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Short communication

The transcriptomic response to viral infection of two strains of shrimp (Litopenaeus vannamei)**

Artur Veloso, Gregory W. Warr, Craig L. Browdy, Robert W. Chapman

**Hollings Marine Laboratory, College of Charleston, Biology Department, Charleston, SC, USA
**Hollings Marine Laboratory, Medical University of South Carolina, Department of Biochemistry and Molecular Biology, Charleston, SC, USA
**Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC, USA

**Keywords:**
cDNA microarray
Gene expression
Disease
Invertebrate immunology
Taura Syndrome Virus
Yellow Head Virus

**Abstract**

The extent to which data-intensive studies of the transcriptome can provide insight into biological responses is not well defined, especially in the case of species (such as shrimp) where much physiological and biochemical knowledge is missing. In this study we took a transcriptomic approach to gain insight into the response to viral infection of two strains of the Pacific whiteleg shrimp (Litopenaeus vannamei) that differ in their resistance to Taura Syndrome Virus (TSV). Changes in gene expression in the hepatopancreas following infection with TSV and Yellow Head Virus (YHV) were assessed using a cDNA microarray containing 2469 putative unigenes. The null hypothesis tested was that significant differences between the transcriptomic responses to viral infection of resistant and sensitive strains would not be detected. This hypothesis was broadly rejected, with the most surprising observation being that the baseline (control, unchallenged) sensitive and resistant strains expressed distinguishable transcriptomic signatures. The resistant line was pre-disposed to lower expression of genes encoding viral (and host) proteins. Many of the genes differentiating resistant and sensitive lines are involved in protein metabolism, cellular trafficking, immune defense and stress response, although it was not possible to clearly identify candidate genes responsible for TSV resistance. In contrast to TSV challenge, YSV either failed to perturb the host transcriptome or created a “confused” response that was difficult to interpret.

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1. Introduction

It is axiomatic that the interactions between pathogens and hosts determine the course of an infection, defining survival or death, sickness or health (Kato-Maeda et al., 2001). In the case of a viral infection this interaction is complex and intriguing because the pathogen will subvert the host’s cellular machinery and bring about changes in the cell’s function at all levels (Novoa et al., 2005). Despite this, there are advantages to studying the effects of viral infection on the transcriptome. Using high throughput methods (such as microarrays) it is possible to measure the expression level of thousands of transcripts in organisms fighting a disease condition, thus potentially identifying genes important to the immune response of the organism as well as genes activated or suppressed by the pathogen (Kato-Maeda et al., 2001). This experimental approach has been undertaken successfully in several organisms, such as human cell lines (Nam et al., 2003), Atlantic salmon (Jorgensen et al., 2008), Drosophila melanogaster (Dostert et al., 2005) and Arabidopsis thaliana (Marathe et al., 2004). The approach can be facilitated by comparison of lineages that show different susceptibility levels to a certain disease, which could point more directly to genes responsible for the resistance to such disease (Ruby et al., 2006; Sarson et al., 2008).

Interest in the immune response of shrimp has been growing due to their worldwide importance in aquaculture and the growing number of emerging pathogens found to affect this group of organisms (Bachere, 2000). In order to fight such pathogens, the shrimp use a wide range of mechanisms, including hemocytes (Johansson et al., 2000); clotting and prophenoloxidase cascade (Sritunyalucksana and Soderhall, 2000); anti-microbial peptides (AMP) (Bachere et al., 2004); and dsRNA (Robalino et al., 2007a,b). Some of the most virulent diseases to affect penaeid shrimp are...
caused by viral pathogens, including the White Spot Syndrome Virus (WSSV), TSV and YHV (Lightner, 2005). The Pacific whiteleg shrimp (Litopenaeus vannamei Boone, 1931) is the most cultured shrimp in the world reaching a revenue close to US$7.5 billion (FAO, 2008). A major reason for the whiteleg shrimp to have become the most cultured shrimp in the world is the existence of both specific pathogen free (SPF) and specific pathogen resistant (SPR) lineages (Wyban et al., 1992; Argue et al., 2002).

