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RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish

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Liu S, Wang X, Sun F, Zhang J, Feng J, Liu H, Rajendran KV, Sun L, Zhang Y, Jiang Y, Peatman E, Kaltenboeck L, Kucuktas H, Liu Z. RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish. *Physiol Genomics* 45: 462–476, 2013. First published April 30, 2013; doi:10.1152/physiolgenomics.00026.2013.—Temperature is one of the most prominent abiotic factors affecting ectotherms. Most fish species, as ectotherms, have extraordinary ability to deal with a wide range of temperature changes. While the molecular mechanism underlying temperature adaptation has long been of interest, it is still largely unexplored with fish. Understanding of the fundamental mechanisms conferring tolerance to temperature fluctuations is a topic of increasing interest as temperature may continue to rise as a result of global climate change. Catfish have a wide natural habitat and possess great plasticity in dealing with environmental variations in temperature. However, no studies have been conducted at the transcriptomic level to determine heat stress-induced gene expression. In the present study, we conducted an RNA-Seq analysis to identify heat stress-induced genes in catfish at the transcriptome level. Expression analysis identified a total of 2,260 differentially expressed genes with a cutoff of twofold change. qRT-PCR validation suggested the high reliability of the RNA-Seq results. Gene ontology, enrichment, and pathway analyses were conducted to gain insight into physiological and gene pathways. Specifically, genes involved in oxygen transport, protein folding and degradation, and metabolic process were highly induced, while general protein synthesis was dramatically repressed in response to the lethal temperature stress. This is the first RNA-Seq-based expression study in catfish in response to heat stress. The candidate genes identified should be valuable for further targeted studies on heat tolerance, thereby assisting the development of heat-tolerant catfish lines for aquaculture.

catfish; heat stress; genome; RNA-Seq; gene expression; gene pathway

TEMPERATURE IS ONE OF THE most prominent abiotic factors influencing ectotherms. Although ectotherms have extraordi-nary ability to adapt to a wide range of temperature changes (23), shifts in temperature may hamper physiological capabil-ities of the organisms to grow or reproduce, cause stress, or even lead to death (10, 39).

The molecular mechanisms underlying temperature adapta-tion have long been of interest. Stress responses have been well studied, especially with model species (84, 87). Stress re-sponses involve expression of a series of evolutionarily con-served stress-responsive genes (42) that include genes control-ling cell cycle, protein folding and repair, DNA and chromatin stabilization and repair, removal of damaged proteins, and energy metabolism (43, 49). While the stress response genes have been well characterized in a few model species (43), studies with nonmodel organisms have been relatively few, especially when dealing with questions of ecological and evolutionary significance (49). In the face of global climate change, it is important to understand the fundamental mecha-nisms for conferring tolerance to temperature fluctuations (49). In recent years, studies have been conducted in several species of fish and shellfish to identify stress induced genes. For instance, Quinn et al. (71) reported that small heat shock protein and HSP90 genes are associated with upper tempera-ture tolerance in Arctic charr, a cold-water fish species. In their study, chicken ovalbumin upstream promoter transcription II was identified as a candidate gene for upper temperature tolerance based on an acute thermal stress experiment. In a second study using chronic, sublethal heat stress treatment, Quinn et al. (72) suggested that ribosomal protein and heat shock protein genes are good candidates for development of genetic markers to assist breeding programs. In eurythermal goby fish, the responses to acute and chronic heat stress and the effects of thermal acclimation on such responses have been examined at the transcriptional level. Buckley et al. (12) observed the induction or repression of over 200 genes in each tissue of gill and liver during an acute heat exposure. These genes were associated with numerous biological processes including homeostasis, cell cycle control, cytoskeletal reorga-nization, and metabolic regulation. Logan and Somero (50) examined the transcriptional responses to thermal acclimation and identified genes involved in protein synthesis, transport,

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and several metabolic processes showing the greatest change in expression (50). Notably, they observed that the stress-induced proteins such as heat shock proteins were not upregulated in the long-term high temperature-acclimated fish and suggested that the acclimation process has largely remedied the effects of acute thermal stress (50). The changes of gene expression in thermal-acclimated goby fish in response to acute heat stress were also examined by Logan and Somero (49). They reported that the severity of stress was indicated by sequential expression of different categories of genes: HSP70 was largely induced during mild stress; the expression of the gene encoding proteolytic protein ubiquitin increased at slightly higher temperatures, and the gene encoding cyclin-dependent kinase inhibitor 1B involved in cell cycle arrest and apoptosis was upregulated only under extreme stress (49). In a study with Antarctic bivalve, transcriptional analysis of response to heat stress revealed that genes involved in antioxidation, Ca^{2+} signaling, and homeostasis could serve as potential biomarkers for heat stress in this species (83).

