Title of the Project: Use of cDNA microarray to isolate differentially expressed genes in White Spot Virus infected shrimp (*Penaeus stylirostris*).

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Project Abstract:

White spot syndrome virus (WSSV), the etiologic agent of white spot disease, is currently the most important viral pathogen infecting penaeid shrimp worldwide. Since the initial report, white spot disease has caused losses of catastrophic proportion to shrimp aquaculture globally. Although considerable progress has been made in characterizing the WSSV and developing detection methods, information on the host genes involved in the immune response in shrimp due to WSSV infection is not available. During this research, the mRNA expression profiles in healthy and WSSV-infected shrimp were determined by analyzing the expressed sequence tags (ESTs) and by microarray analysis. Our data show that WSSV infection alters the expression of a wide array of genes including those that are involved in immune function, signal transduction, structural genes, as well as mitochondrial genes among others. Using EST analysis and real-time RT-PCR, we also identified a candidate receptor gene for another viral pathogen of shrimp, the Taura syndrome virus (TSV). These data show that the potential for using the mRNA expression level of candidate genes as biomarkers for identifying virus-resistant or virus-susceptible lines in shrimp.

Objectives:

The expressed sequence tags (ESTs) and cDNA microarray analyses were used to compare gene expression patterns in hepatopancreas tissues of healthy and WSSV-infected wild shrimp. The hepatopancreas in shrimp is involved in initiating the humoral defense response. Therefore, identification of cellular genes in these tissues, whose expression is altered upon WSSV infection, will help to elucidate the pathways that are critical for WSSV pathogenesis.

The objectives of the project were:

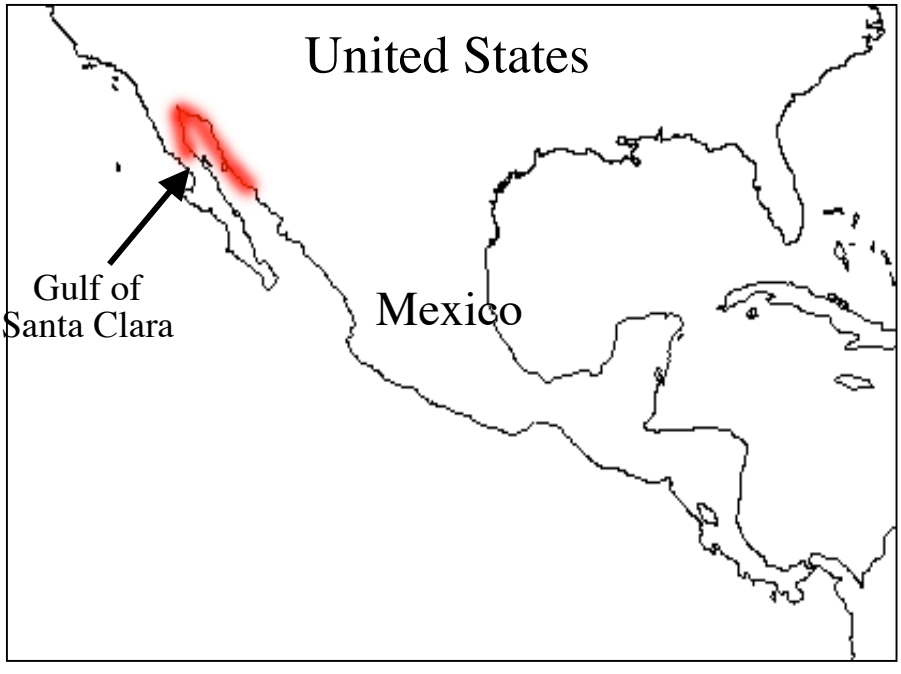
1. Determine the prevalence of the four most important viral pathogens in shrimp collected from the Gulf of California.
2. Isolate expressed sequence tags (ESTs) from hepatopancreas library of *Penaeus stylirostris* shrimp.
3. Compare gene expression profiles in hepatopancreas of healthy and WSSV-infected *P. stylirostris* shrimp and identify differentially expressed genes.

Methods and Results:

Collection of wild shrimp

Wild penaeid shrimp were collected from the Gulf of Santa Clara, located in the Gulf of California, Sonora, Mexico (31° 65' 00'' N and 114° 58' 33'' W) (Fig. 1), in September 2003 and November 2004. During the first year, 66 *P. stylirostris* samples were collected. Twelve out of 66 samples were live animals, while the remaining 54 animals were frozen soon after collection from the ocean and stored at -80ºC. For the live animals, hemolymph was drawn from the ventral sinuses of each shrimp using a 1 mL tuberculin syringe containing 5% sterile sodium citrate as anticoagulant. After hemolymph collection, these animals were kept frozen in dry ice. During the year 2004, 60 samples were collected and all samples were frozen soon after collection from the ocean and stored at -80ºC.

Figure 1. Geographical map of the Gulf of Santa Clara located in the Gulf of California showing location of shrimp samples collection.



Detection of IHHNV, WSSV, TSV and YHV by real-time PCR in wild shrimp

Total genomic DNA was extracted from the tail muscle of *P. stylirostris* shrimp following the DNAzol™ protocol (Molecular Research Center Inc., Ohio) while total RNA was isolated from the tail muscle following the TRI Reagent protocol (MRC, Inc.). DNA was used for the detection of WSV and IHHNV, whereas RNA was used for the detection of TSV and YHV.

The detection of IHHNV, and WSSV was done following a previously published protocol (Dhar *et al.*, 2001). The protocol for the detection of YHV and TSV was the same as described by Dhar *et al.* (2002) and Mouillesseaux*et al.* (2003). The primer sequences used for the detection of IHHNV, WSSV, TSV, and YHV are given in Table 1.

Table 1. List of primers used for the detection of IHHNV, WSSV, TSV and YHV by real-time PCR using SYBR Green chemistry.

Gene	Primer	Primer Sequence (5'-3')	GC%	Amplicon size (bp)
IHHNV	313F	For: AGGAGACAACCGACGACATCA	52	50
	363R	Rev: CGATTTCCATTGCTTCCATGA	42	
	610F	For: TCTGTCACCGGTTCGCATT	51	94
	703R	Rev: TCCCCAACTTGTGACCGTACA	54	
WSSV	110F	For: GATAAGAGAGGTAGACACTAGTAGTGTTATTG CT	38	55
	165R	Rev: CCACTGTGCCAGCTATTGCA	55	
	470F	For: GCAGG AAACATTAAGGGAAATACTAT	53	100
	570R	Rev: TTGCTGCACACGTCAATGAG	52	
TSV	004F	For: ATGAGAGCTTGGTCCTGGACTTC	52	78
	081R	Rev: CCCAATCACTAATCAGAATGTAGTGC	42	
	112F	For: CTGTTTGTAACACTACCTCCTGGAATT	40	88
	199R	Rev: AATTAATCCCTGCTAACCCAGTTG		
YHV	912F	For: TCAATGAGTTCAATGACGTCGAA	39	50
	962R	Rev: GAATGGTATCACCGTTCAGTGTCTT	44	
	399F	For: ATCGGGACAGGAGCAGACA	58	98
	496R	Rev: GTAACCCCGGCCATGACTT	58	
□-actin	178F	For: GGTCGGTATGGGTCAGAAGGA	57	50
	228R	Rev: TTGCTTGGGCCTCATCAC	55	
EF-1□	123F	For: TCGCCGAACTGCTGACCAAGA	57	55
	123R	Rev: CCGGCTTCCAGTTCCTTACC	60	

A summary of the prevalence of these four viruses in wild *P. stylirostris* shrimp collected from the Gulf of Santa Clara in the Gulf of Mexico is given in Table 2. IHHNV prevalence was found to be 100% during both 2003 and 2004, whereas YHV was not detected in any of these samples in any year. The prevalence of WSSV and TSV varied between 2003 and 2004. The prevalence of WSSV was reduced from 46% to 9% from 2003 to 2004, whereas TSV prevalence increased from 6% to 21% during the same period (Table 2). Some samples had a dual infection of IHHNV + WSSV or IHHNV + TSV while others carried infection by all three viruses (IHHNV + WSSV + TSV).

Table 2. Prevalence of IHHNV, WSSV, TSV, and YHV in wild shrimp samples collected from the Gulf of Santa Clara, located in the Gulf of California, Sonora, Mexico (31° 65' 00'' N and 114° 58' 33'' W) in September 2003 and November 2004.

Virus	Number of animals infected (%virus prevalence)	
	1st year (n=66)	2nd year (n=60)
IHHNV	66 (100%)	60(100%)
WSSV	46 (77.7%)	9 (15%)
TSV	6 (9.1%)	0 (0%)
YHV	0 (0%)	0 (0%)
IHHNV + WSSV	46 (77.7%)	9 (15%)
IHHNV + TSV	6 (9.1%)	0 (0%)

The amplification plots and the dissociation curves of IHHNV, WSSV, and β–actin (as an internal control gene) of a representative sample are shown in Fig. 2. Successful amplification was obtained for both IHHNV and WSSV indicating the presence of a dual infection by these viruses in this sample (Fig. 2, top left panel). The dissociation curves of IHHNV and WSSV amplicons are shown in the top right panel. The amplification plot and the dissociation curve for the internal control gene, β–actin, is shown in the bottom panel. The amplification plots and the dissociation curves of TSV and EF-1□, the internal control gene, for a representative sample are shown in Fig. 3.

