adapted from our earlier work with proteomics data (2), with the main change being the use of Parzen kernels to describe the bivariate cumulative distributions of the quantile-quantile plots (41). In brief, the analysis pipeline takes expression data extracted from microarray images using QuantArray software (Perkin Elmer), and raw signal intensities are parsed and analyzed as microarray data structures in the Matlab 7.0 R14 scientific computing programming environment, as defined by their Bioinformatics toolbox. Using these Matlab data structures, the pipeline, which is integrated into the functional genomics infrastructure hosted at <http://www.marinegenomics.org> (36), executes four data analysis steps: 1) filtering, 2) normalization, 3) calibration based on replicate series, and 4) differential expression assessment.

Data filtering. Data were subjected to a series of filters, to exclude two types of spots from analysis: first, spots that are not informative for expression analysis (e.g., landing lights, empty spots), and second, spot pairs for which the ratio of intensity of the contiguous duplicate spots was not within an arbitrary range of 0.66–1.5. This second filter successfully removes spots affected by minor artifacts on the scanned images (data not shown).

Normalization. For each array, each spot was rank-ordered (and thus normalized) based on its corrected intensity (raw intensity minus local background). All analyses were performed on the rank-normalized data represented as quantiles, as previously proposed for proteomics data in Ref. 3.

Calibration. Arrays from the calibration series (biological or technical replicates) were compared after rank normalization, and the cumulative probability distributions of all versus all comparisons were built. The procedural details and conceptual design are similar to those described previously (3), except that in this study a model-free approach was followed to capture the bivariate density distribution: the Parzen window kernel method with a Gaussian distribution function (42) was used here. Every individual spot on every array was compared against every other spot representing the same clone in every other array of the same calibration set, i.e., spots representing the same clone in an array were not combined a priori, but rather considered separately throughout the analyses.

Assessment of differential expression. Differential expression was evaluated by determining for each gene a variable *df*, which assesses the strength of differential expression, accompanied by the corresponding *P* value, to evaluate its statistical significance. The basic function of the Matlab toolbox for these calculations takes three arguments: 1) one or more reference arrays, *X*, 2) one or more test arrays, *Y*, and 3) a series of calibration arrays, typically one or more replicate series. The resulting basic functionality produces two output arguments: 4) the average of the differential expression, *df*, obtained by projecting the reference and test values on the calibrating conditional cumulative distribution plot (generated from 3 as described above in *Calibration*), and 5) the *P* value of its consistency/reproducibility, which is assessed by the Wilcoxon sign-rank test of its deviation from the median response (quantile 1/2). For ease of interpretation and to avoid confusing the strength of differential expression (4) with its reliability (5), the former is represented as: $df = \text{average}[P(YX)*2 - 1]$, which projects the values of *df* between -1 and 1 with the positive values indicating overexpression and the negative values indicating underexpression (relative to the control or reference dataset). Determination of *df* is further illustrated in Fig. 1B. Because each probe is spotted at least four times, there will be at least as many individual values for *P*(*YX*) for each comparison of two arrays. When the test and control groups include multiple arrays (the norm), then the number of *P*(*YX*) values is multiplied by the product of the number of arrays in either group, e.g., $4 \times (\# \text{ control arrays}) \times (\# \text{ test arrays})$. The expression profiles generated in this study are

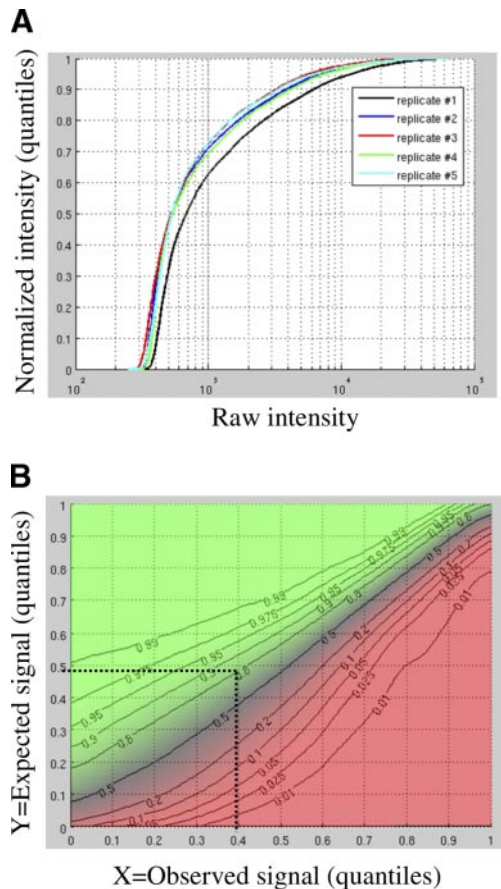


Fig. 1. Characteristics of the *Litopenaeus vannamei* microarray platform and strategy for analysis of differential expression. *A*: normalized vs. raw intensities for every clone in 5 replicate microarrays (technical replicates), illustrating the level of technical reproducibility that can be accomplished in the system (Spearman coefficient = 0.8935) and the importance of normalization for overall intensity during the analysis of the data (i.e., some samples can diverge significantly due to technical error). *B*: based on the model defined by the replicates from *A*, the index of differential expression $df = \text{average}[P(YX)*2 - 1]$ is given by the cumulative conditional probability $P(YX)$ of the occurrence of a normalized intensity in a test sample (Y), given the observed intensity in a reference sample (X), and given the distribution of probabilities shown, which is defined by the calibrator dataset (in this case the 5 technical replicates). The contour lines represent the indicated levels of the probability $P(YX)$. As an example, the values of X and Y that intercept the dotted lines shown would result in $df = 0.6$.

publicly available at NCBI (Gene Expression Omnibus series GSE4949, GSE4954, and GSE4955) as well as at <http://www.marinegenomics.org>.

RESULTS AND DISCUSSION

Characterization of Shrimp cDNA Sequences

Shrimp cDNA sequences were collected from unmodified hepatopancreas, hemocyte, and gill libraries and from libraries constructed by SSH. The SSH cDNA libraries (a total of 5,760 clones, see Table S1 in Supplementary Materials) were designed to enrich for mRNAs regulated by immune challenge using gills, hepatopancreas, or hemocytes as source material. The stimuli used were 1) WSSV infection (early and late times postinfection), 2) injection of heat-killed bacteria and fungal spores, 3) injection of dsRNA, and 4) WSSV infection at 32°C vs. WSSV infection at 27°C. These latter conditions were

chosen because hyperthermia (>31°C) is known to suppress WSSV replication and pathogenicity (20, 55).