Channel catfish is the primary aquaculture fish species in the United States. It has a wide natural habitat ranging from the Gulf of Mexico in the south to Manitoba in the north (32), with coldest temperatures of near 0°C in the winter in the north to >30°C in the summer in subtropical areas, suggesting its great plasticity in dealing with environmental variations in temperature. Therefore, catfish is not only an important aquaculture species but also a good research model for heat stress studies. In Alabama, water temperature in catfish farm ponds can reach to as high as 36–40°C for a short period of time during the hottest summer days. It is observed that outbreaks of bacterial diseases in catfish often closely relate to heat stress during the summer. On the one hand, the high temperature can enhance the virulence of pathogens. For instance, enteric septicemia of catfish (ESC) disease causes the largest economic loss in catfish industry. Pathogen of ESC, *Edwardsiella ictaluri*, presents highest virulence at 28–29°C; on the other hand, elevated body temperature can itself stress the fish and consequently make them vulnerable to be infected by pathogens. However, heat stress in catfish remains largely unexplored. No studies have been conducted at the transcriptomic level to identify and characterize heat stress-induced gene expression in catfish, except that a number of heat shock genes have been characterized (2, 51). The objective of this study was to identify heat stress-induced genes in hybrid catfish, generated from crossing channel catfish (*Ictalurus punctatus*) female and blue catfish (*Ictalurus furcatus*) male, which is now widely used in aquaculture production because of its superior performance. Analysis of such genes should allow identification of biomarkers to be used for the establishment of heat-resistant lines.

MATERIALS AND METHODS

Experimental animals and tissue collection. The heat stress experiment was conducted at Auburn University's Fish Genetics Research Unit in May 2011 using F_1 hybrid catfish fingerlings generated by crossing female channel catfish and male blue catfish, following the procedures conducted in Arctic charr (71, 72) with modifications according to temperature regime of catfish and available equipment (Fig. 1). All procedures involving the handling and treatment of fish during this study were approved by the Auburn University Institutional Animal Care and Use Committee prior to the initiation of the experiments. The fish were reared for 2 wk prior to challenge. Tanks
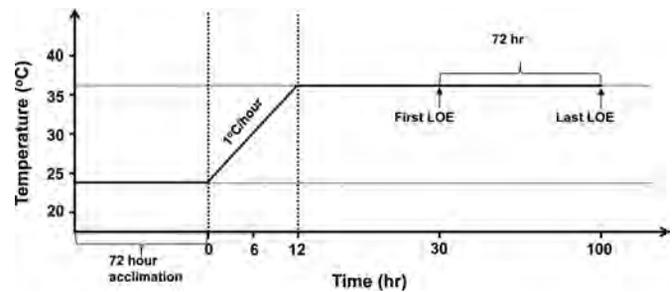

Fig. 1. Schematic presentation of the heat stress experiment. A total of 300 fish were acclimated for 72 h at ambient temperature (24°C) before heat stress treatment. Water temperature was then increased for the experimental fish at a pace of 1°C/h until it reached 36°C. The temperature was then held constant at 36°C, and the fish were closely monitored for signs of stress. The first and last 45 individuals that lost equilibrium (LOE) were quickly removed from the tank and sampled. The 1st fish showing LOE was observed after 30 h at 36°C, and the last fish showing LOE was observed about 72 h thereafter. A total of 50 fish were picked as controls, which were cultured under same conditions but without heat stress and were sampled during the experiment.

were set up with a constant flow-through system (0.45 l/s) with fresh pond water at ambient temperature (~24°C) and oxygen levels (8.0–10.0 ppm). Before the experiment, several trials were conducted to optimize the temperature that can be used to differentiate the phenotypic extremes (intolerant or tolerant). We finalized the temperature of 36°C, the observed lethal temperature for catfish (55). However, mortalities would not occur upon short exposures before the fish lose balance at this temperature. In the experiment, the fish were collected when they showed signs of losing balance, far short of lethality (see below).

In the experiment, a total of 300 fish were transferred to an experimental tank (length 3.2 m, width 0.5 m, and depth 1.0 m) and left to acclimate the fish for 72 h at ambient temperature. Meanwhile, 50 fish from the fish pool were randomly selected as control fish and cultured in another tank under the same conditions except without heat stress treatment. Water temperature was increased for the experimental fish through heat exchangers by approximately 1°C/h until it reached 36°C. Dissolved oxygen levels were allowed to fluctuate naturally and decreased from 8.5 ppm to a minimum of 6.8 ppm during the experiment. Fish were not fed after being transferred for experiment to avoid confounding gene expression changes due to feeding.