Taura Syndrome was first observed in Ecuador and the causative agent (TSV) is a small icosahedral non-enveloped virus with a 10 kb long genome composed of a positive sense single stranded RNA (ssRNA) that belongs to the Dicistroviridae family (Hasson et al., 1995; Bonami et al., 1997; Mayo, 2005). Despite being first identified in 1991 in southeast Asia infecting the black tiger prawn (Panulirus monodon), Yellow Head Syndrome has the potential to infect L. vannamei and other penaeid shrimp (Limsuwan, 1991; Lu et al., 1994; Lightner, 2005). The etiological agent is YHV, a rod-shaped enveloped virus with a helical nucleocapsid containing a long genome composed of a positive sense 22 kb ssRNA genome, belonging to the Roniviridae family (Wongteerasupaya et al., 1995; Nadala et al., 1997; Tang and Lightner, 2005). The Pacific whiteleg shrimp in the world reaching a revenue close to US$7.5 billion (FAO, 2008). A major reason for the whiteleg shrimp to have become the most cultured shrimp in the world is the existence of both specific pathogen free (SPF) and specific pathogen resistant (SPR) lineages (Wyban et al., 1992; Argue et al., 2002).

2. Materials and methods

2.1. Shrimp maintenance and viral challenge

The experiment was conducted using a SPF TSV resistant shrimp strain (R) (Shrimp Improvement Systems Inc., Islamorada, FL) and a SPF TSV susceptible shrimp strain (S) (Kona, Oceanic Institute, HI). The average weight ± standard deviation of the resistant and susceptible shrimp was 2.4 ± 0.84 g and 3.1 ± 0.86 g respectively. The shrimp were kept in an indoor, semi-closed recirculating system composed of 25 transparent 10 L tanks with particulate, biological and UV light filtration, and were allowed to acclimatize for one week prior to the experiment. The shrimp were injected with 60 µL of one of three shrimp extracts prepared as described by Prior et al. (2003): 1. SPF shrimp (diluted 1:10 −4, w/v); 2 moribund shrimp injected with TSV (diluted 1:10 −5, w/v); 3 moribund shrimp injected with YHV (diluted 1:10 −4, w/v). The hepatopancreas of 5 shrimp were sampled from days 0 to 2 and kept in RNA Later® (Ambion, Austin, TX) and the remaining shrimp were kept in the tanks and mortality counts were taken for 14 days. The mortality curve of the different treatments was statistically compared using the log-rank test (Harrington and Fleming, 1982) as implemented on the R package survival (Terry and Lumley, 2007; R Development Core Team, 2008). In order to facilitate the flow of this paper, the shrimp treatment groups will be referred to by acronyms that will be composed of the shrimp strain (R or S) and the pathogen injected TSV, YHV or C for controls.

2.2. RNA extraction, labeling and microarray hybridization

Total RNA was extracted from the hepatopancreas using the RNeasy kit (Qiagen, Valencia, CA) with the on-column DNase step (RNase-Free DNase, Qiagen) following the manufacturer’s instructions. Next, 1 µg of the extracted RNA was reverse transcribed, in vitro transcribed into antisense amino allyl RNA and labeled with the Cy3 dye using the Amino Allyl MessageAmp II aRNA kit (Ambion). Finally, 10 µg of labeled aRNA was hybridized to the microarrays and they were washed and scanned at 70 PMT in a Scanning Array Express (Perkin Elmer, Boston, MA) as described by Robalino et al. (2007a,b) (Supplementary Material). The microarray slides used in this experiment were composed of two arrays occupying the top and bottom of the slide and with spots printed in the same order. Each array contained duplicates of each DNA spot printed side-by-side (Robalino et al., 2007a,b).

2.3. Microarray data analyses

2.3.1. Normalization and data filtering

The foreground and background values were calculated using the QuantArray software (Perkin Elmer) and the output files will be uploaded to the NCBI GEO website. The remaining data analyses were carried out using a set of packages from the open source software R and BioConductor (Gentleman et al., 2004; R Development Core Team, 2008), with an emphasis on functions from the package limma (Smyth, 2005).