A total of 7,021 ESTs collected from both regular cDNA and SSH libraries were found to be of adequate quality for analyses. The CAP3 sequence assembly program (28) was used to organize these sequences into contigs, which resulted in 908 contigs and 2,323 singletons for a total of 3,231 putative unigenes. Sequence homology searching (BLAST, Ref. 4) and gene ontology (GO, Ref. 22) analyses were performed for each unique sequence, and in agreement with most previous EST studies in shrimp, a high number (64%) exhibited no significant similarity to known genes from other organisms (using an arbitrary BLASTx e -value of 1×10^{-4} as threshold to define significant similarity). The remaining predicted unigenes (1,176 or 36%) had significant homology to known genes within the nonredundant GenBank database maintained at NCBI (e -value $< 1 \times 10^{-4}$), and 839 matched GO-annotated sequences from the Gene Ontology database maintained by the Gene Ontology Consortium [<http://www.geneontology.org> (22)] (BLAST-derived e -value $< 1 \times 10^{-6}$). The entire collection of sequences is publicly accessible at <http://www.marinegenomics.org>; here the discussion will be limited to selected genes identified as having potential roles in immune function. We identified 89 unigenes with functions and activities of potential relevance to the immune response (Table 1). These were classified under 10 functions, including antimicrobial and antiviral proteins, intracellular signal transducers, components of the RNAi machinery, transcription factors, regulators of apoptosis, proteases and protease inhibitors, oxidative stress response, and cell adhesion molecules.

Of the ESTs listed in Table 1, two components of highly conserved immune signaling pathways are of note. The first is a homolog of I κ B kinase, a positive regulator of the NF- κ B pathway involved in a broad range of immune responses (reviewed in Refs. 7, 23). The second is a homolog of signal transducer and activator of transcription (STAT), a core component of the interferon response in vertebrates (reviewed in Ref. 43) and of antiviral responses in *Drosophila* (15). Over a dozen kinases (both serine/threonine and tyrosine specific) and transcription factors also were identified from the cDNA libraries enriched in genes differentially expressed upon immune stimulation (Table 1).

Potentially important to antiviral immune pathways in shrimp is the identification of three putative components of the RNAi pathway (Table 1). RNAi has been demonstrated to function as an antiviral mechanism in several invertebrates including shrimp (32, 35, 45, 56). Of the three gene segments identified, the candidate homologs of the *Drosophila* RNA helicase Armitage and of the conserved Tudor-nuclease were isolated by differential expression cloning (SSH). Armitage cDNA may be of particular interest, as it was potentially enriched in gills from shrimp infected with WSSV at 27°C (the permissive temperature for WSSV replication), perhaps suggesting viral induction of Armitage expression in gills of shrimp. Another candidate antiviral gene product was identified from similarity to a Zn-finger-containing protein from mammals, which has been shown to confer resistance to retroviruses and to members of the *Togaviridae* family (6, 17). As was the case for Armitage cDNA, the EST encoding a portion of the putative antiviral Zn-finger protein was isolated from an SSH library designed to enrich for genes induced in

Table 1. *Litopenaeus vannamei* ESTs representing genes with domains, activities, or functions potentially relevant for the immune response

MGID	Predicted Function	Libraries
<i>Antimicrobial</i>		
7431	Histone H2A variant Z	H-I-mic
7381	Histone H4	H-I-mic
5074	Lysozyme	H-N, H-R-dsRNA(24), H-I-32C/W, HP-I-W(48)
6628	Tachylectin-5A	H-I-32C/W, H-R-32C/W
6835	Anti-LPS factor*	G-I-mic, H-I-mic, G-R-dsRNA(24), H-I-dsRNA(24)
<i>Antiviral</i>		
6174	CCCH-type zinc finger antiviral protein	HP-R-32/W
<i>Cell adhesion</i>		
1491	Cadherin 23	HP-R-W(48)
7569	Immunoglobulin domain	H-I-mic
456	Integrin alpha	H-I-dsRNA(24)
408	Integrin beta	H-I-dsRNA(24)
6211	Leucine-rich repeat and PDZ domain protein	HP-R-32C/W
1223	Peroxinectin	G-R-W(48), G-N, H-N
2078	Tetraspanin	G-R-W(9), HP-I-mic
6113	Tetraspanin	HP-R-32C/W, H-N
7097	Tetraspanin, cell surface glycoprotein	HP-I-mic
2376	Unc-112-related, pleckstrin homology domain	H-I-W(9)
<i>Cell death</i>		
2874	Autophagy protein 9	HP-I-W(9)
8531	GULF adaptor protein	H-N
5320	Phosphatidylserine receptor, phagocytosis of apoptotic cells	H-N
<i>Oxidative stress</i>		
8321	Copper chaperone	H-N
1560	Glutathione S-transferase	HP-R-W(48), G-N
4949	Peroxiredoxin	HP-N
5876	Thioredoxin reductase	HP-I-32/W, G-I-mic
4292	Thioredoxin	G-N
<i>Proteases</i>		
6958	Aminopeptidase	G-I-mic, H-I-mic
1538	Astacin protease	HP-R-W(48)
2397	Cathepsin A serine carboxypeptidase	H-I-W(9)
2471	Cathepsin B cysteine protease	H-I-W(9), H-N
6207	Cathepsin D aspartic protease	HP-R-32C/W
6359	Cathepsin L cysteine protease	H-R-32C/W, HP-R-32C/W, G-I-dsRNA(24), H-I-dsRNA(24), G-I-W(48)
1283	Cubilin protease	G-R-W(48)
2397	Lysosomal carboxypeptidase	H-I-W(9)
300	Nepilysin metalloproteinase	G-R-dsRNA(24)
8840	Prohormone and neuropeptide processing protease	H-N
1590	Serine carboxypeptidase	HP-R-W(48)
966	Zn carboxypeptidase	G-I-W(48), G-I-dsRNA(6)
<i>Protease inhibitors</i>		
8678	Serine protease inhibitor	H-N
7686	Serine protease inhibitor	H-N, H-I-mic, H-I-dsRNA(24), H-I-32C/W, H-R-32C/W
8274	WAP domain protease inhibitor	H-N, H-R-dsRNA(24)
<i>RNA interference</i>		
5777	Putative helicase from Moloney leukemia virus, SDE-3/armitage	G-R-32/W
4765	TAR RNA binding protein/Loquacious	H-N
7642	Tudor staphylococcal nuclease	H-I-mic
<i>Signal transduction</i>		
8371	Activator of mitogen-activated protein kinase (MAPK) pathway	H-N
2092	cAMP dependent protein kinase	G-R-W(9)
2477	Casein kinase II, alpha subunit	H-I-W(9), H-I-mic, H-R-mic
2926	COP9 signalosome complex subunit 1	HP-I-W(9)
2489	I κ B kinase	H-I-W(9)
7146	Inositol 1,4,5-triphosphate 3-kinase	HP-I-mic
8670	Protein kinase C, mu	H-N