Figure 2. Amplification plots and dissociation curves of IHHNV (primers used: 610F and 703R, see Table 1), WSSV (110F+165R, see Table 1) and β–actin (primers used: 178F and 228R, see Table 1) genes from a wild *P. stylirostris* shrimp collected from the Gulf of Santa Clara in the Gulf of Mexico. For each gene, the left hand panel shows the amplification plots and the right hand panel shows the dissociation curves. The melting temperature (T_m) of the amplicon is indicated in the dissociation curves.

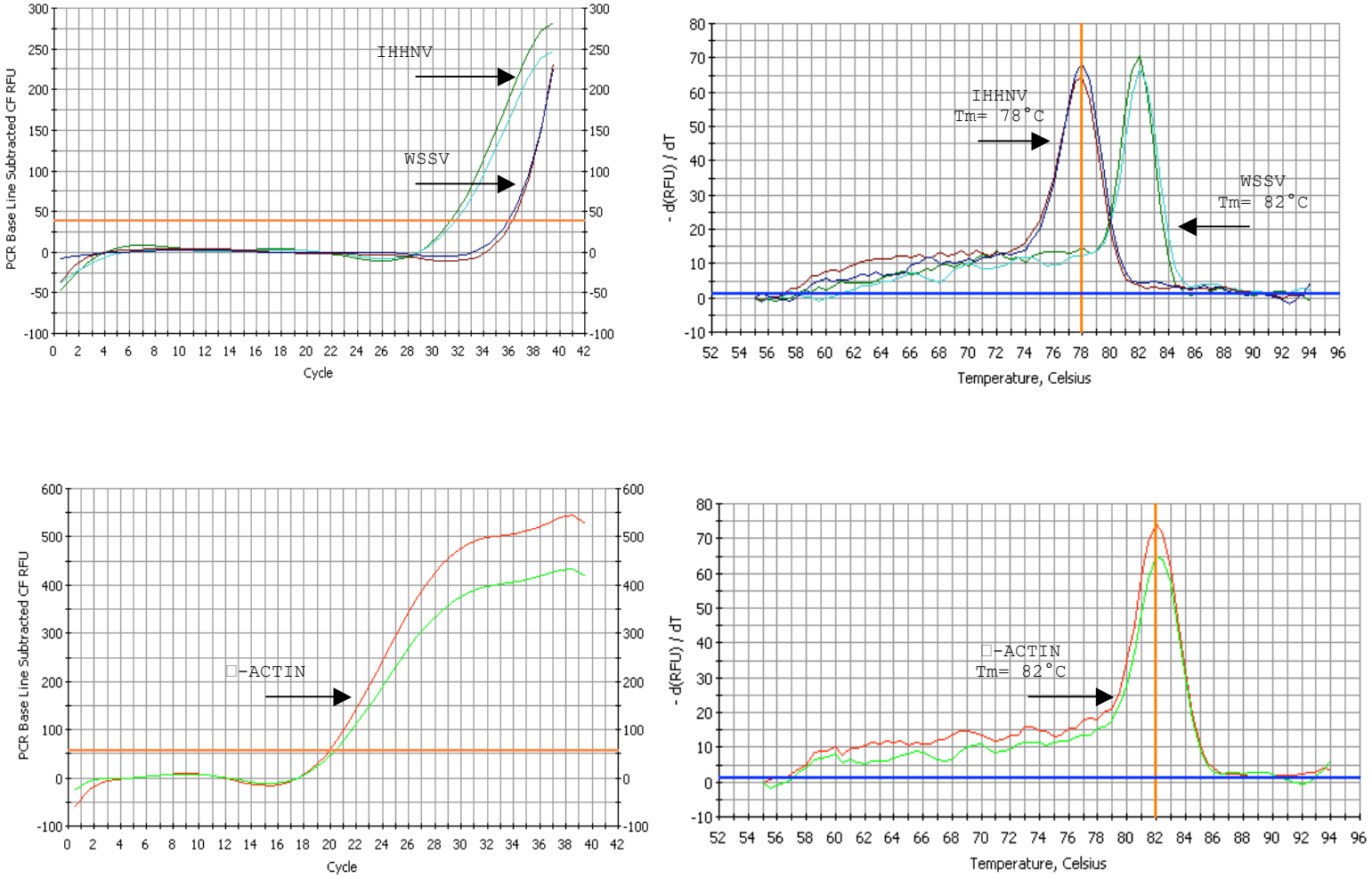
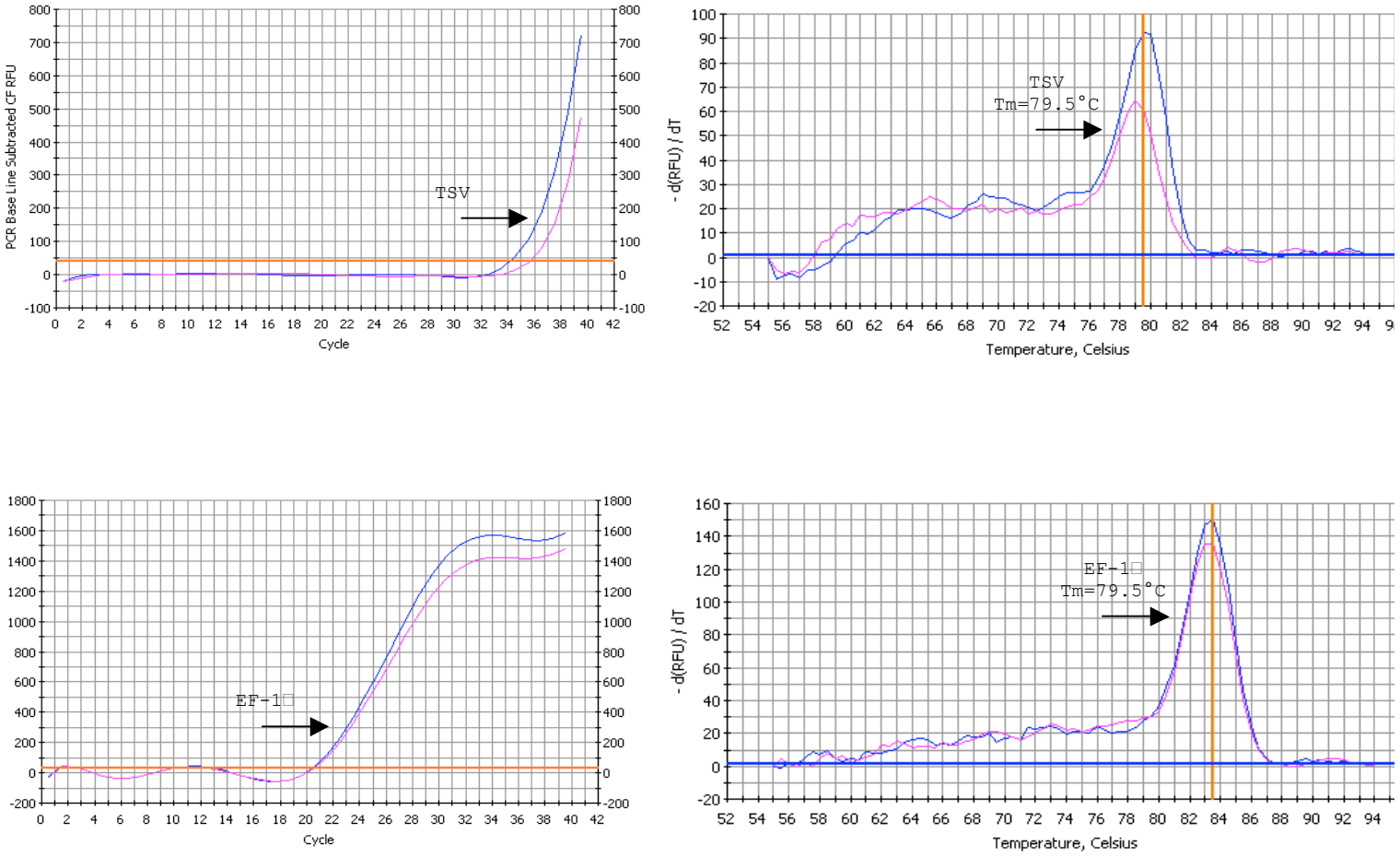


Figure 3. Amplification plots and dissociation curves of TSV (primers used: 112F and 199R, see Table 1) and the internal control gene, EF-1α, (primers used: 123F and 123R, see Table 1) genes from a wild *P. stylirostris* shrimp collected from the Gulf of Santa Clara in the Gulf of Mexico. For each gene, the left hand panel shows the amplification plots and the right hand panel shows the dissociation curves. The melting temperature (T_m) of the amplicon is indicated in the dissociation curves.