The data were initially background corrected using the normexp model which uses maximum likelihood to calculate the parameters to fit a convolution of a normal and an exponential distribution (based on background and foreground respectively) and from that estimate it calculates the expected foreground (Ritchie et al., 2007). As the microarrays used were in-house printed (Robalino et al., 2007a,b) and batches of amplicons presented differences in purity, a print-order loess normalization was applied (Smyth and Speed, 2003). Next, the aroma.light package was used to perform a quantile normalization using a smooth spline that transforms the data to fit an empirical distribution calculated based on the average expression between all arrays (Bengtsson et al., 2008). Genes for which, the 4 replicates did not show intensity higher than three times the average intensity of the non-shrimp controls (16 Tursiops truncatus genes printed in duplicate in both arrays present in each slide) in at least 10% of the slides in the experiment were disregarded. Lastly, the data were variance stabilization normalized using the vsn package (Huber et al., 2002).

2.3.2. Discriminance of treatments

To examine the capacity of our microarrays to discriminate the various treatments we employed a variation of the Artificial Neural Network (ANN) strategy we have described in detail elsewhere (Chapman et al., 2009). Briefly this approach makes pairwise comparisons of treatment groups (regardless of day of sampling) using all the genes passing the filtering process described in Section 2.3.1 with 5 rounds of ANN training and random sampling of the records (individuals) in each group for each training session. The samples not used in the training session were used as a cross validation set. Following the initial training sessions the ANN models with the highest R² were then used to compute the sensitivities of the binary classifications to changes in gene expression. The 250 genes with the highest sensitivities for each comparison were then used in a second round of ANN training accomplished as before but this time with 20 rather than 5 training sessions. To evaluate the power of this reduced set of genes to discriminate treatment groups,
we employed receiver operating characteristic curves (ROC) as described in Chapman et al. (2009). Combining treatment groups regardless of day of collection is less than optimal. However, it was necessary, as our initial analysis (which employed typical linear approaches, e.g. control vs. treatment on day 1), while interesting, did not identify with statistical significance many genes. Further this approach does not really address the most important questions: Whether individual gene expression profiles can collectively discriminate resistant versus sensitive lineages and whether these lineages respond differently to viral challenges. Combining data regardless of day of sampling is not a desirable approach, but it has been our experience that ANNs do not perform well with sample sizes less that 10. We did explore the alternative (comparing each treatment day) and found the results less than compelling. In other words, combining the data regardless of day introduces noise due to time delays in the genomic response, but boosts the signals due to larger sample size.

3. Results and discussion

3.1. Mortality curve

At the end of the 14 days of observation the different group mortalities were very similar to the expected. The RTSV groups showed different mortalities to the STSV group (p-value = 1.53 × 10^-6) and to the SC and RC groups (p-value = 4.89 × 10^-2 and p-value = 1.74 × 10^-2 respectively). The mortality of all treatment groups was extremely similar at day 1 (∼7%). At day 2, however, the mortality in RYHV and SYHV (69.6% and 90% respectively) was much greater than the one seen in RTSV and STSV (7.4% and 34.6% respectively) (Fig. 1). The differences in mortality at day 2 need to be taken in consideration when trying to extract biological information from the microarray experiment. While the YHV infected animals at day 2 were in a final stage of the disease, the TSV infected animals were less sick, such that most of the mortality happened on the following days. It is possible that the differently expressed genes in the YHV treatments at day 2 are more related to the death process, while in the TSV treatments there might be more genes related to immune defense.