The temperature was held constant at 36°C, and the fish were closely monitored for signs of stress. The first and last 45 individuals showing loss of equilibrium (LOE) were quickly removed from the tank and sampled. The intermediate fish were removed and discarded. The sampled fish represented the 15% least heat tolerant and most heat tolerant extremes and were thereafter referred to as the intolerant and tolerant treatment groups, respectively. The first fish showing LOE was observed after 30 h at 36°C, and the last fish showing LOE was observed about 72 h thereafter (Fig. 1). It should be noted that tolerant group fish were exposed to lethal temperature for up to 3 days longer than intolerant group fish. Therefore, it should be recognized that any gene identified with differential expression between tolerant and intolerant fish can be caused by the longer heat exposure, rather than heat tolerance itself (71). Due to the lack of heat-tolerant/intolerant catfish strains and the irreversible nature of heat damage under the lethal temperature, we acknowledge this limitation of the experiment, same as in the previous study with Arctic charr (71). However, the goal of this study was to gain a broad understanding of heat response to provide insights into genome-level gene regulation involved in the processes and pathways in catfish.

Before sampling, fish were euthanized with MS-222 (300 mg/l) and then weighed and measured for body weight and length. Blood was withdrawn from the caudal vein of the fish (300–500 μl) for DNA

extraction, and the gill and liver were collected for RNA. Gill and liver were chosen for this study because of their high metabolic activity and essential physiological roles in response to stress (49). Gill tissue is the primary site of oxygen uptake, osmotic and ionic regulation, and export of nitrogenous wastes in fishes (24, 49). Expression studies in other fishes reveal extensive changes in gene expression in gill tissue in response to heat stress (12, 49, 50, 71, 72). Tissues were placed into RNAlater (Ambion), stored at room temperature for 24 h, and then moved to $-80°C$ for storage until RNA isolation. A total of 45 control fish were collected as control following the same protocol. The first 45 intolerant fish, last 45 tolerant fish, and the 45 control fish were randomly divided into three groups as three replicates, respectively.

RNA extraction, library construction, and sequencing. Total RNA was isolated from tissues using the RNeasy Plus Mini Kit (Qiagen) and treated with RNase-free DNase I (Qiagen) to remove genomic DNA following manufacturer's instructions. The concentration and integrity of total RNA were measured on an Agilent 2100 Bioanalyzer using an RNA Nano Bioanalysis chip. For each group, equal amounts of RNA from the three replicates were pooled for RNA-Seq library construction.

RNA-Seq was carried out by the HudsonAlpha Genomic Services Lab (Huntsville, AL) with the total mRNA from gill and liver. Briefly, the mRNA was fragmented and copied into first-strand cDNA using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. The cDNA fragments then went through an end repair process, the addition of a single "A" base, and then ligation of the adapters. The products were then purified and enriched with PCR to create final cDNA library. The cDNA libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina) according to the TruSeq protocol with modifications to capture total mRNA. The libraries were amplified with 15 cycles of PCR and contained TruSeq indexes within the adaptors, specifically *indexes 1–3*. Finally, amplified library yields were quantified. After KAPA quantitation and dilution, the libraries were clustered three per lane and sequenced on an Illumina HiSeq 2000 instrument with 100 bp paired-end reads.

De novo assembly of sequencing reads. Raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides before assembly. Reads with quality scores <30 and length <30 bp were trimmed. The resulted high-quality reads were used in subsequent assembly.

The de novo assembly was performed using a de Brujin graph assembler to obtain the best assembly result (76, 88). We used publicly available programs ABySS (version 1.3.0) (76) and Trans-ABySS (version 1.2.0) (74) to produce the assembly. Briefly, the clean reads were first hashed according to a predefined k-mer length, the "k-mers." After capturing overlaps of length k-1 between these k-mers, we assembled the short reads into contigs. The clean reads from all the three groups of both tissues were used for the de novo assembly. The ABySS assemblies were performed at various k-mers of every four ranging from 50 to 94. The assemblies from all k-mers were merged into one assembly by running the first stage of Trans-ABySS pipeline. The multiple-k approach produces redundant assemblies that may cause problems in subsequent analyses. Despite Trans-ABySS having built-in redundancy elimination solutions, additional steps were required to identify unique sequences (45). We passed the assembly to CD-HIT-EST (version 4.5.4) (38) for multiple comparisons after trimming contigs <200 bp to reduce redundancy.

Gene annotation and ontology. The assembled contigs were used as queries against the National Center for Biotechnology Information (NCBI) RefSeq zebrafish protein database and the UniProt-SwissProt (UniProt) database using the BLASTX program. The E-value cutoff was set at 1E-6 and only the top hits were initially assigned to each contig. The unannotated catfish contigs from zebrafish RefSeq and UniProt databases were annotated based on BLASTX searches against nonredundant protein (Nr) database. The same criteria were used for

BLASTX search against these protein databases. Gene ontology (GO) annotation analysis was performed using Blast2GO (version 2.5.0), an automated tool for the assignment of GO terms (19). The annotation result was categorized with respect to biological process, molecular function, and cellular component at *level 2*.