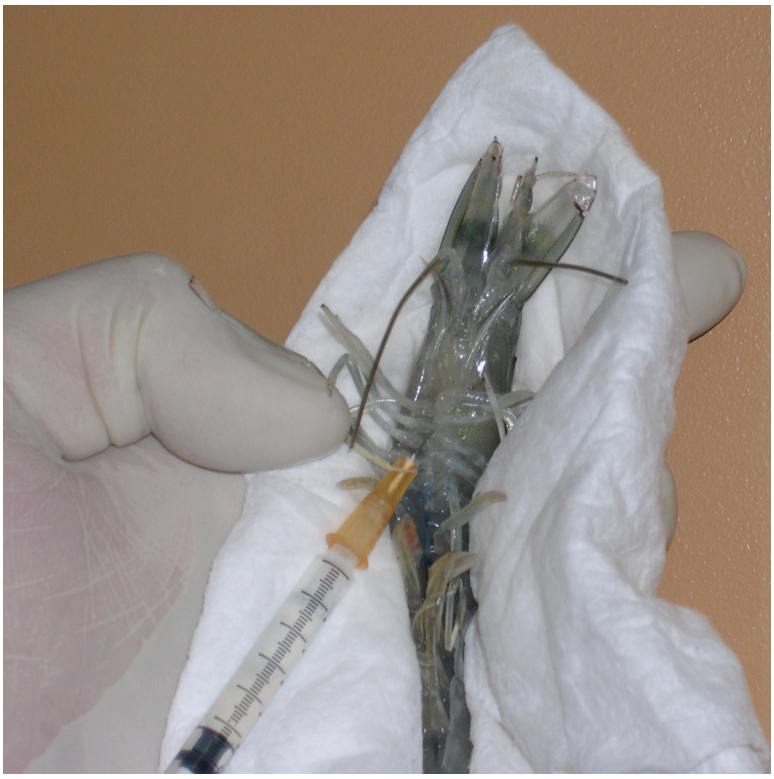


Laboratory challenge of Specific Pathogen Free (SPF) *P. stylirostris* shrimp with WSSV

In order to isolate expressed sequence tags (ESTs) from *P. stylirostris* shrimp, cDNA libraries were constructed using laboratory-challenged shrimp. Two cDNA libraries were constructed: (1) hepatopancreas cDNA library (not normalized) from a WSSV-infected shrimp, and (2) hepatopancreas suppression subtractive hybridization (SSH) library (reciprocal subtraction) using healthy and WSSV-infected shrimp. For the first library, *P. stylirostris* shrimp from Super Shrimp Inc. were used, and for the SSH library, Specific Pathogen Free (SPF) *P. stylirostris* shrimp (average wt. 15 g) were purchased from Dr. James Wyban, High Health Aquaculture Inc., Hawaii.

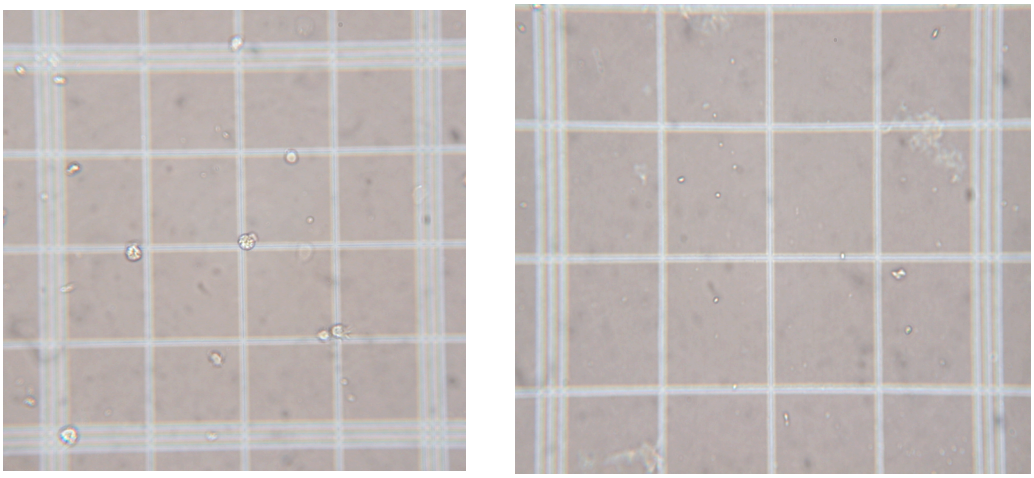
SPF shrimp from High Health Aquaculture were challenged with WSSV in the laboratory by feeding the animals WSSV-infected shrimp tail tissue at 10% of the biomass. Control animals were fed healthy shrimp tissue at the same rate. Hemolymph samples were drawn from WSSV-challenged and control animals (Fig. 4) at 0, 6, 12, 24, and 48 h post-challenge before freezing the animal in liquid nitrogen.

Figure 4. Drawing of hemolymph from *Penaeus stylirostris* shrimp challenged with WSSV in the laboratory.



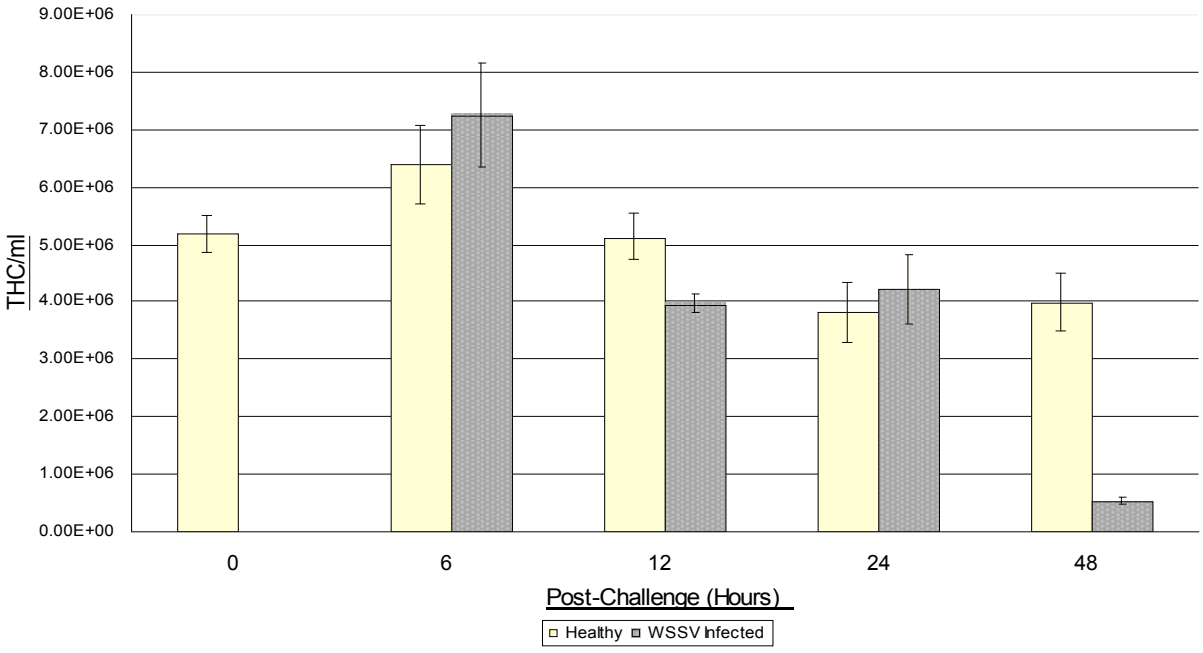
There were 6 animals for the WSSV-challenged and 5 animals for the control treatment for each time point, thus a total of 49 animals. Total hemocyte count (THC) was determined for each animal. A representative figure, showing the hemocytes from a healthy and a WSSV-infected shrimp at 48 h post-challenge, is shown in Figure 5.

Figure 5. Hemocytes from a healthy (Left panel) and a WSSV-infected (Right panel) *Penaeus stylirostris* shrimp.



The THC in the healthy and the WSSV-infected animal at different time points after WSSV challenge is shown in Figure 6. Our data showed that 6 h post-challenge there is an increase in the THC in both WSVV-challenged and control animals. At 12 and 24 h post-challenge, the THC was less compared to 6 h for both treatments. By 48 h post-challenge the THC was dramatically reduced in the WSSV-challenged animals but, in the control animals, THC did not show any significant change compared to 24 h.

Fig. 6. Total hemocyte count (THC) in the healthy and WSSV-challenged animals at different time points after the virus challenge.



Detection of WSSV in the laboratory challenged SPF *P. stylirostris* shrimp by real-time PCR

Total genomic DNA was extracted from the tail muscle of each laboratory challenged SPF *P. stylirostris* shrimp following the DNAzol protocol . The real-time PCR for determining the viral load was performed following a previously published protocol (Dhar *et al.*, 2001). WSSV was detected in the samples from all time points. The viral load was lowest at 6 h and highest at 48 h post challenge, indicating that as time progressed the WSSV load increased significantly. The increase in the WSSV load coincided with the reduction of the THC in the hemolymph.

Construction of a cDNA library (not normalized or subtracted) and isolation of expressed sequence tags (ESTs)

A cDNA library was constructed from hepatopancreas mRNA of WSSV-infected *Penaeus stylirostris* shrimp in Uni-Zap XR vector using the ZAP-cDNA® Synthesis kit, and ZAP-cDNA® Gigapack® III Gold Cloning Kit (Stratagene, La Jolla, California). One hundred and twenty one recombinant clones containing inserts >350 bp were sequenced in an automated DNA sequencer (Model ABI 373A) using the *Taq* DyeDeoxy terminator cycle sequencing kit and T3 primer (Applied Biosystems, Foster City, California). Similarity searches were performed by comparing the shrimp EST sequences to those in the GenBank database using BLASTX and BLASTP search protocols (http://blast.genome.ad.jp). Multiple alignments were performed using ClustalW Multiple Alignment program (http://searchlauncher.bcm.tmc.edu/) and BOXSHADE. Of the 121 ESTs, 99 (81.8%) showed similarity with the database entries, whereas the remaining 22 (18.2%) showed no similarity. The ESTs were categorized into 6 groups that include ESTs with homology to receptors or immune function genes, enzymes or endocrine system, structural genes, mitochondrial genes, genes with unknown function or showing no similarity with GenBank entries, and WSSV encoded genes (ORF 9, van Hulten *et al.*, 2001) (Table 3). These ESTs have been deposited in the GenBank database.