3.2. Variability in gene expression

3.2.1. Resistant versus sensitive lineages

In Table 1 we present the results of the ROC curves that report the discrimination between the transcriptomic signatures of the control and experimental shrimp groups. These values can be considered as the probability of correctly assigning individuals to the appropriate class. The first comparison was between the control (C, unchallenged) transcriptomes of the shrimp lines resistant (R) and sensitive (S) to TSV infection. The RC versus SC comparison in Table 1 shows that the probability of correctly assigning a resistant or susceptible individual to the correct group is better than 98%; this value differs from random allocations (50:50) by a highly significant (p < 0.001) margin. We interpret this result to suggest that resistance to infection by TSV may be associated not only with the induction of genes by infection, but by a pre-existing pattern of gene expression reflecting a relatively resistant constitutive state. Supplementary Table S1 presents lists of genes that lead to this distinction between the transcriptomic signatures of the RC and SC groups, as well as the fold differences in their mean expression. The numbers in the column should be considered baseline differences between the strains. In this gene list more than half of the genes are unknowns, reflecting our ignorance of the functions of most genes in shrimp. However, of the annotated genes nearly all are involved in cellular trafficking and motility, protein synthesis, energy production or control of energy production, growth, and lipid metabolism/transport. It is significant that the bulk of the known genes, are involved in protein synthesis and cellular trafficking and are down regulated in the TSV resistant strain. The down regulation of energy and growth related genes in the resistant line is consistent with the results of Argue et al. (2002) who reported that the TSV resistant line grew more slowly than control lines and that there was a negative correlation between growth and TSV resistance. The results present here provide a molecular underpinning for this observation.

As the protein synthesis and cellular trafficking machinery are precisely the components of cellular activity that are essential for viral replication, it is logical that down-regulation of these systems would inhibit viral induced mortality as was evident in Fig. 1. The translationally controlled tumor protein (TCTP), also called fortilin (Tongsnunt et al., 2008) is slightly upregulated in resistant lines. This protein has been shown to play a role in protecting shrimp from white spot syndrome virus (WSSV) (Tongsnunt et al., 2008). The inhibition of the transactivation response (TAR) RNA binding protein (TRBP) has been shown to inhibit replication of HIV in human cells, presumably through the action of TRBP on protein kinase R activation which is essential for HIV replication (Christensen et al., 2007). It would, however, be premature to suggest that the mechanism of TSV resistance in shrimp is analogous to HIV resistance in human cells, although genes that play a major role in trafficking mRNA to the rough endoplasmic reticulum for translation, are down regulated in the resistant line. Three transcription initiation factors and one elongation factor are also down regulated in the resistant line. This is significant in that TSV is a ss(+), RNA virus whose genome effectively behaves as mRNA. This suggests that the resistant line has a reduced capacity for translating the TSV messages (as well as it own messages) compared to the sensitive line. Overall, comparisons of transcriptomes from unchallenged resistant and sensitive lines suggest that the resistance to TSV is at least in part due to a predisposition of the resistant lineage to slow viral replication.

3.2.2. Response of the resistant line to TSV challenge

The transcriptomic changes resulting from TSV infection in the resistant line provided some discrimination of the treatment groups (AUC = 0.73, RC vs. RTSV, Table 1) and while this classification precision was significantly greater than random expectations, it was substantially less (p > 0.001, one tailed z-test of the AUC's) than the correct classification level for the resistant versus sensitive control comparisons. The implication is that resistance to TSV may involve some transcriptomic adjustments, but it reinforces the notion (discussed above) that preprogramming is a major feature of resistance to TSV infection.

| Treatment | Auc | sd  | p-value | Auc | sd  | p-value |
|-----------|-----|-----|---------|-----|-----|---------|
| RC        | 0.987 | 0.0091 | -     | 0.087 | 0.0350 | -     |
| RTSV      | 0.731 | 0.0116 | -     | 0.589 | 0.0017 | -     |
| STSV      | 0.840 | 0.0016 | -     | 0.914 | 0.0367 | -     |
| RYHV      | NA   | NA   | -     | NA   | NA   | -     |
| SYHV      | 0.609 | 0.00513 | -     | 0.914 | 0.0184 | -     |
The genes discriminating the transcriptomic responses of the resistant control shrimp versus resistant shrimp challenged with TSV include a long list of energy metabolism related proteins, some secretory systems (sec61, sec24, and other translocators), initiation factors, protein synthesis, and cytokinesis genes (actin and related factors including spaghetti squash and spindle pole body), which are slightly more represented in the up regulation than down regulation classes. While it is tempting to infer some role of these genes in viral responses, it has been our experience and that of others (Chapman et al., 2009; Gracey, 2007; Gracey et al., 2008; Hofmann and Place, 2007; Place et al., 2008; O'Donnell et al., 2009; Evans and Somero, 2008) that energy metabolism, cytokinesis and protein synthesis genes are general indicators of organismal stress (including chemical, pH, temperature, land use and disease) in a variety of organisms including oysters, mussels, shrimp, fish and marine mammals. Thus the bulk of the known genes that discriminate viral infection from the control probably reflect general stress responses rather than a specific response to viral challenge. Genes down regulated by TSV infection were dominated by unknowns, particularly those with the higher fold changes in expression. Absent from this gene list is the TRBP gene mentioned above, which indicates that this gene was not a major contributor to discrimination of the impact of TSV on the resistant line.