Continued

Table 1—Continued

MGID	Predicted Function	Libraries
2746	Putative regulator of MAPK pathway	HP-I-W(9), H-N
661	Ribosomal protein S6 serine/threonine kinase	G-I-dsRNA(24)
4172	Serine/arginine-rich serine protein kinase	G-N
199	Serine/threonine protein kinase 25, oxidant stress response	G-R-dsRNA(6)
6858	Serine/threonine protein kinase checkpoint	G-I-mic
7507	Serine/threonine protein kinase Misshapen	H-I-mic
6264	Serine/threonine protein kinase, transduction of mitogenic signals	H-R-32C/W
8922	Serine/threonine protein kinase	H-N
4013	Serine/threonine protein kinase Pk61C	G-N
1723	Serine/threonine protein kinase polo	HP-R-W(48)
4233	Serine/threonine protein phosphatase 1B	G-N
6184	Serine/threonine protein phosphatase 2A	HP-R-32C/W
6671	Serine/threonine protein phosphatase PP1	H-I-32C/W
6089	src-family tyrosine protein kinase	HP-R-32C/W
6622	Trio protein, spectrin repeat, nucleotide exchange factor, serine/threonine kinase	H-I-32C/W
4523	Tyrosine kinase, signaling	G-N
7781	WD40 domain	H-I-mic
9606	WD40-domain	G-R-mic
4145	WD40-domain	G-N
9222	WD40-domain	H-N
7740	WD40-domain	H-I-mic
7058	WD40-domain	HP-I-mic
1567	Zn finger protein, associates with PKC-related kinases	HP-R-W(48)
5787	Platelet derived growth factor-like	G-R-32C/W, HP-I-mic
<i>Transcriptional control</i>		
7457	Brahma associated protein 60 kDa, chromatin remodeling	H-I-mic
6898	dsDNA-binding, chromatin organization, transcriptional regulation	G-I-mic
1250	Hormone receptor transcription factor	G-N, G-R-W(48)
6047	Interleukin enhancer binding factor 2	HP-R-32C/W
4197	Lola-like transcription factor	G-N
9180	Repressor of E1A-stimulated genes, transcriptional repression	H-N
5447	Rhombotin-like transcription factor	G-I-32C/W
8767	Signal transducer and activator of transcription	H-N
6058	Similar to cAMP responsive element binding protein-like 2, transcriptional regulator	HP-R-32/W, G-I-dsRNA(6), HP-I-32/W
1868	SMAD transcription factor	G-I-W(9), HP-I-32/W
2445	ssRNA/DNA binding protein, chromatin regulation	H-I-W(9)
2226	Thyroid hormone receptor-associated protein TRAP240	H-I-W(9)
4950	Transcription factor slowmo	HP-N, G-N
558	Transcription factor E3	G-I-dsRNA(6)
8897	Transcription factor, related to enhancer of yellow 2	H-N
8658	Transcriptional repressor CRTR-1	G-N

Only expressed sequence tags (ESTs) with Gene Ontology (GO) annotation were included (with the exception of EST marked with *). Relevance to immune response was considered based on functions predicted by comparison with sequences in GenBank. Marine Genomics identification (MGID) is a unique identifier in the *Litopenaeus vannamei* database at <http://www.marinegenomics.org>. Full sequence information, basic local alignment search tool (BLAST) and GO annotations, and contig information can be retrieved at this site via the MGID identifier. Predicted function is based on BLAST matches (an e -value of 1×10^{-4} was used as threshold) and/or on the presence of known domains. Library refers to the cDNA library in which the reported EST was found, as well as other libraries in which overlapping ESTs were found. H, hemocytes; HP, hepatopancreas; G, gills; I, potentially induced [suppression subtractive hybridization (SSH)]; R, potentially repressed (SSH); N, library from nonstimulated normal shrimp; W, infected with white spot syndrome virus (WSSV); 32C/W, infected with WSSV at 32°C. These libraries were generated by subtracting against the corresponding cDNA from animals infected with WSSV at 27°C (24 h postinfection); Mic, injected with a mixture of heat-inactivated *Aerococcus viridans*, *Vibrio parahaemolyticus*, and *Fusarium oxysporum* (a total of 1.3×10^8 cells/spores per shrimp, based on Ref. 14, samples were taken 6–8 h postinjection). dsRNA, dsRNA for a 309-bp portion of duck Igu (NCBI accession no. AJ312200); t, time postinjection (h).

animals infected at temperatures permissive to WSSV replication (Table 1). Also of interest for the study of virus-host interactions is the identification of genes involved in programmed cell death, as apoptotic responses have been suggested to play roles in viral pathogenicity and/or in the anti-WSSV response of shrimp (48, 57).

Among genes of broad relevance to immune function identified in this collection were regulators of cell shape, cell adhesion, and cell mobility, as well as proteases and protease inhibitors. Processes regulated by these genes are generally

thought to modulate phagocytic events, recruitment of immune cells to sites of insult, cellular remodeling, and extracellular immune cascades such as the melanization response. In the protease group, several lysosomal proteases (e.g., cathepsins) are of special interest, because they were present in libraries enriched for transcripts induced by WSSV infection and by dsRNA, an inducer of antiviral immunity in shrimp (46). It may be that changes in cathepsin expression reflect activation of lysosomal functions for antiviral purposes, as discussed further in this report in the context of microarray data.