Identification of differentially expressed genes. To identify differentially expressed genes, the clean reads from each sample were mapped to the de novo assembly using CLC Genomics Workbench software (version 4.9, CLC bio). During mapping, at least 95% of the bases were required to align to the reference and a maximum of two mismatches were allowed. The number of total mapped reads for each transcript was counted and then normalized to determine RPKM (reads per kilobase of exon model per million mapped reads). Differentially expression analyses were performed using the RNA-Seq analysis and the Expression analysis modules in CLC Genomics Workbench. The proportions-based tests were conducted to identify the differentially expressed genes among control, tolerant, and intolerant groups with false discovery rate (FDR)-corrected P value < 0.05. The tests compared read counts by considering the proportions that they made up in the total sum of counts in each sample rather than raw counts, thereby correcting the data for sample size. The Benjamini and Hochberg method was used to control FDR for multiple testing (9). After quantile normalization of the RPKM values, fold changes were calculated. Transcripts with absolute fold change values of >2.0 were included in the analysis as from differentially expressed genes. Contigs with previously identified gene annotations were carried forward for further analysis.

Gene set enrichment analysis. To identify overrepresented GO terms in the differentially expressed gene set compared with the broader reference assembly, we performed enrichment analysis of significantly expressed GO terms using Ontologizer 2.0 (6) by the Parent-Child-Intersection method with a Benjamini-Hochberg multiple testing correction (31). The difference of the frequency of assignment of GO terms in the differentially expressed gene sets were compared with the overall catfish transcriptome assembly. The threshold was set as FDR value < 0.1. Functional groups and pathways encompassing differentially expressed genes were identified based on GO analysis, and pathway analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (40), with manual reannotation based on the several databases and literature search.

Experimental validation by quantitative real-time PCR. We selected 15 differentially expressed genes for validation using quantitative real-time PCR (qRT-PCR) with gene specific primers designed using Primer3 software (75). RNA samples from control, tolerant, and intolerant groups following heat stress (with three replicate samples each group) were used for qRT-PCR. First-strand cDNA was synthesized by iScript cDNA Synthesis Kit (Bio-Rad) using the blend of oligo(dT) and random hexamer primers, following manufacturer's protocol. All the cDNA products were diluted to 250 ng/μl and utilized for the qRT-PCR reaction using the SsoFast EvaGreen Supermix on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling profile consisted of an initial denaturation at 95°C (for 30 s), followed by 40 cycles of denaturation at 94°C (5 s), an appropriate annealing/extension temperature (58°C, 5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 to 95°C. The housekeeping gene 18S rRNA was tested and set as the reference gene (77); relative fold changes were calculated in the Relative Expression Software Tool version 2009 (67) based on the cycle threshold (Ct) values generated by qRT-PCR.

RESULTS

Fish sizes are not correlated with heat tolerance. Fish weight per group were as follows (average $\pm$ SD): control fish 13.30 $\pm$ 3.49 g, intolerant fish 13.56 $\pm$ 2.25 g, and tolerant fish 12.94 $\pm$ 2.33 g. Fish length per group were: control fish 13.18 $\pm$ 1.23 cm,

Table 1. *Illumina expressed short reads generation and trimming*

Sample	Group	Number of raw reads, million	Read length, bp	Number of reads after trim, million	Average length after trim, bp	Total bases after trim, Gb
Gill	control	46.58	100	40.16	95.2	3.82
	intolerant	66.62	100	56.90	95.0	5.40
	tolerant	63.81	100	55.00	95.2	5.23
Liver	control	56.64	100	47.93	95.0	4.55
	intolerant	72.87	100	62.10	95.1	5.91
	tolerant	51.87	100	44.08	94.8	4.18

intolerant fish 13.31 ± 0.93 cm, and tolerant fish 13.11 ± 0.95 cm. There were no significant difference in fish size (both body weight and length) among groups (one-way ANOVA followed by least significant difference test $P = 0.317$ and $P = 0.407$ for weight and length, respectively).

Sequencing of short expressed reads from catfish gill and liver. RNA-Seq was carried out on gill and liver samples from intolerant and tolerant catfish groups as well as from the control catfish group. Reads from group-specific samples were distinguished through the use of multiplex identifier tags for each tissue. A total of 177 million 100 bp paired-end reads were generated for gill, and a total of 181 million 100 bp paired-end reads were generated for liver samples. Greater than 46 million reads were generated for each of the three groups of gill tissue, and over 51 million reads were generated for each of the three groups of liver. After trimming, 152 million clean reads for gill and 154 million quality reads for liver were obtained for further analysis (Table 1). Raw reads data have been deposited at the NCBI Sequence Read Archive under accession number SRA049427.