Table 3. List of ESTs isolated from a hepatopancreas cDNA library of a white spot syndrome virus-infected *Penaeus stylirostris*.

Clone ID	Insert Size (bp)*	Homologous Gene	Species	Similarity (%)	Amino acid/ nucleotide overlap**	Probability	Frequency
Receptors or immune function genes							
Ps EST 85b	525	Endocytic receptor	Human	40%	48/117	1e-04	2
Ps EST 112	671	Fatty acid binding protein	Bovine	46%	60/129	1e-10	5
Ps EST 705	599	Fatty acid binding protein	Rat				1
Ps EST 117	681	Langerin, C-type lectin	Human	40%	48/118	4e-06	2
Ps EST 640	7776	Macrophage lectin 2	Human				1
Ps EST 160	860	Low density lipoprotein receptor	Human	51%	101/194	1e-36	1
Ps EST 257	863	Macrophage mannose receptor	Human	46%	77/169	8e-07	2
Ps EST 289	808	Lipopolysaccharide and β-1, 3 glucan binding protein	Crayfish	83%	164/195	8e-84	1
Ps EST 983	513	PTH-responsive osteosarcoma D1 protein	Human				1
Enzymes/ endocrine system							
Ps EST 288a	393	Serine protease	Shrimp	98%	82/91	2e-45	6
Ps EST 110	575	Cathepsin L-like cystein proteinase	Shrimp	99%	135/ 135	5e-76	2
Ps EST 146	601	Trypsin protease	Shrimp	77%	82/105	5e-39	1
Ps EST 672	812	Trypsin protease	Shrimp				1
Ps EST 454	850	Trypsin protease	Shrimp				1
Ps EST 120	989	Adenosine kinase	Arabidopsis	69%	162/229	9e-68	1
PS EST 484	814	Catalase	Campylobacter jejuni				1
Ps EST 213	1178	Methionine adenosyltransferase	Drosophila	84%	179/211	2e-89	1
Ps EST 229	342	Zinc proteinase	Crayfish	72%	70/96	3e-29	1
Ps EST 246	466	Dehydrogenase/ reductase	Mouse	55%	43/77	5e-05	1

Ps EST 171	946	GTP binding protein	Drosophila	80%	171/211	6e-81	1
Ps EST 249	439	Diazepam binding inhibitor	Frog	84%	57/67	4e-24	1
Ps EST 255	912	Adenosyl homocysteinase	Xenopus	89%	177/198	6e-91	1
Ps EST 258	1175	Biphenyl hydrotase	Human	67%	123/180	1e-46	1
Structural genes							
Ps EST 710	1038	Hemocyanin	Shrimp				8
Ps EST 247	605	Crustacyanin A2 subunit	Lobster	80%	141/173	6e-69	1
Ps EST 137	792	16S Ribosomal RNA	Shrimp	97%	763/786**	-	4
Ps EST 185a	576	18S Ribosomal RNA	Shrimp	95%	469/489**	-	1
Ps EST 172a	306	40S ribosomal protein	Rat	68%	57/83	2e-20	1
Ps EST 279	1007	40S Ribosomal protein	Catfish	70%	229/326	e-100	1
Ps EST 701	665	40S Ribosomal Protein S 10	Catfish				1
Ps EST 103	612	60S Ribosomal protein	Rat	56%	109/192	9e-34	2
Ps EST 715	960	40S Ribosomal protein S17	Gallus gallus	70%	75/106	2e-30	3
Ps EST 689	885	Ribosomal protein S21	Fruit fly				1
Ps EST 175c	372	Ribosomal protein S25	Fall armyworm	67%	78/114	5e-33	1
Ps EST 727	700	Ribosomal protein S26	Rattus norvegivus	86%	97/112	7e-46	2
Ps EST 486	885	40S Ribosomal protein S26-2	Ictalurus punctatus	90%	92/101	1e-44	1
Ps EST 411	1092	Ribosomal protein	Homo sapiens	43%	33/76	8e-5	1
Ps EST 450	831	Ribosomal protein L31	Ictalurus punctatus	49%	58/116	1e-14	1
Ps EST 450	821	Ribosomal protein L31	Ictalurus punctatus				1
Ps EST 690	880	60 S Ribosomal Protein P2	Cryptochiton stelleri				1
Ps EST 994	825	Developmental embryonic B protein	Fruit fly				1

Ps EST 1025	791	LD47508P protein	Fruit fly				1
Ps EST 293	579	Mucin protein	Human	36%	55/148	2e-05	1
Ps EST 346	1003	EP 37-A1	Cynops pyrrhogaster				1
Ps EST 367	950	ZK 1127.9A.P	C. elegans				1
Ps EST 659	940	Collagen alpha 1	Gallus gallus				1
Ps EST 645	1119	Actin 2	Penaeus monodon				1
Ps EST 617	1131	CG7433	Fruit fly				1
Ps EST 328	1080	SCO-spondin glycoprotein	Cattle				1
Ps EST 992	876	TC0130 Protein	Chlamydia				1
Ps EST 953	944	GMFP5	Glycine max				1
Mitochondrial genes							
Ps EST 109	679	Cytochrome c	Shrimp	94%	200/211	1e-105	5
Ps EST 684	602	Cytochrome c oxidase subunit 1	Shrimp				1
Ps EST 920	870	Cytochrome c oxidase subunit 1	Shrimp				1
Ps EST 686	914	Cytochromo c oxidase subunit 3	Shrimp				1
Ps EST 118	551	Cytochrome b	Shrimp	95%	176/183	2e-93	1
Ps EST 123	815	ATPase	Shrimp	84%	174/203	5e-81	1
Ps EST 344	1005	ATPase subunit 6	F. notialis				1
Ps EST 174	573	Cyt. C oxidase, subunit VIb	Yeast	70%	54/76	6e-21	1
Ps EST 244	526	ATP synthetase	Rat	66%	58/86	2e-16	1
Ps EST 251	649	COA dehydrogenase	*C. elegans*	74%	78/103	5e-33	1
PS EST 158	1164	Hypothetical 18k protein.	Goldfish	56%	36/63	8e-05	5
Ps EST 271	496	Mitochondrial protein	Mouse	69%	89/128	2e-31	1
Genes with unknown function or showing no similarity with GenBank entries							
Ps EST 114	1128	Unknown	Drosophila	40%	74/178	6e-10	1
Ps EST 180b	582	Unknown	Shrimp	89%	230/258**	1e-69	1
Ps EST 126	359	No similarity	-	-	-	-	2
Ps EST 140a	339	No similarity	-	-	-	-	1

Ps EST 156d	359	No similarity	-	-	-	-	1
Ps EST 161b	547	No similarity	-	-	-	-	1
Ps EST 162a	388	No similarity	-	-	-	-	1
Ps EST 165b	292	No similarity	-	-	-	-	1
Ps EST 173c	378	No similarity	-	-	-	-	1
Ps EST 194	425	No similarity	-	-	-	-	1
Ps EST 203	625	No similarity	-	-	-	-	1
Ps EST 259	705	No similarity	-	-	-	-	1
Ps EST 287	1438	No similarity	-	-	-	-	1
Ps EST 353	1695	No similarity	-	-	-	-	1
Ps EST 376	1200	No similarity	-	-	-	-	1
Ps EST 474	1151	No similarity	-	-	-	-	1
Ps EST 481	832	No similarity	-	-	-	-	1
Ps EST 546	1440	No similarity	-	-	-	-	1
Ps EST 620	1196	No similarity	-	-	-	-	1
Ps EST 873	913	No similarity	-	-	-	-	1
Ps EST 979	233	No similarity	-	-	-	-	1
Ps EST 1068	1160	No similarity	-	-	-	-	1
White spot virus gene							
Ps EST 108	1188	ORF 9 White Spot Virus	WSV	83%	271/322	e-142	1

*When more than one clone showed similarity with the same gene, the insert size of the largest clone was indicated. The largest clone was also taken for GenBank database similarity search.

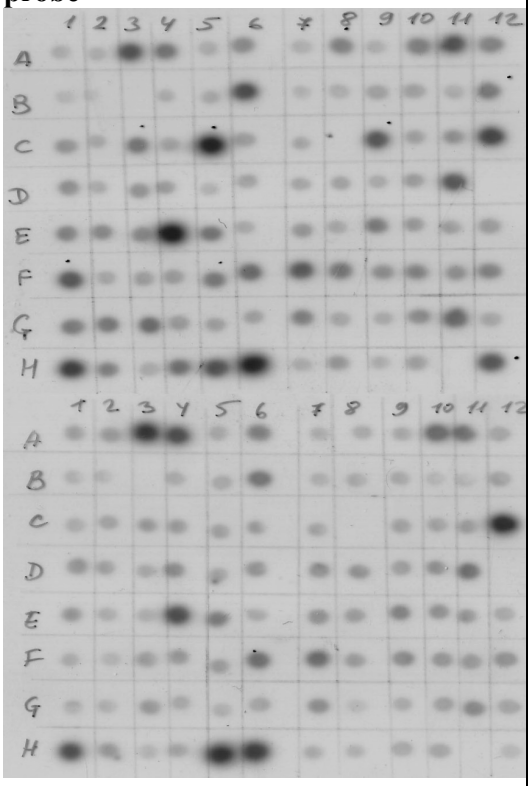
**Clones that showed similarity to the non-coding region

Construction of SSH cDNA libraries using healthy and WSSV-infected shrimp

Reciprocal subtractive hybridizations were performed using healthy and WSSV-infected shrimp (12 h post challenge) following published methods (Diatchenko et al., 1996, 1999). Two subtracted cDNA samples enriched with differentially expressed sequences (healthy shrimp-specific and WSSV-infected shrimp-specific) were used for library construction. The cDNAs were cloned into a plasmid vector, pAL9 vector (Evrogen, Inc.) and transformed into *E. coli*. The titer of each library was $1x10^6$ CFU/μl. The quality of the subtracted libraries were evaluated by differential screening of randomly picked 96 clones from each library by hybridizing with [P^{32}]-labeled subtracted healthy and subtracted WSSV-infected cDNA probes. Differential screening yielded 15 healthy-specific and 26 WSSV-specific clones (Fig. 7). The differential expressions of the candidate healthy- and virus-specific genes were validated by virtual Northern blot analysis (Fig. 8).