### 3.2.4. Response of the sensitive line to viral challenge

Unlike the resistant lineage, the TSV sensitive line differed dramatically in the transcriptomic response to TSV and YHV. The response to TSV could not be distinguished from random effects, as the area under the curve of 0.59 was not statistically significant (Table 1). This suggests that TSV infection in the sensitive line does not initiate large changes in the transcriptome and that the virus manages to evade discovery by the antiviral defense mechanisms. In contrast the transcriptomic response to YHV was highly discriminated (area under the curve 0.91, p < 0.001), indicating that YHV initiates a substantial modification of the transcriptome.

YHV infection influences the expression of a variety of ribosomal, transllocator, elongation and initiation factors as well and energy metabolism genes. However, there does not appear to be a consistent pattern of up or down regulation as genes in all of the classes are found in both up and down states relative to the control. It is as if YHV is not escaping notice, but creating confusion in the host as to what the appropriate response should be. One gene of interest is endoribonuclease U (endo U), which is widely distributed in metazoans and involved in intron excision (Renzi et al., 2006). A similar protein has been discovered in the Nodoviridae order and is essential for viral translation and replication of the coronavirus which causes severe acute respiratory syndrome (SARS) in humans (Ivanov et al., 2004). As YHV is in the related Roniviridae family, it is possible that the signal on the microarrays is confounded by a combination of endogenous shrimp response and viral genes. We doubt this is the case, as 18 kb of the YHV genome has been sequenced (Senapin et al., 2010) and no match to endo U is apparent. Overall, the sensitive line does not provide a great deal of information regarding the mechanisms of shrimp response to viral infection other than the tantalizing notions that
TSV manages to escape detection (an ideal ability for any virus, Hay and Kannourakis, 2002) and YHV appears to create confusion which may be next best choice.

4. Conclusions

In this report we have examined the transcriptomic response of TSV sensitive and resistant lines to two viral pathogens, concluding that TSV resistance is to some degree preprogrammed in the resting state of the resistant shrimp. However, there is likely also to be some genetically determined response that also contributes to resistance. In the resistant line we see some indication of genes that may be involved in resistance to TSV including genes with known capacity to limit apoptosis and viral amplification. However, we should note that inhibiting apoptosis is the Sword of Damocles. In the early stages of infection apoptosis favors the host by destroying the cellular machinery necessary for viral replication. Once the infective viirions have been produced, apoptosis favors the virus by encapsulation of the viirions in host vesicles, which facilitates transmission to other cells (Hay and Kannourakis, 2002). In other words, timing, while it may not be everything, is important. As significant TSV resistance can be generated in as little as one generation, it is likely that a small number of genes are responsible for the resistance. It is therefore unlikely that the large number of genes discussed here are directly responsible for resistance, but rather some master regulator of their expression amenable to selection is at the heart of the response. Further, we can, on the basis of this study, dismiss a large number of known genes as likely candidates underpinning resistance, as they are known to be involved in general stress response in shrimp and other organisms. We should also dismiss at this point the fortilin, fortilin binding protein, TRBP and others. Our reasoning is that these are effectors and not gene regulators, and (2) there are too many of them to respond to selection in a single generation unless they are tightly linked. We view it as more likely that resistance to TSV is conferred by some combination of genes of unknown function and/or genes not represented on this array.

Acknowledgments

This research was supported by the USDA CRRESP U.S. Marine Shrimp Farming Program and by a Marine Genomics fellowship support from the College of Charleston to A.V. and was conducted at the Hollings Marine Laboratory, Charleston, SC. This paper has a contribution number 676 for MRD SCDNR and 54 for Marine Biomedicine and Environmental Sciences Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.10.001.

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