The differential abundance of the mRNAs identified by SSH in this study has not been systematically confirmed, and thus genes reported here as identified through SSH should be considered only as potentially regulated by immune stimuli. In fact, the occurrence of some cDNAs in reciprocal SSH libraries indicates at least some level of background cloning, as might be expected for the SSH method (Table 1 and data not shown). Future high-throughput expression profiling studies will be necessary to confirm differential regulation of most of these genes, although the set of experiments described below represents a first step toward this goal.

cDNA Microarray Platform for *L. vannamei*

The shrimp cDNA microarray was designed to contain a set of genes biased toward immune function. This was accomplished mainly by including a high number of clones isolated by SSH (64% of the amplicons on the array originated from SSH libraries, while 22% were from normal EST libraries, and the remaining 14% were from EST libraries depleted of redundant sequences as described in MATERIALS AND METHODS). The validation of this tool included 1) assessment of its technical reproducibility, 2) assessment of its value in differentiating gene expression between four tissues, three of which were used as source tissues for array construction (hemocytes, hepatopancreas and gill) and the fourth served as a technical out-group (muscle), and 3) evaluation of the experimental utility of the microarray for analyzing differences in gene expression between uninfected and WSSV-infected shrimp.

Technical validation of the array was addressed by dye-labeling five independent aliquots from a sample of total RNA isolated from a single shrimp and hybridizing these independently to five microarrays. In an all-vs.-all comparison (Fig. 1A), a Spearman correlation coefficient of 0.8935 was obtained, a level of reproducibility adequate for robust assessment of differential gene expression, as supported by the observation that three biological replicates from the same tissue type (i.e., gill samples from three different shrimp) yielded a correlation coefficient of 0.7693 in an independent experiment. This indicates that the variance observed due to technical factors is unlikely to mask biological variance when using the *L. vannamei* microarray to assess differential expression. Given a model based on this set of five technical replicates, the distribution of conditional probabilities that define the strength of differential gene expression (df) is represented in Fig. 1B, which is shown to illustrate the statistical approach used to derive df .

Tissue-specific Transcriptional Signatures

Multiple tissues may play immune roles in shrimp *in vivo*, and thus the ability of a microarray to detect differential gene expression in several tissues is important for the transcriptomic study of immune responses. The microarray was tested for its efficacy in differentiating between the gene expression profiles of hemocytes, hepatopancreas, gills, and muscle. Three individual samples for each tissue were analyzed, with the results represented in the form of a double cluster of differential gene expression (df) in Fig. 2A. The hepatopancreas dataset was used as the calibrator for biological/technical variability in gene expression and also as the reference for determination of differential expression. These data demonstrate that distinct profiles of gene expression in each of

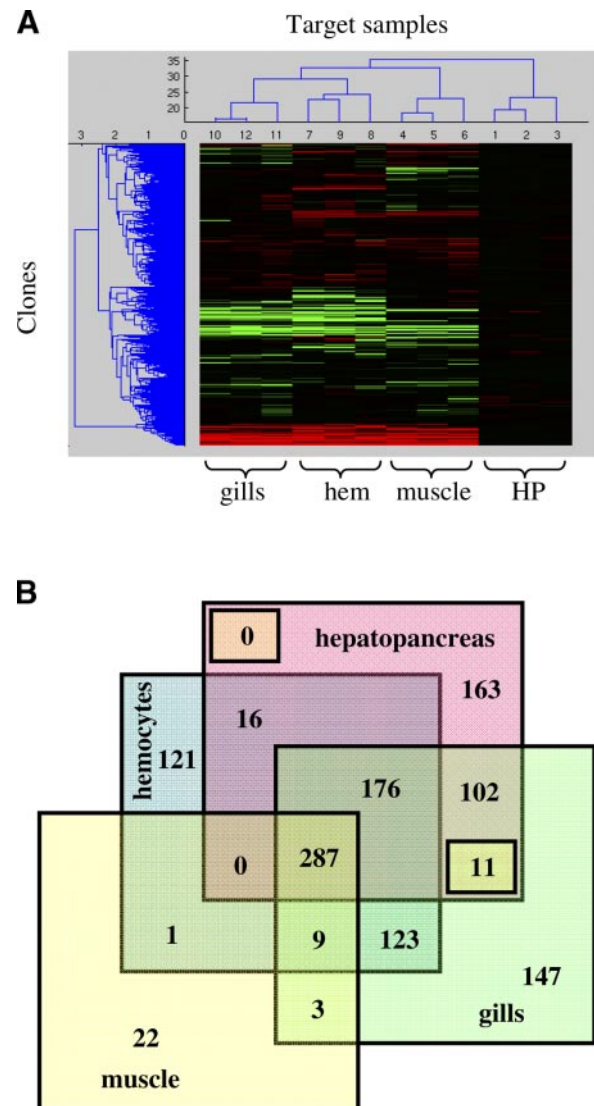


Fig. 2. Transcriptional profiling in different tissues of individual shrimp. **A:** heat map representation of df values for all clones on the microarray after hybridization to three samples of hepatopancreas (HP; 1, 2, 3), muscle (4, 5, 6), hemocytes (hem; 7, 8, 9), or gills (10, 11, 12). Clones are clustered in rows according to common patterns of expression along the 12 samples, while samples are clustered in columns according to similarities in global expression profiles. The hepatopancreas samples were used for calibration of the model and as reference dataset. Green indicates positive df values (overexpression), and red indicates negative df values (underexpression), relative to the hepatopancreas reference. **B:** clones that showed signal above background in at least 67% of the replicate spots along the 3 biological replicates in each tissue were considered positive. Each clone was assigned as positive in only 1 of the categories illustrated in the diagram.