Figure 7. Dot blots showing differential screening results of clones randomly picked from subtracted libraries of healthy (Panel A) and WSSV-infected (Panel B) shrimp (*Penaeus stylirostris)*. Ninety-six clones from healthy (tester-specific) subtracted library and ninety-six clones from WSSV-infected (driver-specific) subtracted library were subjected to differential screening using tester-specific and driver-specific subtracted probes.

Panel A: Healthy-specific (Tester) probe



Panel B: WSSV-Specific (Driver) Probe

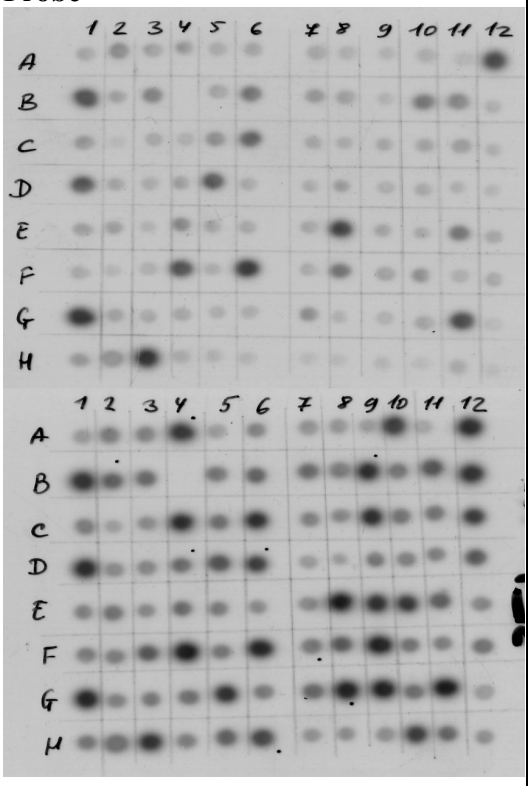
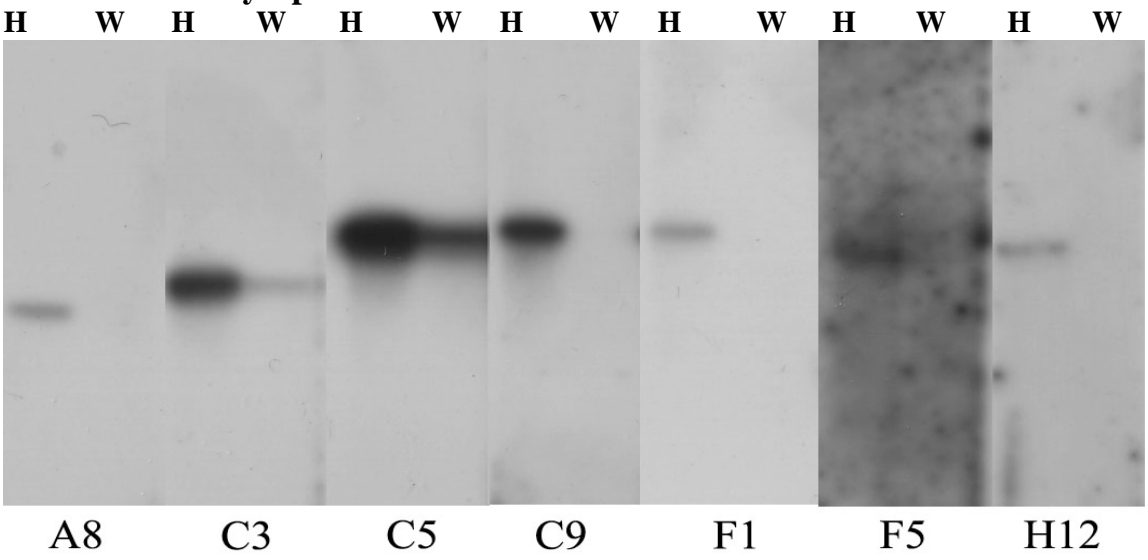
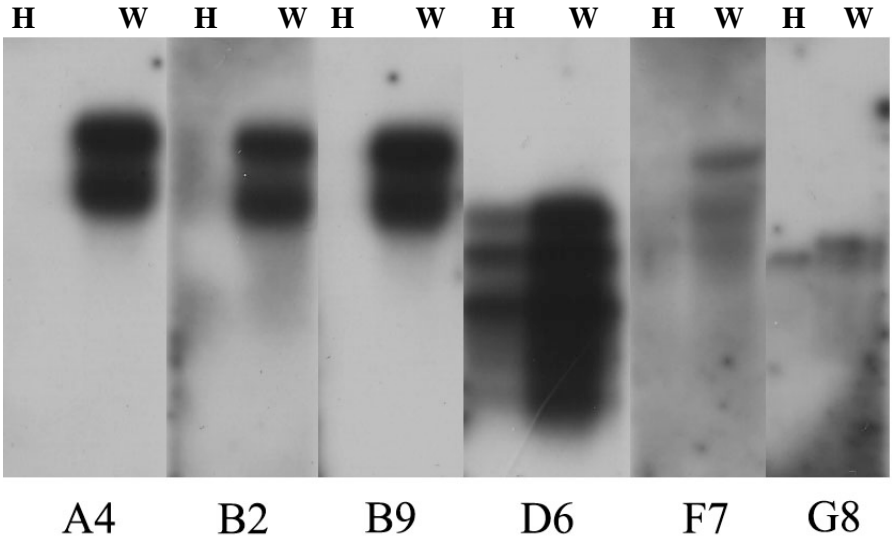


Figure 8. Virtual Northern blot analysis of differential clones obtained from healthy (H, Panel A) and WSSV- (W, Panel B) subtracted libraries. Panel A shows the healthy-specific clones (A8, C3, C5, □9, F1, F5, H12) and Panel B shows the WSSV-specific clones (A4, B2, B9, F7, G8, D6).

Panel A. Healthy-Specific Genes



Panel B. WSSV-Specific Genes



Isolation of expressed sequence tags (ESTs) from subtracted cDNA libraries of shrimp hepatopancreas

A total of 479 EST clones from healthy-specific and 479 clones from WSSV-specific library were sequenced. Vector sequences were trimmed from these sequences before assembling into contigs and singletons. A summary of the EST clones isolated from subtractive libraries is presented in Table 4.

Table 4. Summary of cDNA clones isolated from subtractive libraries of healthy and white spot syndrome virus (WSSV)-infected shrimp.

	Healthy	Infected
Number of cDNA clones sequenced	479	479
Number of clones taken for analysis (Phred>20)	479	479
Average EST sequence length	972	696
Number of contigs*	43	57
Number of unassembled clones/ singletons	42	64
Number of unigenes	85	121

The ESTs were annotated based on similarity with the database entries and categorized into receptors or immune function genes, enzymes or endocrine system, structural genes, mitochondrial genes, and genes with unknown function or showing no similarity with GenBank entries.

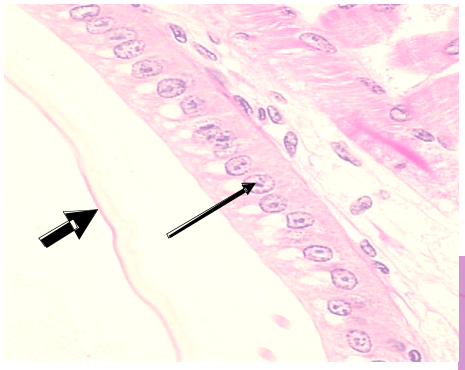
Measuring the expression of low density lipoprotein receptor (LDLr) gene in TSV-susceptible and TSV-resistant shrimp

During the isolation of ESTs from a hepatopancreas cDNA library of *P. stylirostris* shrimp, we isolated a cDNA clone that showed similarity with the low-density lipoprotein receptor (LDL_r) gene of human, mouse, *Drosophila,* and *Caenorhabditis elegans* (see Table 3). The LDL_r gene is a member of an evolutionarily conserved family of multifunctional receptors that bind to rhinoviruses (Family *Picornaviridae*) and a variety of other ligands. Upon binding to the ligands, LDL_r transports the macromolecules through receptor-mediated endocytosis. Although the overall goal of this project was to isolate differentially expressed genes during WSSV infection, we were curious to examine if the LDL_r expression in shrimp is modulated by Taura syndrome virus (TSV) infection since TSV is closely related to LDLr. TSV causes Taura syndrome (TS) disease in shrimp. Taura syndrome disease is an OIE notifiable disease of penaeid shrimp that continues to pose a threat to shrimp mariculture in both Western and Eastern hemispheres. Although considerable progress has been made in elucidating the organization of the TSV genome and developing TSV-specific diagnostic methods, information on shrimp cellular genes involved in TSV pathogenesis and cellular immunity remains unknown.