De novo assembly of catfish transcriptome. Numerous de novo assemblers have recently been developed to address assembly of RNA-Seq short reads (53). The performance of some widely used programs was often compared (45, 82). By considering assemblies with long, accurate contigs to capture catfish genes and correctly identify differential expression, we chose the Trans-ABySS for good performance for catfish transcriptome assembly (45, 48, 81). The initial assembly provided 395,968 contigs with minimum length of 200 bp. After the redundant sequences were removed, the final assembly provided 144,746 contigs with an average length of 834 bp and N50 size of 1,341 bp (Table 2). A total of 37,665 contigs were longer than 1 kb, allowing for reliable representations of entire genes. The Nr set of contigs was used for subsequent gene discovery and differential gene expression analysis. The final assembly has been deposited in the National Animal Genome Research Program Aquaculture Genomics Data Repository (http://www.animalgenome.org/repository/aquaculture/Catfish_RNA-Seq_assembly.gz).

Gene identification and annotation. BLASTX was performed to annotate the catfish transcriptome assembly and inform downstream differential gene expression analysis. After BLASTX annotation, a total 57,710 contigs had a significant BLAST hit against 17,014 unique zebrafish genes (unigenes, Table 3) with an e-value cutoff of 1E-6. To obtain a complete annotation, we carried out the same BLASTX with the UniProt database and Nr database. The number of matches to the UniProt database was 51,319 contigs with a putative 14,473 unigene matches. The number of matches to the Nr database was 62,391 contigs with 22,868 putative unigenes (Table 3). Notably, a fraction of contigs with unigene hits from protein databases were still not informative because they are hypothetical proteins in the databases.

Identification of differentially expressed genes. A total of 2,260 unique genes showed significant differential expression between control fish and intolerant and/or tolerant fish in gill and/or liver after heat stress (Table 4). In gill, a total of 1,507 (66.7%, 1,507/2,260) genes showed significant differential expression between control fish and intolerant fish and/or tolerant fish. Of these, there were 1,120 (45.6%) genes differentially expressed in the tolerant fish relative to control fish and 1,001 (44.3%) genes differentially expressed in the intolerant fish relative to control fish. A total of 399 (17.7%) genes differentially expressed between the tolerant fish and intolerant fish, of which 274 genes were regulated in the same direction (either increase or decrease), and 125 genes were regulated in the opposite direction (up in one group but down in the other group) in tolerant fish and intolerant fish relative to control fish. Similar numbers of genes were upregulated and downregulated in the tolerant and intolerant groups. In liver, a total of 1,099 (44.6%) genes showed significant differential expression between control fish and intolerant and/or tolerant fish (Table 4). Of these, 645 (28.5%) genes were differentially expressed in the tolerant fish relative to control fish, and 680 (30.1%) genes were differentially expressed in the intolerant fish relative to control fish. A total of 432 (19.1%) genes were differentially expressed between the tolerant fish and intolerant fish, of which 210 genes were regulated in the same direction (either increase or decrease), and 222 genes were regulated in the opposite direction (up in one group but down in the other group) in tolerant fish and intolerant fish relative to control fish. The majority of genes displayed tissue specificity in response to heat stress with 1,161 (51.4%) genes differentially expressed only in the gill and 753 (33.3%) genes differentially expressed

Table 2. *Summary of de novo assembly statistics of expressed reads*

Assembly	Contigs ≥ 200 bp, n	Average Contig Length, bp	N50, bp	Contigs ≥ N50, n	Contigs ≥ 1 kb, n	Total Size, Mb
Initial assembly	395,968	843	1,316	73,440	105,710	333.8
Final assembly	144,746	834	1,341	25,873	37,665	120.8

Initial assembly was obtained by running Trans-ABySS with multiple k-mers and was subjected to CD-Hit-EST for redundancy removal to generate the final assembly.

Table 3. *Summary of gene identification and annotation of assembled catfish contigs*

Databases	Contigs With Protein Hits, n	Unique Protein Hits, n	Hypothetical Gene Matches, n
RefSeq	57,710	17,014	2,211
UniProt	51,319	14,473	328
Nr	62,391	22,868	3,084

only in the liver, and a group of 346 (15.3%) genes differentially expressed in both tissues (Fig. 2).

Validation of RNA-Seq results by qRT-PCR. To validate the differentially expressed genes identified by RNA-Seq expression analysis, we randomly selected 15 genes from those with differing expression patterns and from genes of interest based on their function, for qRT-PCR validation. Primers were designed based on contig sequences. Melting-curve analysis revealed a single product for all tested genes indicating the high reliability of transcriptome assembly. Fold changes from qRT-PCR were compared with the RNA-Seq expression analysis results (Fig. 3). As shown in Fig. 3, qRT-PCR results were significantly correlated with the RNA-Seq results (correlation coefficients $0.896-0.979$, P value < 0.001). In general, the RNA-Seq results were confirmed by the qRT-PCR results, indicating the reliability and accuracy of the RNA-Seq expression analysis.

