Fish and Shellfish Immunology 82 (2018) 32–40

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transcriptomic responses to heat stress in rainbow trout *Oncorhynchus mykiss* head kidney

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ARTICLE INFO

Keywords:
Rainbow trout
Heat stress
RNA-seq
Transcriptome

ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) are widely cultured throughout the word for commercial aquaculture. However, as a cold-water species, rainbow trout are highly susceptible to heat stress, which may cause pathological signs or diseases by alleviating the immune roles and then lead to mass mortality. Understanding the molecular mechanisms that occur in the rainbow trout in response to heat stress will be useful to decrease heat stress-related morbidity and mortality in trout aquaculture. In the present study, we conducted transcriptome analysis of head kidney tissue in rainbow trout under heat-stress (24 °C) and control (18 °C) conditions, to identify heat stress-induced genes and pathways. More than 281 million clean reads were generated from six head kidney libraries. Using an adjusted P-value of $P < 0.05$ as the threshold, a total of 443 differentially expressed genes (DEGs) were identified, including members of the HSP90, HSP70, HSP60, and HSP40 family and several cofactors or cochaperones. The RNA-seq results were confirmed by RT-qPCR. Gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis of DEGs were performed. Many genes involved in maintaining homeostasis or adapting to stress and stimuli were highly induced in response to high temperature. The most significantly enriched pathway was "Protein processing in endoplasmic reticulum (ER)", a quality control system that ensures correct protein folding or degradation of misfolded polypeptides by ER-associated degradation. Other signaling pathways involved in regulation of immune system and post-transcriptional regulation of spliceosome were also critical for thermal adaptation. These findings improve our understanding of the molecular mechanisms of heat stress responses and are useful to develop strategies for the improvement of rainbow trout survival rate during summer high-temperature period.

1. Introduction

Accelerated climate change poses a significant threat to the environment and to biological organisms, including poikilothermic fish, for which water temperature is a critical environmental factor. Like other organisms, fish have a preferred temperature, and, when faced with changes in ambient temperature, fish undergo different aspects of physiological adaption, including changes in metabolism and growth rates of individual fish [1,2], and alterations in sociality and activity [3]. Fish are able to cope with daily and seasonal variations in water temperature or quality, but their health and survival are threatened when temperatures reach close to or beyond species-specific thermal tolerances [4]. As a result of global warming, mean water temperatures are increasing globally, according to a report of the Intergovernmental Panel on Climate Change [5]; this will inevitably cause challenges to farmed fish, especially cold-water species, in semi-open aquaculture. The alterations in physiology and behavior of fish in response to heat stress vastly differ, even among similar species or species with comparable geographic distribution. Elucidation of these changes at the transcriptome level would facilitate our understanding of the biological and physiological mechanisms by which fish adapt to thermal stress, or fail to respond to it.

Transcriptomics has been used to study complex responses by organisms to the environment, interpret functional elements of the genome, and understand the biological processes that occur in natural populations. DNA microarray was the dominant method used for ecological transcriptomics studies for the past decade [6]. However, as mRNA sequencing using next-generation sequencing technologies (RNA-seq) becomes more affordable, RNA-seq has more often been the method of choice to investigate transcriptional response to environmental stress. Abundant RNA-seq reads can construct a complete transcriptome, provide a wealth of information on differential gene

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https://doi.org/10.1016/j.fsi.2018.08.002
Received 17 April 2018; Received in revised form 24 July 2018; Accepted 1 August 2018
Available online 02 August 2018

expression, and can be used to identify biological pathways involved in response to thermal stress. Fish are widely used as a model system for medical and genetic research; in addition to their ecological importance and economic value, a rapidly growing body of literature has emerged regarding identifying mechanisms of temperature acclimation in different fish species using RNA-seq [7]. These studies revealed that changes occur in the expression of many genes in response to heat stress, including heat shock protein (HSP) genes and immune-related genes; these are associated with either long-term or short-term heat stress responses. In the majority of species studied, HSP genes are upregulated, as is the case for hybrid catfish [8], crimson spotted rainbow fish [9], rainbow trout [10], redband trout [11], snow trout [12], half-smooth tongue sole [13], Iberian freshwater fish [14], and genetically modified farmed tilapia [15]. However, HSP genes were downregulated after short-term heat exposure in *Pagothenia borchgrevinki* [16]; this suggested that a variety of heat coping mechanisms exist among different fish species. Immune related genes were upregulated in crimson spotted rainbow fish [9], snow trout [12], redband trout [11], and tubenose goby [17]. These results were consistent analyses using microarrays and qPCR. These studies also identified important regulatory pathways involved in the response to heat stress, including metabolism, protein folding and degradation, and immune response; modulation of these pathways were observed in a variety of fish species under different stress conditions, indicating that these biological pathways are critical for thermal adaptation.