Two separate strains of *P. vannamei* juveniles (one TSV-resistant (SPR) and one Fast Growth (FG) TSV-susceptible line) were *per os* exposed to TSV (Texas 2004 TSV strain). Moribund acutely infected shrimp, surviving chronically infected shrimp, and unexposed negative control shrimp were preserved in Davidson's (AFA) for histological analysis. Mild to moderate multifocal pathodiagnostic acute phase epithelial necrosis was detected in moribund FG shrimp, which suffered 20-36% mortality. Moderate to severe lymphoid organ spheroids were detected in chronically infected FG survivors. No mortality or acute TSV lesions were detected among the TSV-exposed SPR groups (Fig. 10).

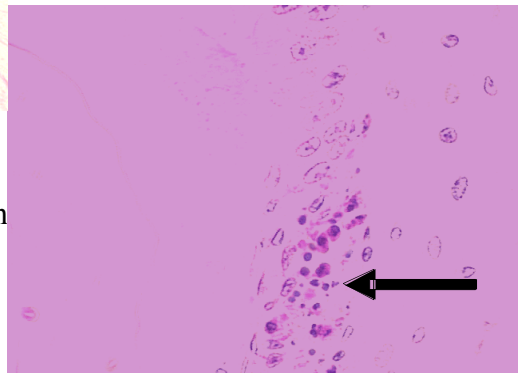
Figure 10. Histopathology of a healthy and acute TSV-infected (Panel A) and chronic TSV-infected (Panel B) *Penaeus vannamei* shrimp (FG-line).

A.

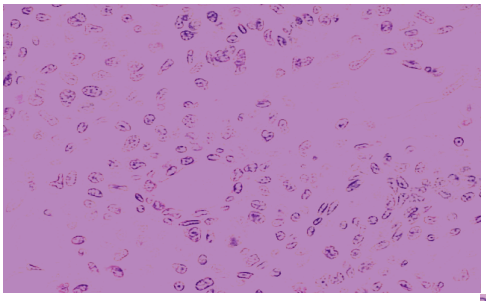


FG *P. vannamei* juvenile. Normal stomach cuticular epithelium (thin arrow) and cuticle (thick arrow). H&E, 600X

FG *P. vannamei* juvenile. **Acute phase TSV infection (arrow)** with the stomach cuticular epithelium. Nuclear pyknosis, karyorrhexis & cytoplasmic eosinophilia are evident. H&E, 600X

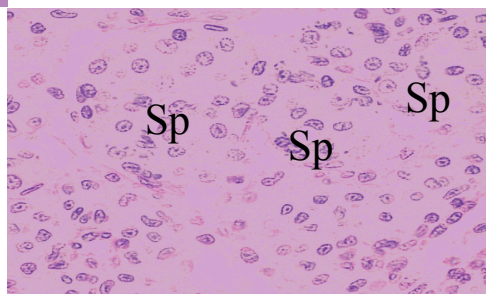


B.



FG *P. vannamei* juvenile. Normal LO showing a prominent arteriole with centralized lumen. H&E, 600X

FG *P. vannamei* juvenile with a **chronic TSV infection** w/i the lymphoid organ (LO). **LO** displays prominent **spheroids (Sp)** . H&E, 600X



We measured the TSV load and the expression of the LDL_r gene in both healthy and TSV-infected (acute and chronically infected) shrimp by real-time RT-PCR (Fig. 9). The sequences of the primers used to measuring the LDLr expression and the expression of the

internal control gene, EF-1α is given in Table 5. The cycle threshold values of the target genes (LDLr) were normalized with respect to the internal control gene, EF-1α and expressed as delta Ct (ΔCt). The fold changes in LDLr expression was measured as $2^{\Delta Ct}$.

LDL_r mRNA expression was almost 4-fold higher in the healthy TSV-resistant SPR shrimp compared to the healthy FG TSV-susceptible line. In the SPR animals, LDL_r expression increased upon TSV challenge (3.3 to 6.6-fold higher expressions depending on the TSV load). In the FG TSV-susceptible acute phase animals, there was no increase in the LDL_r expression. However, in the FG TSV-susceptible chronic phase animals, LDL_r expression was 5-fold lower compared to the healthy control animals (Table 6).

Table 5. List of primers used to measuring the mRNA levels of immune-related genes in shrimp by real-time RT-PCR.

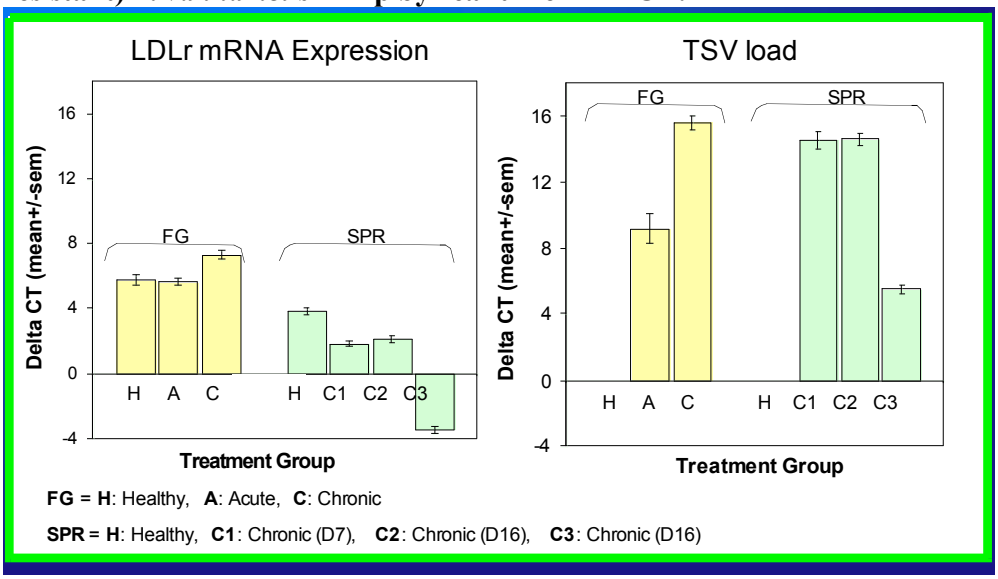
Gene	Primer	Primer Sequence (5'-3')	GC%	Amplicon size (bp)
LDLr	PvH005F	For: CATCTCGCTGAGTACCGCTAC	55	95
	PvH005R	Rev: TGACGCTTTACATTCCCACAGA	45	
Chitinase	366F	For: ACTACCTGTGCTCGCTCAACAC	55	105
	470R	Rev: AAGCCCAATCGCAGTAGTAGCT	50	
Ras	54F	For: AGGTACGCGGGACAGCC	71	60
	113R	Rev: CTCAGGTCGAGGACTTCGATG	57	
Proteinase	94F	For: GACTCCAACGGCTGCATCTAC	57	52
	145R	Rev: CGTGCATGAGCTCGTGGAT	58	
Internal control (EF-1α)	123F	For: TCGCCGAACTGCTGACCAAGA	57	55
	123R	Rev: CCGGCTTCCAGTTCCTTACC	60	

Table 6. Comparison of LDL_r gene expression in FG (TSV-susceptible) and SPR (TSV-resistant) lines of shrimp.

Comparison groups	Fold changes in LDLr expression*
Healthy FG vs. Healthy SPR	↑3.7-fold
Healthy FG vs. FG acute phase (days 3-8 post challenge)	↑1.1-fold
Healthy FG vs. chronic phase (day 16 post-challenge)	↓5.3-fold
FG acute vs. FG chronic phase	↓5.0-fold
Healthy SPR vs. SPR chronic (day 7 post-challenge)	↑4.1-fold
Healthy SPR vs. SPR chronic (day 16 post-challenge)	↑3.3 to 6.6-fold

*'↑' indicates up-regulation of the LDLr expression and '↓' indicates the down-regulation of LDLr expression.

Figure 9. Measuring the LDL_r expression and TSV load in FG-(TSV-susceptible) and SPR (TSV-resistant) *P. vannamei* shrimp by real-time RT-PCR.