Gene set enrichment and pathway analysis. The differentially expressed genes were then used to perform GO annotation. Analysis of GO term distribution at *level 2* showed that cellular process (GO:0009987), binding (GO:0005488), and cell (GO:0005623) were the most common annotation terms in each of the three GO term categories, separately. Parent-child GO term enrichment analysis detected a total of 83 significantly overrepresented GO terms, with FDR-corrected P value < 0.1. Around 10 higher level GO terms from each category were retained for further pathway analysis. In brief, the GO terms for biological processes include protein and carbohydrate metabolic process, proteolysis, protein translation, folding and polymerization, and response to stress. The GO terms for cellular component include cytoplasm, endoplasmic reticulum and protein synthesis complex such as ribosome complex and protein degradation complex such as proteasome complex. The molecular function terms include macromolecule stability such as rRNA/RNA/cofactor binding and unfolded protein binding, ion and molecule transporting such as calcium/iron ion binding, and lipid/calcium-dependent phospholipid binding.

The incomplete annotation of reference proteins in the databases complicated the enrichment analysis and downstream

pathway analysis. Unlike in well-characterized species such as human, the available pathway analysis software often could not resolve annotations into large functional categories or pathways for catfish. Therefore, we used a combination of KEGG pathway analysis, manual reannotation based on the several databases, and manual literature searches to identify broad functional categories of the differentially expressed genes (Fig. 4). As listed in Table 5, we identified six functional categories: *1*) protein folding, *2*) protein degradation, *3*) protein biosynthesis, *4*) energy metabolism, *5*) molecule and ion transport, and *6*) cytoskeleton reorganization. Putative functional roles and interactions of these genes involved in mediating the catfish response to heat stress are discussed in detail below.

DISCUSSION

Fish exposed to temperature higher than the optimal range show signs of stress, such as decreased immune response, growth, and reproduction (65, 72). This is an issue of increasing concern as temperature may continue to rise as a result of global climate change (11). Catfish experience serious heat stress during the summer, which induce disease outbreaks, and pond flooding coincides with hypoxia stress. The molecular mechanisms underlying the response to heat stress in catfish have not been studied. In the present study, we conducted a transcriptomic-level analysis of the response to heat stress in catfish using RNA-Seq.

RNA-Seq analyses for transcriptome profiling have been accepted as a robust approach to assess transcriptional responses to different experimental conditions (60, 62). Comparable to the results in our previous work (45, 81), use of 100 bp paired end reads and multiple k-mer assembly generated a high-quality reference transcriptome assembly under heat stress conditions containing 144,746 contigs with an average length of 834 bp. The assembled contigs had significant matches to $\sim$23,000 unique proteins in the most comprehensive protein database searched. The in-depth sequencing of transcripts expressed in gill and liver allows for the assessment of expression profiles of less abundant transcripts.

In the present study, we used master-pooled samples, created by combining equal amounts of RNA from three replicate pools (15 fish per pool), to assess expression profiles in individual pools. Obviously, pooled samples mask individual variation. However, the goal of this study was to gain a broad understanding of heat response in catfish and to provide early insights into the important processes and pathways. Follow-up work will use the results here as a foundation for more targeted studies. Notably, no previous studies have examined the transcriptional response to heat stress in catfish. Future studies will

Table 4. *Number of genes differentially expressed following heat stress challenge*

	Gill			Liver		
	T vs. C	I vs. C	T vs. I*	T vs. C	I vs. C	T vs. I*
Upregulation	431	395	246 (184)	341	337	182 (122)
Downregulation	689	606	153 (90)	305	343	250 (88)
Subtotal	1,120	1,001	399 (274)	645	680	432 (210)
Total genes from each tissue		1,507			1,099	
Total genes from both tissues			2,260			