these tissues can be readily discriminated by the *L. vannamei* microarray, as the clustering algorithm could unequivocally group expression profiles based on tissue type. Interestingly, the cluster analysis suggests that gene expression profiles in hemocytes and gills are more closely related to each other than they are to those of hepatopancreas and muscle. Clustergrams with similar branch structures were produced regardless of the tissue dataset used as the calibrator/reference (data not shown), indicating the robustness of the association between the profiles observed in hemocytes and in gills. This may reflect the fact that circulating

hemocytes readily infiltrate gill tissues in shrimp (40). In the four tissues tested, expression can be readily assessed for only a fraction of the genes spotted on the array. The percentage of hybridizing clones in this experiment ranged from 13% in muscle to 30, 31, and 35% in hemocytes, hepatopancreas, and gills, respectively (Fig. 2B). This is likely reflective of the fact that muscle was not a tissue used for cDNA library construction during EST collection, whereas libraries from the other three tissues were each exploited to approximately the same extent. The highest overlap of positive clones between tissues was seen between gills and hemocytes (123 clones) compared with any other pair-wise comparison (Fig. 2B), supporting the cluster analysis (Fig. 2A) that showed gills and hemocytes to have the most closely related transcriptomes. As might be expected, since cDNA from muscle was not examined as a source of unigenes for the microarray, few clones hybridized exclusively with muscle targets, and most of the muscle-positive clones (287, or 89%) represent ubiquitously expressed transcripts (Fig. 2B).

To investigate the functional diversity of gene expression detected by the microarray in the different shrimp tissues, GO analysis was performed on the data summarized in Fig. 2B. When comparing the total complement of genes detected by the microarray in hepatopancreas, hemocytes and gills, we found the distributions of GO annotations within the three tissues remarkably similar [the categories “cellular physiological process” (GO:0050875) and “cellular metabolism” (GO:0044237) are shown in Fig. 3A to illustrate this point]. This similarity extends to essentially every GO category for which a significant number of annotations (>50) exist for each tissue profile (not shown, full dataset available at: http://www.marinegenomics.org/go.php?&s=y&organism=est_l_vannamei&abbrev=L.%20vannamei&srn=Litopenaeus%20vannamei). As might be expected, more divergent distributions of functional GO annotations could be observed when the analysis was performed on those subsets of genes that hybridized exclusively in only one of the tissues, as illustrated by comparison between gills and hepatopancreas at the levels of cellular physiological process (GO:0050875) and cellular metabolism (GO:0044237) (Fig. 3B). This divergence, however, did not apply to all the hierarchies of the GO annotation system (full dataset available at: http://www.marinegenomics.org/go.php?&s=y&organism=est_l_vannamei&abbrev=L.%20vannamei&srn=Litopenaeus%20vannamei).

It seems likely, based on these results, that tissue-specific functional specializations in shrimp are reflected in the predominant fraction of the transcriptome that escapes annotation with the current GO system. This underscores the need for a more comprehensive cataloguing of the transcriptome of the shrimp and emphasizes the importance of expanding the universal GO annotation system to enhance its value in studying organisms (such as shrimp) with scarce molecular information. The *L. vannamei* microarray reported here should be adequate for studying immune gene expression in at least three tissues: hemocytes, hepatopancreas, and gills.

Changes in Gene Expression in Response to Immune Challenge

To test the ability of the microarray to assess transcriptomic responses of shrimp undergoing an immune challenge, differential gene expression was investigated in animals infected with a lethal dose of WSSV, a prevalent pathogen of crustacea.

Forty hours after infection, RNAs were isolated from the hepatopancreas of eight control shrimp and of eight WSSV-infected shrimp and profiled by microarray analysis. After calibrating the model using the eight control uninfected samples, we analyzed the dataset to reveal the genes whose expression was significantly regulated in WSSV-infected hepatopancreas. In Table 2, clones that met all of the following criteria for differential expression are listed: 1) a value of *df* between 0.5 and 1 (induced) or between -0.4 and -1 (repressed, see explanation below), 2) a Wilcoxon *P* < 0.001, and 3) a projected fold change >1.30 (induced) or <0.77 (repressed). We detected 25 clones corresponding to WSSV genes and 36 clones corresponding to shrimp genes in the upregulated group, while 28 clones for shrimp genes were identified in the downregulated set. Not surprisingly, there is at least partial concordance between sequences recovered by SSH and microarray results (Table 2).

As mentioned above, 41% (25 clones) of the transcripts induced in infected shrimp correspond to WSSV-encoded genes. These WSSV genes were isolated as part of the SSH screen for cDNAs differentially expressed during WSSV infection. Their detection in WSSV-infected samples validates the experimental and analytical approaches used here. A function can be predicted for only a few of the induced shrimp genes, as 38% of these have no matches in public databases. This result was not unexpected, since a large fraction (48%) of the genes represented on the microarray has no predictable function based on the sequence information available. We predict that many genes important for the immune response of the shrimp are included in this group and that functional genomic approaches such as the one applied here are likely to pave the way to uncovering their specific roles. Among genes of unknown function with homologs in other organisms, shrimp MGID 8371 may particularly warrant further investigation, as its homolog, the *Drosophila melanogaster* gene CG10103, has been reported to be induced by *Drosophila* C virus (DCV) infection (15), perhaps suggesting broad viral responsiveness of this gene in arthropods.

Perhaps the most intriguing group of virus-induced genes corresponded to bona fide antimicrobial proteins. The mRNAs for lysozyme, a homolog of tachylectin (from horseshoe crab), and antilipopolysaccharide factor were significantly upregulated in infected hepatopancreas. The antimicrobial activity of these proteins (against bacteria and fungi) has been well established (12, 24, 29, 50), but their potential involvement in antiviral responses has remained largely unexplored. Overall, these results suggest that infection with WSSV activates responses that overlap (at least partially) with those induced by challenge with bacteria (51, 52). The upregulation of antimicrobial proteins as a response to viral infection has also been reported in *Drosophila*, where infection with *Drosophila* X virus and DCV induce the expression of some antimicrobial peptides (15, 59).