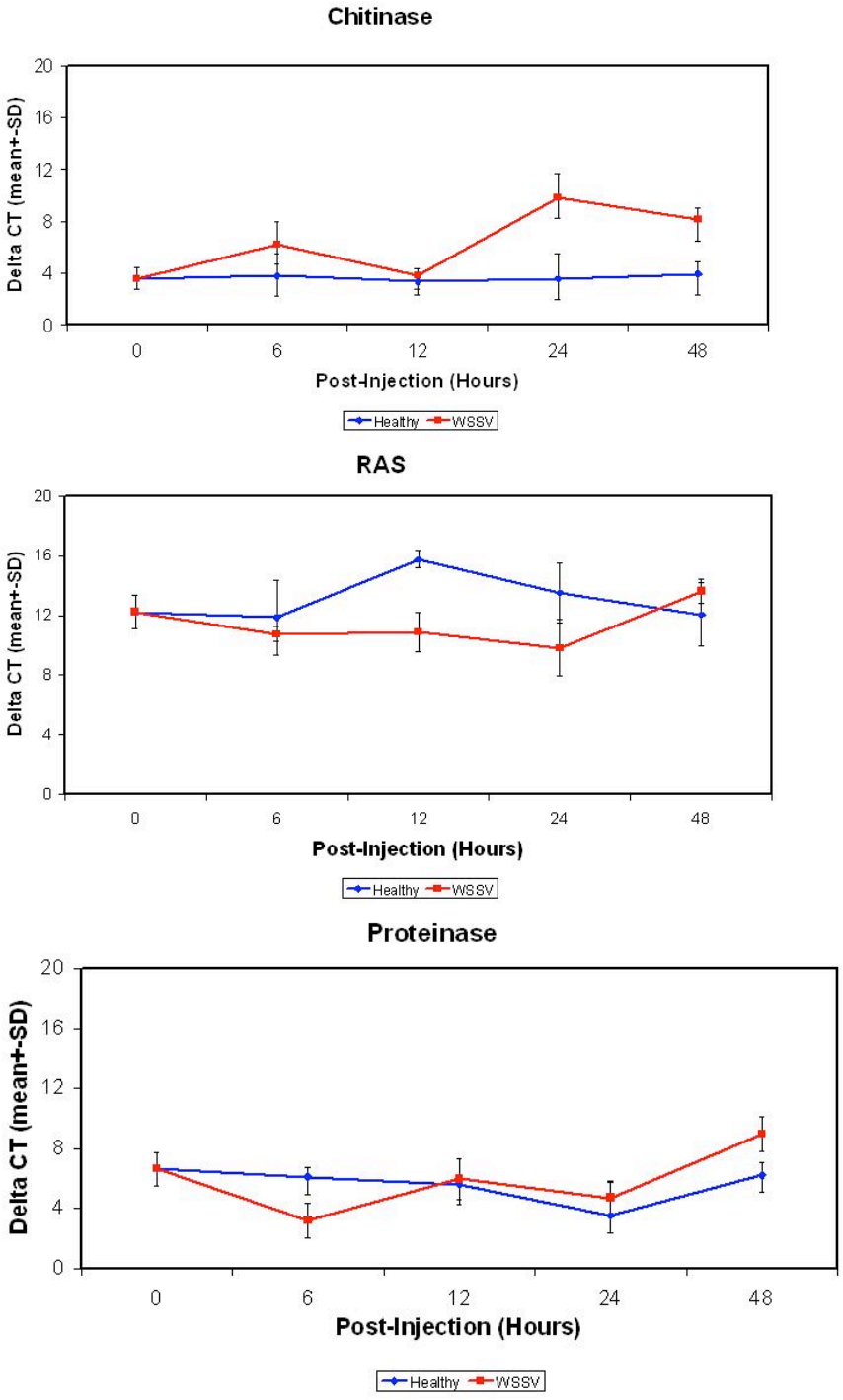


These data indicate that LDL_r expression is differentially modulated in the TSV-resistant and susceptible animals. To our knowledge, this is the first report of a shrimp cellular gene involved in TSV pathogenesis, and opens up a possibility of using LDLr expression as a marker for identifying TSV-resistance in shrimp. However, it is unknown if LDLr gene is in involved in WSSV pathogenesis.

Measuring the temporal expression of immune-related genes (Chitinase, Ras and Proteinase) in healthy and WSSV-infected *P. stylirostris* shrimp by real-time RT-PCR.

We also measured the temporal expression of three immune-related genes (chitinase, Ras, and a proteinase) in healthy and WSSV-infected shrimp by real-time RT-PCR. These genes were isolated by SSH hybridization (Table 4). The real-time RT-PCR was performed uaing DNase treated total RNA from healthy and WSSV-infected *P. stylirostris* shrimp collected at 0, 6, 12, 24 and 48 hr post-injection. The sequences of the primers used for the real-time RT-PCR assay are given in Table 5. The cycle threshold values of the target genes (Chitinase, Ras and Proteinase) were normalized with respect to the internal control gene, EF-1α and expressed as delta Ct (ΔCt).

Figure 10. Measuring the temporal expression of immune-related genes (Chitinase, Ras and Proteinase) in healthy and WSSV-infected *P. stylirostris* shrimp by real-time RT-PCR.



Microarray Analysis using cDNA probes

Initially to evaluate the potential of microarray analysis in WSSV pathogenesis study, we printed a low-density array containing 100 elements (cDNA) was used for preliminary microarray analysis. This included 47 unique ESTs from Table 3 (GenBank accession numbers CD526659-

CD526706), 37 differentially expressed cDNAs previously identified by the mRNA differential display technique by Dhar and colleagues (Astrofsky *et al*., 2002; Dhar *et al*. 2001), and a panel of 16 controls elements. The details of the sample preparation, slide printing, and hybridizations were described in Dhar *et al*. (2003). Of the 84 unique cDNA clones analyzed, 15 gene sequences exhibited up- or down-regulation by greater than 2-fold, relative to the healthy control, at the 95% confidence level. Subtler, yet still statistically significant, differences in gene expression were noted in 9 other genes that exhibited up- or down-regulation of 1.3 to 1.8-fold at the 95% confidence level (Table 7). Although a limited set of elements was used, this study showed the potential of microarray analysis in identifying differentially expressed genes in WSSV pathogenesis in shrimp.

Table 7. List of differentially expressed genes in WSSV-infected shrimp (*P. stylirostris)* identified by cDNA microarray analysis.

Clone ID#	Gene	Induction Ratio (fold changes)
Up-regulated genes		
Ps EST 288a	Serine protease	+4.4
Ps EST 161b	Unknown	+4.3
Ps EST 114	Drosophila unknown gene product	+4.3
Ps EST 117	Langerin, C-type lectin	+3.8
Ps EST 257	Macrophage mannose receptor	+3.7
Ps EST 185a	18S ribosomal RNA	+3.3
Ps EST 160	Low density lipoprotein receptor	+3.0
Ps EST 289	Lipopolysaccharide and β-1, 3 glucan binding protein	+2.9
Ps EST 287	Unknown	+2.6
Ps EST 156d	Unknown	+2.2
Ps EST 203	Unknown	+2.2
Ps EST 279	40S ribosomal RNA	+1.6
DD 156*	Unknown	+1.5
Ps EST 173c	Unknown	+1.4
Ps EST 10d	Hemocyanin	+1.4
Down-regulated genes		
Ps EST 162	Unknown	-1.3
Ps EST 249	Diazepam binding inhibitor	-1.4
DD197	Unknown	-1.6
DD149	Unknown	-1.8
DD106	Unknown	-2.1
Ps EST 213	Methionine adenoslyltransferase	-2.4
Ps EST 247	Crustacyanin A2 subunit	-2.9
Ps EST 246	Dehydrogenase	-4.0

*DD = Differential display clone

Microarray analysis using oligonucleotide probe

Based on our initial success of microarray analysis, we decided to design a high-density oligonucleotide array containing over 10K elements to determine the expression of gene during WSSV pathogenesis.

Design of the array

High-density oligonucleotide arrays (60-mer) were custom made by Nimblegen Systems, Inc. using a proprietary Maskless Array Synthesizer (MAS) technology. *Penaeus stylirostris* EST

sequences from our work as well as EST sequences from other *Penaeus* spp. from the GenBank database were utilized for probe construction. The list contains ESTs from *P. stylirostris, P. vannamei, P. japonicus, P. setiferus, P. monodon, P.* schmitt and ESTs from crayfish. A total of 1:2 of 11271 shrimp genes/ests were printed onto the slide. Each gene/ EST had 9 probes and each probe was printed two times. The probe design includes random GC, truncated at 148 cycles and all probes were perfect match probes. These probes were printed on a glass slide using MAS technology.

In order to check the quality of the array several control genes were printed on the array. These control genes include include CPK5 (Uniformity 42 total), CPK6(calcium-dependent protein kinase isoform 6 (CPK6), identical to calmodulin-domain protein kinase CDPK isoform 6 (Arabidopsis thaliana) gi|1399275|gb|AAB03246; contains protein kinase domain, Pfam:PF00069; contains EF hand domain (calcium-binding EF-hand), Pfam:PF00036, INTERPRO:IPR002048) and randomers There were a total of 1605 control genes per array.

Hybridization and data analysis

Shrimp Chips were hybridized with biotinylated cRNA derived from a WSSV-time course study. Healthy and WSSV-infected shrimp were collected at 0, 6, 12, 24 and 48 hr, see "Laboratory challenge of Specific Pathogen Free (SPF) *P. stylirostris* shrimp with WSSV" section for details of the time course study). The double-stranded cDNA was synthesized using the Super Script Choice System (Invitrogen, Inc.) and pooled total RNA for each time course sample. For each pool, RNA from three animals was combined in equal concentration. The cDNA was transcribed *in vitro* using T7 RNA polymerase and biotinylated UTP was incorporated into the cRNA during *in vitro* transcription. Hybridizations were carried out in duplicate for each time point. Thus a total of 14 hybridizations were carried out (8 for healthy, 0h, 6h, 12h and 24 h) and six for the WSSV-infected (6h, 12h, and 24h) samples. In order to normalize the data set for each time point, fluorescent values were normalized to the median values for each timepoint and treatment (Table 8).

Table 8. Normalization of fluorescent values of shrimp oligonucleotide array.