C, control; I, intolerant; T, tolerant. Values indicate contigs/genes passing cutoff values of fold-change $\geq$2.0 and $P < 0.05$. *Genes differentially expressed in T relative to I, where upregulation indicates higher gene expression in T than I, and vice versa. Numbers in parenthesis indicate genes regulated in the same direction in T and I relative to C.
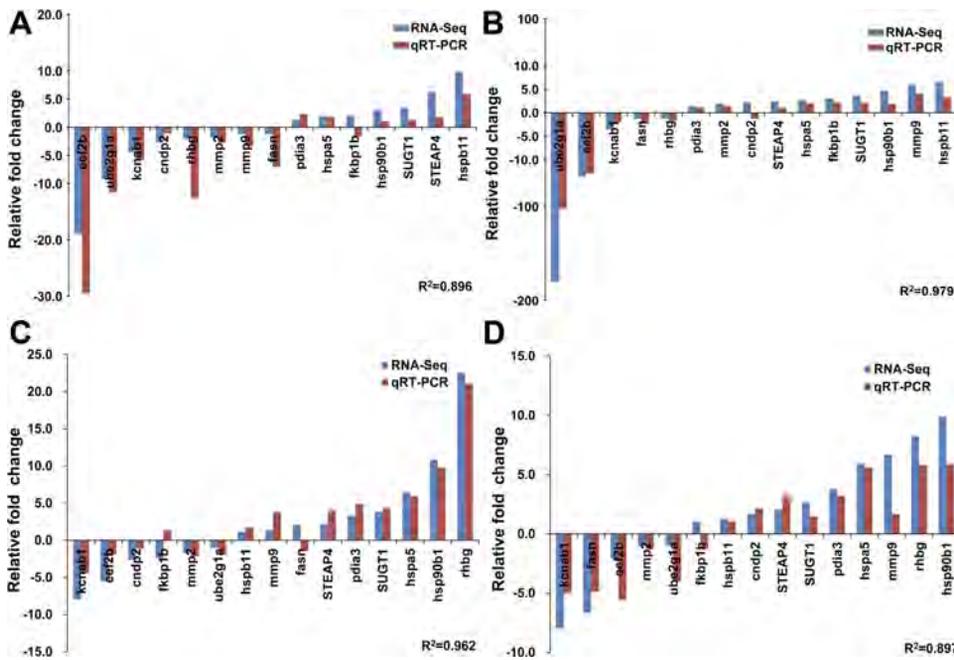
Fig. 2. Validation of RNA-Seq results using qRT-PCR. *A*: gill intolerant group, *B*: gill tolerant group, *C*: liver intolerant group and *D*: liver tolerant group. Gene abbreviations: kcnab1, potassium voltage-gated channel, shaker-related subfamily, beta member 1; ube2g1a, ubiquitin-conjugating enzyme E2G 1a; eef2b, eukaryotic translation elongation factor 2b; SUGT1, SGT1, suppressor of G2 allele of SKP1; mmp9, matrix metalloproteinase 9; fasn, fatty acid synthase; STEAP4, STEAP family member 4; hsp90b1, heat shock protein 90, beta (grp94), member 1; rhbg, Rhesus blood group, B glycoprotein; hspa5, heat shock protein 5; pdia3, protein disulfide isomerase family A, member 3; hspb11, heat shock protein, alpha-crystallin-related, b11; cndp2, CNDP dipeptidase 2; mmp2, matrix metalloproteinase 2 and fkbp1b, FK506 binding protein 1b. Fold changes represented gene expression changes in each group relative to control group.

utilize target genes identified here to examine individuals, strains, and families. To validate the RNA-Seq results, we performed qRT-PCR to determine whether the expression profiles in the master-pooled samples were consistent with those of the component replicate pools. Differentially expressed genes with various expression patterns from both groups were tested in both gill and liver. Significant correlations between qRT-PCR and RNA-Seq based expression profiles were observed (R^2 of 0.896–0.979, *P* value < 0.001; Fig. 3), suggesting the reliability of RNA-Seq for gene expression analysis.

A total of 2,260 genes were identified differentially expressed in gill and/or liver of intolerant and/or tolerant catfish in response to heat stress. These differentially expressed genes were enriched and categorized into broad functional categories based on GO annotation and manual reannotation through literature search of studies in vertebrate model species (Table 5). Several important processes or pathways were likely involved in mediating the catfish response to heat stress, as discussed below.

Heat stress resulted in transcriptional activation of genes involved in protein folding and related factors. Proteotoxic stressors such as heat can cause denatured proteins, which become cytotoxic by forming aggregates (25). Given the evidence that denatured proteins are increased at elevated temperatures, it is expected that constitutive protein-folding machinery would increase as well, which is indicated by higher expression of genes with chaperoning activities. These chaperone proteins are pivotal in maintaining protein homeostasis during cellular response to heat stress by interacting with

Fig. 3. Venn diagram of tissue-specific and mutually differentially expressed genes in catfish gill and liver.
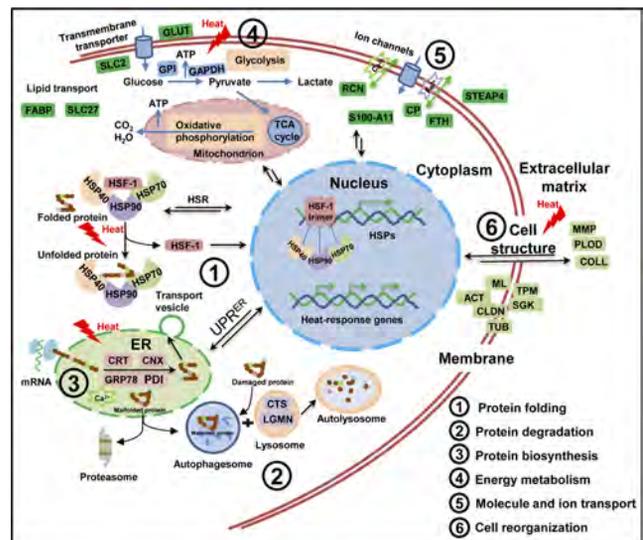
Fig. 4. Schematic presentation of gene pathways involved in heat stress responses based on RNA-Seq expression signatures in catfish gill and liver. Six pathways are indicated, as circled numbers 1–6. Gene pathway names are indicated at *bottom right* corner, and all the gene names are indicated in their relevant pathways. Full gene names are shown in Table 5. Abbreviations: HSP, heat shock response; UPR, unfolding protein response; ER, endoplasmic reticulum; TCA cycle, tricarboxylic acid cycle.