Rainbow trout (*Oncorhynchus mykiss*), a member of the Salmonidae family, is rapidly becoming an important fish in aquaculture, and is a biologically relevant model to study adaptation and response to heat stress. Farmed fish experience numerous stressors, especially thermal stress, when temperatures are elevated during the summer. Suitable water temperatures for rainbow trout range from 12 to 18 °C. In water at 21 °C, pathological changes were observed in liver tissue, and inflammation resulted from heat stress; moreover, the immune function of the trout was significantly reduced, and tissues were severely injured past 24 °C [18,19]. Previous reports have demonstrated that the primary gene expression changes in heat stress response include molecular chaperone genes (e.g., members of the HSP gene family) [20–23]. Responses to temperature change in rainbow trout has been evaluated using microarray [24], and microarray technology has been used to compare heat stress responses between different rainbow trout strains [25–27]. Under either acute or mild heat stress, many HSP genes were highly expressed in the high-temperature selected strain, or in fish challenged with higher temperatures; similar but distinct tissue-specific expression patterns were also observed, and the links of different temperature-dependent pathways and gene networks were revealed between different breeding lines. A recent study used RNA-seq to discover that several pathways are influenced by heat stress in response to heat stress, including protein metabolism, energy metabolism, and immune system function [9]. These results provide abundant insights and comparable datasets to study heat adaptation processes. It still remains of great interest to perform more comprehensive analyses of tissue-specific responses to heat stress in rainbow trout in order to elucidate the molecular mechanisms of thermal adaptation in fish.

The head kidney is a crucial organ that contains cortisol-producing interrenal cells. Cortisol is the primary glucocorticoid that is released when teleost fish are exposed to a stressor, is the end product of activation of the hypothalamic-pituitary-interrenal axis, and has long-term effects on stress adaptation [28–31]. In a study by Li et al., serum cortisol was significantly upregulated in rainbow trout under conditions of heat stress (25 °C for 8 h) [21]. Although the head kidney is associated with the thermal response, the transcriptome data are very limited for stressed kidney tissue in rainbow trout. We previously reported on a heat stress response that is specific for the liver, a crucial metabolic organ [9]. In the present study, we determine transcriptome changes that occur in response to heat stress in rainbow trout head kidney. To simulate a natural aquatic environment, moderate heat stress was performed by increasing the temperature at a relatively constant speed (increments of 1 °C per 24 h) from 18 °C to 24 °C. In a previous study, we show that 24 °C is a "key high-temperature point" that commits rainbow trout from adaptive regulation to injury, according to non-specific immune and metabolism parameters, [18]. Therefore, we selected 18 °C and 24 °C as control and heat stress treatment temperatures, respectively. The aim of this study was to identify kidney-specific genes and relevant pathways in response to heat stress in rainbow trout.

2. Materials and methods

2.1. Animals and ethics approval

All methods used in this study were conducted according to the guiding principles of the Chinese Legislation on the Use and Care of Laboratory Animals. The animal protocol was approved by the institutional ethics committee of Gansu Agricultural University. Full-sib rainbow trout were purchased from a trout farm in Yongjing, Gansu Province, China. Fish with a mean weight 400 ± 10.5 g were transferred into a 3000 L aerated water tank and were cultured at 18 °C for seven days. Prior to the experiment, the fish were randomly divided into six groups in 300 L water tanks, and allowed to acclimate for another seven days. To simulate temperature conditions in natural environment, the water temperature in the heat stress groups was increased from 18 °C to 24 °C at a constant rate of 1 °C per 24 h using a temperature control system. After anesthetizing with a lethal dose of MS-222 (Sigma Aldrich Co., St. Louis, USA), the head kidneys were harvested from three female fishes from both the 18 °C control group and the 24 °C heat stress group. Tissues were immediately flash frozen in liquid nitrogen and stored at −80 °C for gene expression profiling analysis.

2.2. RNA isolation and library preparation

Head kidney tissue samples were homogenized and total RNA was extracted using a TRIzol reagent, according to standard protocol (Invitrogen, Carlsbad, CA, USA). The purity of the isolated RNA was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and agarose gel electrophoresis. The quality and quantity of the extracted RNA were assessed using a Qubit 2.0 fluorometer (Life Technologies, CA, USA) and a Bioanalyzer 2100 System (Agilent Technologies, CA, USA).

Six sequencing libraries were created by reverse-transcription from ~3 μg of RNA from each sample using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® sequencing (NEB, USA), according to a published protocol [9]. In brief, mRNA was purified using poly-T oligo-attached magnetic beads and broken into short fragments using divalent cations in NEBNext® First Strand Synthesis Reaction Buffer (5 ×). First-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H⁻) and random hexamer primers, reaction buffer, RNase H, and DNA polymerase I. Remaining overhangs were converted into blunt ends by exonuclease/polymerase activities. Before hybridization, the 3′ends of the DNA fragments were mono-adenylated, and the NEBNext® hairpin loop adaptor was ligated. Next, the library fragments were purified using the AMPure XP System (Beckman Coulter, Beverly, USA) to select 150–200 bp cDNA fragments. The PCR products from enriched fragments with ligated sequencing adapters were purified using the AMPure XP System. An Agilent Bioanalyzer 2100 System was used to assess the library quality. According to the manufacturer's instructions, the cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, CA, USA) was used to cluster index-coded samples. Six library preparations of cluster generation were sequenced on the Illumina Hiseq™ 4000 platform to generate 150-bp paired-end raw reads.