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Research paper

Identification of cold responsive genes in Pacific white shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridization

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ABSTRACT

The Pacific white shrimp (*Litopenaeus vannamei*) is one of the most widely cultured shrimp species in the world. Despite *L. vannamei* having tropical origins, it is being reared subtropically, with low temperature stress being one of the most severe threats to its growth, survival and distribution. To unravel the molecular basis of cold tolerance in *L. vannamei*, the suppression subtractive hybridization (SSH) platform was employed to identify cold responsive genes in the hepatopancreas of *L. vannamei*. Both forward and reverse cDNA libraries were constructed, followed by dot blot hybridization, cloning, sequence analysis and quantitative real-time PCR. These approaches identified 92 cold induced and 48 cold inhibited ESTs to give a total of 37 cold induced and 17 cold inhibited contigs. Some of the identified genes related to stress response or cell defense, such as *tetraspanins* (*TSPANs*), *DEAD-box helicase*, *heat shock proteins* (*HSPs*) and *metallothionein* (*MT*), which were more abundant in the forward SSH library than in the reverse SSH library. The most abundant Est was a *tetraspanin-8* (*TSPAN8*) homolog dubbed *LvTSPAN8*. A multiple sequence alignment and transmembrane domain prediction was also performed for *LvTSPAN8*. *LvTSPAN8* expression was also examined in the gills, muscle, heart and hepatopancreas following cold exposure and showed the highest expression levels in the hepatopancreas. Overall, this study was able to identify several known genes and novel genes via SSH that appear to be associated with cold stress and will help to provide further insights into the molecular mechanisms regulating cold tolerance in *L. vannamei*.

1. Introduction

Shrimp farming is a major industry in some Asian and Latin America countries, with annual production in China reaching 1,000,000 metric tons in 2005 (Hennig and Andreatta, 1998). When rearing shrimp, various abiotic stresses such as pH, salinity, cadmium or temperature change can lead to a reduced growth rate, declined survive rate, increase disease susceptibility and even death (Ponce-Palafox et al., 1997; Kumlu et al., 2010; Miura and Furumoto, 2013). Of the commercially farmed shrimp, the Pacific white shrimp (*Litopenaeus vannamei*) is the most heavily farmed and makes up over 80% of shrimp in production. While *L. vannamei* naturally occur in tropical waters, expansion of farming in subtropical climates where temperature variations are normative is of interest. While some studies have examined the effect of salinity on cold tolerance in shrimp and tried to optimize the cold acclimation process (Miura and Furumoto, 2013; Zhou et al., 2011), very little research has focused on the molecular mechanisms occurring during cold stress in *L. vannamei*.

While little is known regarding the molecular underpinnings of cold tolerance in *L. vannamei*, the molecular mechanisms behind a cold stress response have been examined in other aquatic forms. For example, transcriptomic examination of cold challenged larval zebrafish revealed that low temperature acclimation was in large attributed to transcriptional and post-transcriptional alterations (Chinnusamy et al., 2010; Guan et al., 2013). Additionally, a cold tolerant transgenic zebrafish model showed that energy metabolic processes, lipid transport and cell death regulation were all essential to low temperature acclimation (Owttrim, 2012). Moreover, one study examining barramundi (*Lates calcarifer*), populations originating from a cooler environment found that they grew significantly faster at a lower temperature, with transcriptomic analysis during cold exposure revealing expressional changes relating to the regulation of peptidase activity, microtubules, cytoplasmic and cellular metabolic based processes and especially genes involved in cytoskeletal element reorganization (Liu et al., 2010). When examining cold stress responses in tilapia (*Oreochromis niloticus*), genes pertaining to lipid and carbohydrate content and types, material transport and apoptosis were all differentially expressed; in addition to two quantitative trait loci affecting cold tolerance being detected (Long and Gl, 2013; Long et al.).

Abbreviations: SSH, suppression subtractive hybridization; cDNA, complementary DNA; mRNA, messenger RNA; EST, expressed sequence tags; HSP, heat shock protein; PCR, polymerase chain reaction; RACE, rapid-amplification of cDNA ends; ORF, open reading frame; UTR, untranslated regions; kD, kilodalton; SPF, specific pathogen free; bp, base pair; dNTP, deoxyribonucleotide triphosphate.

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In *L. vannamei*, low temperature was found to potentially induce oxidative stress (Qian Wang et al., 2014), DNA damage, lipid peroxidation and changes in osmolality (Newton et al.); in addition to several differentially expressed proteins being identified in cold treated hemocytes (Cnaani et al., 2003). However, the molecular mechanisms of cold tolerance are complicated and poorly understood in aquatic animals, especially warm water species. To gain a better understanding of genes pertaining to cold stress in *L. vannamei*, this study employed suppression subtractive hybridization (SSH) as a means of identifying differentially expressed genes following cold stress. SSH has been successfully used to identify and characterize differential expressed genes involved in sexual development (Yang et al., 2015; Zhou et al., 2010), disease-resistance (Qiu et al., 2011; Fan et al.; Preechaphol et al., 2010; Qu et al., 2014; James et al., 2010) and stress-resistance (Prapavorarat et al., 2010; Wang et al., 2013) in aquatic animals. More specifically, this study identified differential expressed genes in the hepatopancreas of cold challenged shrimp using a SSH platform in conjunction with quantitative real-time PCR (qPCR).