Timepoint	Treatment	Mean	Median	Std Dev	Minimum	Maximum	Dynamic Range
Zero1	Healthy	28.84	11.89*	235.67	8.38	13278.55	4
Zero2	Healthy	28.52	11.89*	237.87	8.92	10642.70	4
Six1	Healthy	32.68	11.89*	326.60	7.79	23815.70	4
Six2	Healthy	31.56	11.89*	284.59	8.98	16650.23	4
Twelve1	Healthy	29.24	11.92*	215.01	8.44	9765.88	3
Twelve2	Healthy	28.77	11.91*	212.46	8.15	9902.71	3
Twenty-four1	Healthy	28.30	11.86*	224.81	8.63	15878.23	4
Twenty-four2	Healthy	27.15	11.86*	193.84	8.45	7641.29	3
Six1	Infected	28.91	11.90*	205.57	8.55	8957.95	3
Six2	Infected	28.42	11.89*	199.85	8.77	8898.38	3
Twelve1	Infected	27.86	11.90*	196.37	8.44	9703.09	3
Twelve2	Infected	27.08	11.88*	183.45	8.79	10721.52	4
Twenty-four1	Infected	26.99	11.88*	196.35	8.48	8402.23	3
Twenty-four2	Infected	26.89	11.88*	200.65	8.35	9799.07	3

The correlation coefficient of the fluorescent values for each slide of different time points (0, 6, 12, 24 hours) for each treatment (Healthy and Infected) were determined using SAS version 8.1. The correlation coefficient values of these treatments ranged from 0.96-0.82 (Table 9).

Table 9. Correlation of shrimp microarray data sets from WSSV-time course study.

Chip ID	Timepoint	Treatment	N	Correlation	P-value
43096	Zero hour	Healthy	2	0.95	<.0001
43292	Six hour	Healthy	2	0.96	<.0001
43304	Twelve hour	Healthy	2	0.96	<.0001
43132	Twenty-four hour	Healthy	2	0.95	<.0001
43295	Six hour	Infected	2	0.82	<.0001
47520	Twelve hour	Infected	2	0.84	<.0001
43098	Twenty-four hour	Infected	2	0.93	<.0001

Pearson's correlation analysis (K-means) was performed to determine the expression pattern of the healthy (where animals were fed healthy tissue) and WSSV-infected (where animals were fed WSSV-infected tissues) groups at different time point post-challenge. A total of 368 genes showed differential expression (≥2-fold change) at 6, 12 and 24 hrs post-challenge compared to 0 hr time point in both healthy and WSSV-infected shrimp. The mRNA expression patterns of these differentially expressed genes (as determined by K-means) showed 8 clusters (Fig. 11). In the healthy animals, out of 368 differentially expressed genes, 42 genes were down-regulated for the entire time course (at 6, 12 and 24 hr) compared to 0 hr; 207 genes were up-regulated and 119 genes showed variable regulation across the time course. In WSSV-infected animals, 45 genes were down-regulated in for the entire time course (at 6, 12 and 24 hr) compared to 0 hr, 152 genes were up-regulated, and 171 genes showed variable regulation across the time course. When we compared the expression of the differentially expressed genes between healthy and WSSV-infected groups across the entire time course of the study (6 hr. healthy vs. 6 hr. infected; 12 hr. healthy vs. 12 hr. infected, 24 hr. healthy vs. 24 hr. infected), a total of 36 genes were found to be up-regulated, and 44 genes were down-regulated in the healthy compared to the infected animals. The dendrograms showing the expression patterns of the up- (n=36) and the down-regulated (n=44) genes are shown in Figure 11.

Figure 11. The mRNA expression patterns (K-means) of differentially expressed genes (≥2-fold change, log infected-log healthy) in the WSSV-infected shrimp, 8 clusters, 500 iterations.

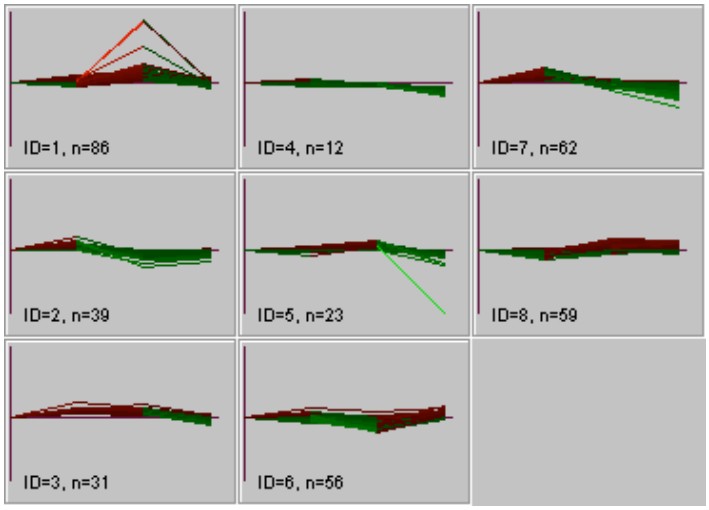
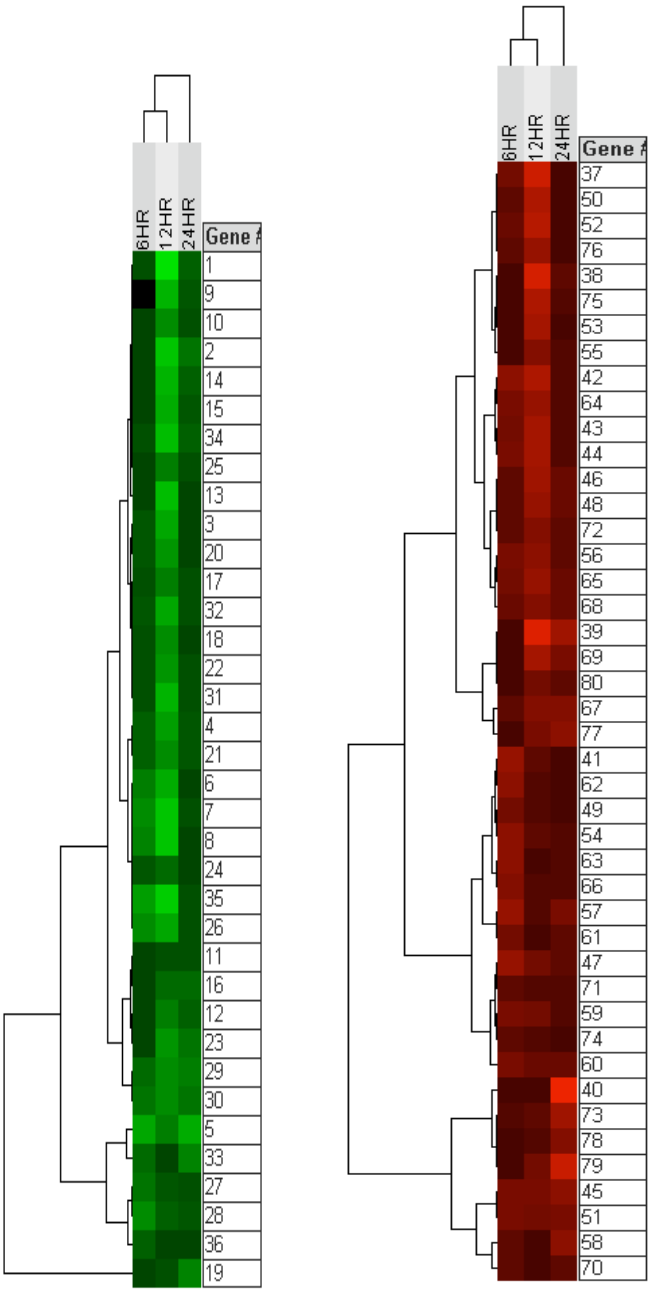


Figure 12. A dendogram showing the temporal changes in gene expression of the up-regulated (Panel A) and the down-regulated (Panel B) genes in shrimp during WSSV infection in shrimp.



The potential role of these candidate genes in WSSV-pathogenesis in shrimp should be the focus for future studies. Our studies indicate that in species like shrimp, where the number of ESTs available in the GenBank databases are limited, cross-species microarray analysis could provide a nice platform in identifying candidate genes involved in viral pathogenesis.

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Publications came out of this project so far:

1. Dhar, A. K., Dettori, A., Roux, M. M., Klimpel, K. R., and Read, B., 2003. Identification of differentially expressed genes in white spot syndrome virus infected shrimp (*Penaeus stylirostris*) by cDNA microarrays. Arch. Virol. 148, 2381-2396.

2. Dhar, A. K., Licon, K. S., Hasson, K. W., Varner, P. W., and Allnutt, F. C. T. 2005. LOW DENSITY LIPOPROTEIN RECEPTOR (LDLR) IS DIFFERENTIALLY EXPRESSED IN TAURA SYNDROME VIRUS (TSV) INFECTED SHRIMP *Penaeus vannamei*. In: World Aquaculture Society Meeting Shrimp Genetics Session, May 9-13, Bali, Indonesia.

3. Dhar, A. K., Licon, K. S., Robles-Sikisaka, R., Zhang, X., Bullis, R. A., and Read, B. 2006. Differential Gene Expression Profiling in Healthy and White Spot Syndrome (WSSV) Virus-Infected Shrimp (*Penaeus stylirostris*) by EST Analysis Isolated by Suppression Subtractive Hybridization. In: Plant and Animals Genome Mapping Conference XIV, Jan 14-18, San Diego, California.