Among the WSSV-induced genes it is interesting to observe a homolog of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) from *Drosophila*. In *Drosophila*, the PDGF/VEGF receptor system is known to regulate cell migration, hemocyte proliferation, and embryonic hemocyte survival (8, 16, 39). Specifically, PDGF receptor signaling is known to promote hemocyte survival by activating antiapoptotic responses (8). The possible involvement of a PDGF-related shrimp factor

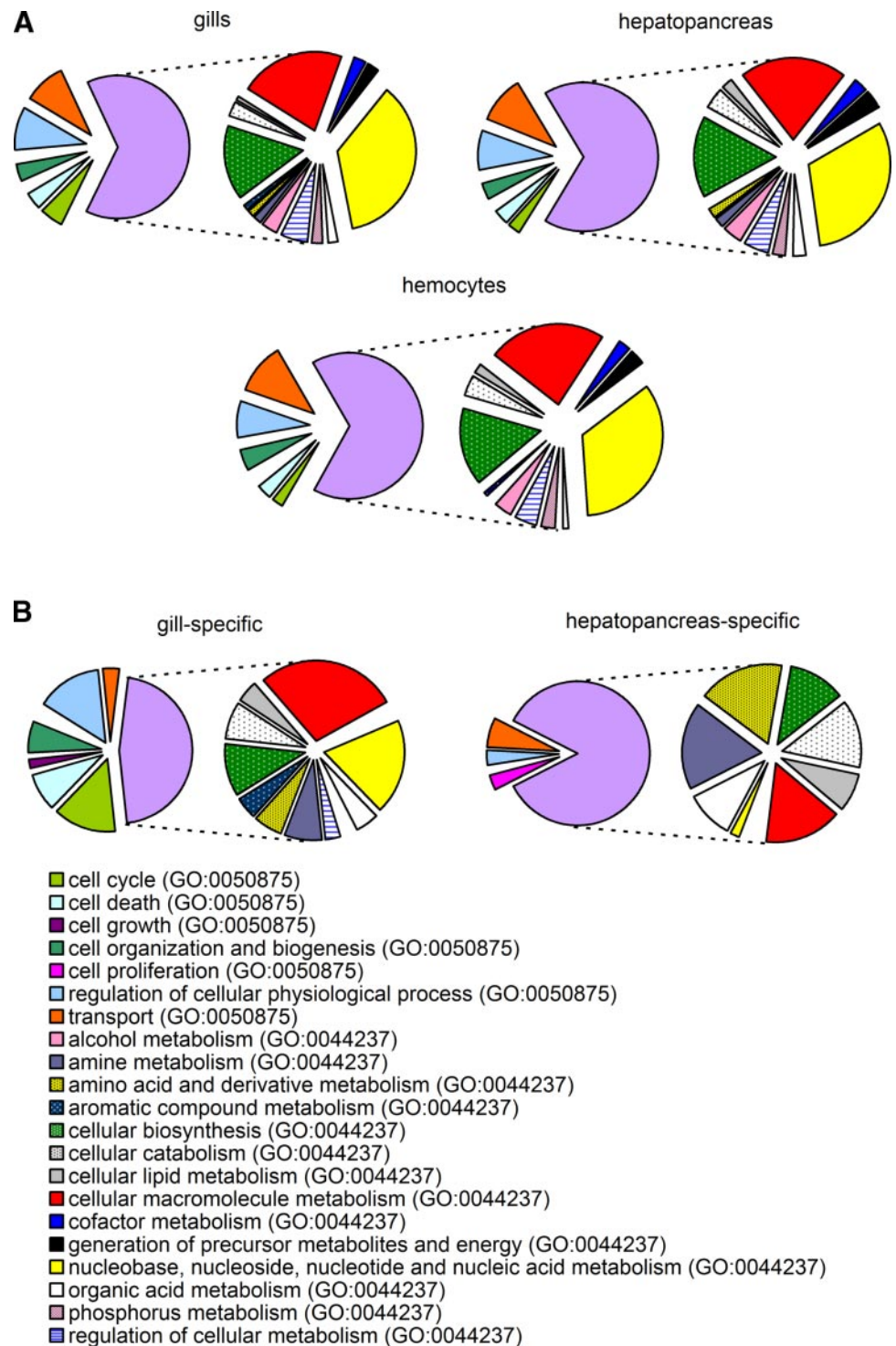


Fig. 3. Gene Ontology (GO) analyses of transcripts associated with different shrimp tissues. Distribution of GO annotations at the levels of cellular physiological process (GO: 0050875, charts on the *left*) and cellular metabolism (GO:0044237, charts on the *right*) for the clones that reacted positively to gills, hepatopancreas, or hemocytes (A) or for the clones that reacted exclusively to gills or hepatopancreas (B). The dotted lines connect the slice that corresponds to cellular metabolism within the cellular physiological process chart to the description of subcategories in cellular metabolism. The legend describing the color-coded GO categories is shown at the *bottom*.

in cell to cell signaling during antiviral responses deserves examination.

At least one of the cathepsins (a cathepsin-L homolog) predicted to respond to WSSV infection based on analyses of SSH libraries (Table 1) was also detected as induced by the virus via microarray analysis (Table 2). Cathepsins are endosomal/lysosomal proteases involved in the regulation of cellular processes as varied as the cell cycle, hormone maturation, and autophagy (18, 19, 25). In terms of immune response, the

best described functions of cathepsins are related to peptide processing during antigen presentation in vertebrates (reviewed in Ref. 26). It is currently difficult to provide context to the apparently widespread induction of cathepsins in response to WSSV infection in shrimp (Tables 1 and 2), but it is possible that endosomal or secreted cathepsins play roles in antiviral immunity.