2. Materials and methods

2.1. Low temperature treatment

A total of 66 experimental shrimp (3 months old, weight of 10–15 g) were reared at Fangchenggang aquaculture base, Guangxi Academy of Fishery Science and adapted to conditions at 28 °C, 32–35% salinity and a dissolved oxygen level ≥ 5 mg/L for one week prior to experimentation. Shrimp were feed with Lucky Star shrimp feed (Initial #4) three times daily, with a daily ration being about 5% of the total body weight of each shrimp. Three groups of shrimp (21 shrimp per group) were cooled to specific experimental temperatures and then maintained at those temperatures for sampling at different points, with a control group maintained at 28 °C. To acclimate the experimental groups to a low temperature, the temperature was lowered to 18 °C and maintain for 24 h, with feeding halted only in the experimental groups form this point forward. Experimental groups were cooled at a rate of −1 °C/4 h until reaching 15 °C, 13 °C or 11 °C. Once the desired temperatures were reached, samples were obtained at 12, 24, 36, 48, 60 and 72 h for each group, with the exception of the 11 °C group that had no live shrimp remaining at 72 h. Three shrimp from each group were sampled at each time point, with shrimp sacrificed by destroying the main nerve center and the muscle, gill, heart and hepatopancreas were rapidly isolated and stored in RNAlater (Ambion) solution for subsequent RNA isolation.

2.2. Construction and identification of SSH libraries

Total RNA was isolated from the hepatopancreas of the control (28 °C) and the 13 °C for 36 h treatment group, with this group selected as an intermediate response group that showed a degree of impairment without leading to hypothermia, using TRizol (GIBCO) and RNA was qualified via electrophoresis on a 1% agarose gel. For each group, RNA was isolated and pooled from 3 shrimp, with RNA purified using Oligotex mRNA Kits (QIAGEN) according to the manufacturer's protocols and quantified via spectrophotometry. The obtained mRNA (2 μg) was then reverse-transcribed using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocols.

SSH was carried out using a PCR Select cDNA Subtraction Kit (BD Clontech) according to the manufacturer's protocols. Briefly, after RsaI digestion and ligation of the adaptors, differentially expressed cDNAs were normalized and enriched by two rounds of hybridization and PCR amplification. The PCR products were purified by ethanol precipitation, cloned into pGEM-T Easy vectors (Promega) and transformed into *Escherichia coli* DH5α (Takara). In the forward library, the 13 °C for 36 h was the tester while the control was the driver, with the reverse done in the reverse library. The efficiencies of the SSH libraries were evaluated by amplification of the house keeping gene *β-actin* (primers: BF/BR; Table 1). Insertions were verified via PCR amplification using nested primers 1 and 2R provided in the PCR Select cDNA Subtraction Kit, with 1 μl PCR product from each positive clone subjected to dot blot hybridization as previously described (Kumar et al., 2015).

2.3. EST analysis

Following dot blot hybridization, the two hybridization images were scanned using a Cyclone PacKard scanner. The individual clone signal intensities were determined with PDQuest 2-D analytical software (Bio-Rad, USA) and the clones showing the greatest differential expression relative to the control were sequenced using ABI 3730XL genetic analyzer by BGI. EST nucleotide sequences were assembled into contigs using SeqMan and aligned in GenBank using BLASTN and BLASTX, with matches considered significant with an E-value $<1^{e-04}$.

2.4. RACE PCR and sequence analysis

Total RNA was isolated from shrimp treated at 13 °C for 36 h as described above and cDNAs were synthesized using a SMART cDNA Library Construction Kit (Clontech, USA) according to the manufacturer's instructions followed by directional (5′-3′ direction) cloning into a pBluescript II SK vector. Of the 57 identified contigs, contig1 (*Tetraspanin-8*; Table 2) from the forward library contained the most ESTs and thus was selected for further cDNA clone and expressional analysis. The contig1 sequence was also used as a template for designing both forward (TetF, located near the 5′ terminus of the contig1) and reverse (TetR, located near the 3′ terminus of the contig1) gene specific RACE primers. Common M13R and TetF primers were used for 3′-RACE and common M13F and TetR primers were used for 5′-RACE amplification under the following PCR conditions: 94 °C for 3 min, followed by 35 cycles at 94 °C for 20 s, 61 °C for 30 s and 72 °C for 2 min 30 s. PCR products of the expected size were cloned into pGEM-T easy vectors (Promega) and sequenced. The obtained sequences were then assembled into a cDNA containing the entire *Tetraspanin-8* (*TSPAN8*) gene ORF. After BLASTX analysis of the cDNA sequence, representative proteins that shared identity with the *L. vannamei TSPAN8* (*LvTSPAN8*) protein, yet were from species of different taxonomic status, were downloaded for multiple sequence alignments. The alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and shaded for enhanced visibility using the BoxShade server. Transmembrane helices within the protein sequence were predicted using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), with predictions of the most probable locations and orientations based on an N-best algorithm that sums over all paths through the model with the same location and direction of the helices.

Table 1
Primers used in this study.

Genes	Primer name	Primer sequence (5′-3′)
β-actin	BF	GGACTTCGAGCAGGAGATGACCAC
	BR	ACGTCGCACTTCAT GATGGAGTTG
Tetraspanin-8	TETF	TGTGCCAATGAAAAGGTAAC
	TETR	AATCGGCTGAAGAATCCC
	TETF2	CACTCGCATACCTGTTGA
	TETR2	TTCCATATTCGCCGTTCTT
Contig4	NGT4F	GTGAGTGAGATTGAACATCG
	NGT4R	GTTAGAAGGTAGGCGTTAGA
Contig7	NGT7F	CGAGAACTGAAGGTGAGAC
	NGT7R	GACTGACTTGTGGCTACTC
Contig38	NGT38F	TCTTGGATATGGTTGCTGTT
	NGT38R	ATGTCTGTCTTGAGGAATGG