Comparison of genes up- and downregulated by WSSV infection suggests that fewer genes are downregulated than are

Table 2. *L. vannamei* clones showing differential abundance in control vs. WSSV-infected hepatopancreas by microarray analysis

MGID	Putative Function/Homology	df	P	Fold Change	Libraries
Induced in WSSV-infected Hepatopancreas					
<i>Shrimp genes with significant matches in GenBank</i>					
6835	Anti-lipopolysaccharide factor	0.76097	3.60E-40	2.9257	G-I-mic, H-I-mic, G-R-dsRNA(24), H-I-dsRNA(24)
5787	PDGF and VEGF related	0.69714	7.03E-38	1.6579	G-R-32C/W, HP-I-mic
906	Zn carboxypeptidase	0.65845	4.80E-37	1.4154	G-I-W(48), G-I-dsRNA(6)
4699	Orphan sodium- and chloride- dependent neurotransmitter transporter	0.61744	1.73E-38	1.7127	HP-N
4425	Ubiquitin-like fused to 40S ribosomal protein S30	0.60416	1.16E-35	3.1387	G-N, H-N
6628	Tachylectin-5A	0.58918	4.28E-35	1.4172	H-I-32C/W, H-R-32C/W
921	Cathepsin-like Cys protease	0.58166	2.02E-32	1.3855	H-R-32C/W, HP-R-32C/W, G-I-dsRNA(24), H-I-dsRNA(24), G-I-W(48)
4297	Histone deacetylase complex component	0.56403	2.71E-27	1.8923	G-N
4912	<i>D. melanogaster</i> CG11686/unknown function	0.56359	5.07E-74	1.5723	HP-N
7146	Inositol-trisphosphate 3-kinase	0.55426	2.98E-30	1.5958	HP-I-mic
1944	Phosphoenolpyruvate carboxykinase	0.54507	1.19E-32	1.9009	G-R-W(9)
8371	<i>D. melanogaster</i> CG10103/unknown function	0.53824	1.15E-37	2.3382	H-N
6501	Lysozyme	0.52674	1.58E-30	1.3065	H-N, H-R-dsRNA(24), H-I-32C/W, HP-I-W(48)
<i>Shrimp genes with no significant matches in GenBank</i>					
908	No match	0.82486	2.80E-43	1.9458	G-I-W(48)
12173	No match	0.79686	1.87E-40	2.4446	G-I-W(48), G-I-32/W
5667	No match	0.79429	2.10E-40	3.9605	G-R-32/W, HP-I-W(48)
6027	No match	0.75447	4.36E-40	2.0765	HP-R-32/W
5144	No match	0.72562	5.40E-37	2.8095	H-N
5595	No match	0.70683	1.26E-40	1.8751	G-I-32/W
6747	No match	0.6974	8.82E-39	2.9217	H-R-32/W, H-I-mic
734	No match	0.68854	9.71E-41	1.3055	HP-I-W(48)
6611	No match	0.66379	4.98E-40	1.8196	H-I-32/W
7386	No match	0.59801	6.30E-35	1.5828	H-I-mic
8610	No match	0.58935	2.02E-37	1.7758	H-N
6333	No match	0.58489	5.27E-35	1.441	H-R-32C/W
5168	No match (overlaps MGID 5144)	0.57444	4.12E-27	2.0491	H-N
6546	No match	0.57253	3.34E-38	1.515	H-I-32C/W
7728	No match	0.56658	1.48E-30	1.6552	H-I-mic
2771	No match (overlaps MGID 7020)	0.549	1.73E-34	1.6023	HP-I-W(9), HP-R-W(9), HP-I-mic
6508	No match	0.54764	3.67E-31	2.4266	H-I-32/W
7500	No match	0.54204	2.93E-32	1.7844	H-I-mic
8915	No match	0.52956	1.19E-38	1.8493	H-N, G-I-32/W
7020	No match	0.52343	3.93E-34	1.9053	HP-I-W(9), HP-R-W(9), HP-I-mic
1617	No match	0.51041	3.26E-33	1.4012	HP-R-W(48), G-R-32/W
6134	No match	0.50828	2.53E-27	2.4835	HP-R-32/W, HP-I-W(48)
6033	No match (overlaps MGID 6134)	0.50527	5.64E-25	2.2537	HP-R-32/W, HP-I-W(48)
<i>White spot syndrome virus genes</i>					
6059	wsv179	0.92604	9.99E-44	13.8254	HP-R-32C/W, G-R-32C/W, G-I-W(48)
984	wsv178	0.90043	5.39E-43	26.9171	HP-R-32C/W, G-R-32C/W, G-I-W(48)
5734	wsv051	0.87604	2.77E-42	4.831	G-R-32C/W, G-I-W(48), HP-I-W(48)
5656	wsv311	0.86114	7.11E-42	5.1029	G-R-32C/W, H-R-32C/W, G-I-W(48)
5645	wsv482	0.85753	2.55E-42	3.9024	G-R-32C/W, HP-I-W(48), G-I-W(48)
915	wsv482	0.8455	8.78E-43	2.4265	G-I-W(48), HP-I-W(48), G-R-32C/W
931	wsv051	0.84097	1.08E-41	4.2115	G-I-W(48), G-R-32C/W, HP-I-W(48)
900	wsv311	0.78761	1.38E-40	2.8133	G-I-W(48), H-R-32C/W, G-R-32C/W
965	wsv215	0.78551	8.77E-41	2.9691	G-I-W(48), G-R-32C/W, H-R-32C/W, H-I-W(48)
968	wsv421	0.76686	2.63E-40	3.432	G-I-W(48), G-R-32/W, H-R-32C/W, HP-I-W(48)
6321	wsv421	0.76506	4.44E-39	4.2585	G-I-W(48), G-R-32/W, H-R-32C/W, HP-I-W(48)
947	wsv108	0.76397	5.63E-41	1.5401	G-I-W(48), G-R-32/W
945	wsv207	0.72626	4.86E-41	1.4298	G-I-W(48), HP-I-W(48)
881	wsv465	0.70986	1.10E-36	1.67	G-I-W(48)
884	wsv128	0.67949	1.17E-35	1.8509	G-I-W(48)
5704	wsv215	0.67132	4.51E-36	1.7121	G-I-W(48), G-R-32C/W, H-R-32C/W, H-I-W(48)
875	wsv188	0.66979	3.13E-38	1.5091	G-I-W(48), HP-I-W(48)
695	wsv215	0.65713	3.72E-39	1.4836	G-I-W(48), G-R-32C/W, H-R-32C/W, H-I-W(48)
5671	wsv310	0.65707	1.73E-40	1.4565	G-R-32C/W, H-R-32C/W
5694	wsv285	0.62883	1.92E-36	1.3403	G-R-32C/W

Continued

Table 2—Continued

MGID	Putative Function/Homology	df	P	Fold Change	Libraries
882	wsv179	0.62232	2.09E-34	1.4479	HP-R-32C/W, G-R-32C/W, G-I-W(48)
876	wsv230	0.60939	5.38E-30	1.4174	G-I-W(48), G-R-32C/W
5711	wsv077	0.59369	1.71E-33	1.3875	G-R-32C/W
6390	wsv186	0.57236	1.57E-32	1.324	H-R-32C/W
5643	wsv244	0.52559	3.69E-30	1.3428	G-R-32C/W
Repressed in WSSV-infected Hepatopancreas					
<i>Shrimp genes with significant matches in GenBank</i>					
4299	Glutathione-S-transferase	-0.72219	1.32E-42	0.37589	G-N, HP-R-W(48)
7145	Translation initiation factor eIF-2B alpha subunit	-0.62869	1.66E-37	0.67236	HP-I-mic
2911	BCS-2	-0.5588	1.95E-29	0.62694	HP-I-W(9)
1505	Integral membrane protein of unknown function	-0.53727	9.37E-33	0.57715	HP-R-W(48)
5270	Nucleoplasmin-like	-0.52797	1.56E-34	0.49038	H-N
1509	Putative transmembrane protein	-0.52351	1.25E-30	0.61502	HP-R-W(48)
7732	Thioredoxin related	-0.51367	5.99E-31	0.74974	H-I-mic
8767	Signal transducer and activator of transcription (STAT)	-0.48618	3.54E-32	0.67894	H-N
5916	Calreticulin	-0.46251	3.73E-28	0.55454	HP-I-32C/W, H-I-32C/W, G-I-32C/W, G-R-32C/W
7650	Fibrillarlin	-0.46162	2.42E-30	0.67124	H-I-mic
6919	Extracellular protein ARMET	-0.45085	7.7E-22	0.69824	G-I-mic, G-N
1574	Alpha esterase	-0.44564	2.06E-22	0.61311	HP-R-W(48)
4685	Translocon-associated protein, delta subunit	-0.41954	6.85E-32	0.57042	HP-N, H-N
2004	Calreticulin	-0.41887	2.00E-25	0.68456	G-R-W(9)
7734	Seryl tRNA synthetase	-0.41319	1.93E-28	0.74256	H-I-mic
<i>Shrimp genes with no significant matches in GenBank</i>					
5960	No match	-0.61393	1.39E-32	0.46819	HP-I-32/W
5838	No match	-0.55785	6.14E-37	0.56347	HP-I-32/W
4991	No match	-0.54002	4.14E-71	0.68049	HP-N
7859	No match	-0.47949	5.73E-22	0.72677	H-R-mic, H-I-mic
7439	No match (overlaps 7859)	-0.46012	6.83E-25	0.59578	H-R-mic, H-I-mic
5373	No match	-0.43791	2.14E-29	0.72757	H-N
8143	No match	-0.4375	1.88E-22	0.64659	H-N
5015	No match	-0.43387	2.49E-30	0.60136	HP-N, H-I-32C/W
1451	No match	-0.4261	7.20E-23	0.65063	HP-R-W(48)
9084	No match	-0.4193	4.03E-23	0.58796	H-N, G-I-mic, H-I-32C/W
6543	No match	-0.41741	1.80E-22	0.75826	H-I-32C/W
6545	No match	-0.40514	7.17E-16	0.73624	H-I-32C/W, H-I-mic, HP-I-mic, H-I-W(9)
2666	No match	-0.40288	3.47E-25	0.76878	H-R-W(9)

See text for description of criteria used to select differentially expressed clones.

induced (Table 2), an effect that could be related to a biased sampling of the transcriptome by the microarray generated in this study. Given this possible bias, we consider in our discussion below genes for which the strength of downregulation was as low as -0.4 *df*, since it may be that relatively modest downregulation mediated by WSSV infection is of functional significance.

Two genes likely involved in the response to oxidative stress, encoding glutathione-S transferase and a thioredoxin-related protein, were among the WSSV-repressed transcripts (Table 2). It has been observed in multiple tissues (including hepatopancreas) of the shrimp *Fenneropenaeus indicus* that a marked increase in lipid peroxidation and depletion of antioxidant activities accompany WSSV infection (38). It is possible that at least some of these effects are mediated by transcriptional repression of genes involved in antioxidant responses, as suggested by the data presented here. Two genes with roles in Ca^{2+} -dependent protein folding and transport in the endoplasmic reticulum (ER) were also downregulated by WSSV infection, namely calreticulin and the delta subunit of the translo-

con-associated protein complex. The biological significance of these changes is difficult to envision from expression data alone, but a simple explanation is that protein folding and secretion may be compromised in WSSV-infected cells.

One prominent immune function gene, the shrimp homolog of STAT, was found to be downregulated by WSSV infection. The roles of STAT in the immune responses of *Drosophila* are well established (1), and recent evidence for the involvement of STAT-mediated transcriptional activation in the antiviral response of *Drosophila* has been reported (15). Interestingly, STAT DNA binding activity is inhibited in mosquito cells infected with Japanese encephalitis virus (34), perhaps suggesting targeted suppression of STAT-mediated signaling for the purpose of bypassing antiviral mechanisms. This hypothesis would be consistent with the decrease in STAT mRNA observed in WSSV-infected hepatopancreas.

In conclusion, molecular studies on immunity in Crustacea are hindered by the lack of permanent cell lines and of genomic, transcriptomic, and proteomic resources. The present study represents a step forward in this regard, by providing an

extensive catalogue of expressed genes from shrimp (including information regarding their tissue distribution) and by identifying a number of conserved genes that are likely involved in immune responses (Table 1). Furthermore, analyses of changes in gene expression associated with WSSV infection in hepatopancreas of shrimp suggests, broadly, the activation of antimicrobial responses together with the repression of antioxidant functions and of ER-dependent protein processing in infected animals. The EST sequences generated in the present study are available at the NCBI EST repository and integrated into the databasing and analysis platform hosted at <http://www.marinegenomics.org>. The microarray data are also publicly available at NCBI (Gene Expression Omnibus series GSE4949, GSE4954, and GSE4955) and at <http://www.marinegenomics.org>. These functional genomic resources should provide the bases for generating hypotheses to guide future research in crustacean host-virus interactions.

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For information regarding availability of the microarray, contact P. S. Gross at grossp@musc.edu.

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DISCLOSURES

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the supporting bodies mentioned herein.

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