Table 5. *Enriched catfish genes differentially expressed following heat stress challenge involved in six molecular pathways*

Genes and Pathways	Gene Name	Contig ID	Gill		Liver	
			T vs. C	I vs. C	T vs. C	I vs. C
Protein folding						
DnaJ homolog subfamily A member 1	DNAJA1	k82:1035821	**70.7**	**33.3**	**51.2**	**53.3**
DnaJ homolog subfamily A member 4	Dnaja4	k86:809477	**43.1**	**26.3**	**128.7**	**146.0**
DnaJ homolog subfamily B, member 1	dnajb1	k86:846920	**11.9**	**6.6**	**13.4**	**8.8**
Heat shock protein 47	hsp47	k86:263021	**108.3**	**33.5**	**19.9**	**9.9**
Heat shock protein, alpha-crystallin-related, 1	hspb1	k86:131430	**24.2**	**28.4**	3.6	3.1
Heat shock protein beta-11	hspb11	k86:802606	**32.8**	**19.7**	1.3	1.1
Heat shock 60 kDa protein	hspd1	k78:1172625	1.6	1.8	**2.6**	**4.2**
Heat shock 70 kDa protein 1	HSPA1	k78:1282572	**403.1**	**146.9**	**308.2**	**36.4**
Heat shock cognate 71 kDa protein	Hspa8	k86:834390	**14.6**	**20.0**	3.5	2.4
Heat shock cognate 70 kDa protein, like	HSC73L	k86:91142	**301.8**	**107.5**	**80.2**	**9.7**
78 kDa glucose-regulated protein	hspa5	k86:369242	**2.7**	**2.0**	**5.9**	**6.4**
Heat shock protein HSP 90-alpha 1	hsp90AA1	k70:704082	**18.5**	**16.3**	**12.8**	**14.5**
Heat shock protein 90-alpha 2	hsp90AA2	k86:310147	**13.3**	**9.3**	**11.7**	**14.6**
Heat shock protein HSP 90-beta	hsp90ab1	k74:1392914	**2.3**	**2.7**	**2.7**	**4.7**
Endoplasmin	hsp90b1	k86:230807	**4.8**	**3.1**	**9.9**	**10.9**
Hsp90 co-chaperone Cdc37	cdc37	k86:835445	**3.1**	**2.7**	**2.3**	**2.6**
Activator of 90 kDa heat shock protein ATPase homolog 1	ahsa1	k58:421783	**5.2**	**4.6**	3.1	**4.6**
Hsc70-interacting protein	st13	k82:1036915	1.5	1.2	**2.5**	**2.5**
Stress-induced-phosphoprotein 1	stip1	k86:823276	**2.8**	2.6	**3.9**	**5.3**
Calreticulin	crt	k86:641264	**5.7**	**2.5**	**6.3**	**5.2**
Calreticulin like	crtl	k82:584598	**7.1**	**3.8**	**7.4**	**6.4**
Calnexin	cnx	k78:1255865	2.1	1.8	**3.1**	**3.0**
T-complex protein 1 subunit delta	cct4	k82:1065713	1.9	**2.3**	1.4	**2.9**
Calumenin-B precursor	calub	k74:1414840	**2.8**	1.3	1.7	**2.1**
Cysteine and histidine-rich domain-containing protein 1	CHORDC1	k86:828025	**32.4**	**29.2**	1.6	1.5
Peptidyl-prolyl *cis*-trans isomerase FKBP4	fkbp4	k86:844065	**4.6**	**3.7**	5.0	**6.1**
Peptidyl-prolyl *cis*-trans isomerase FKBP5	fkbp5	k82:82465	**2.9**	**16.9**	2.2	**4.6**
Protein disulfide-isomerase family A, member 4	pdia4	k82:26328	**3.0**	1.4	**10.8**	**7.8**
Protein disulfide-isomerase family A, member 6	pdip6	k86:90926	**2.9**	1.7	**5.9**	**5.0**
2-peptidylprolyl isomerase A	ppia	k86:792081	**7.1**	**4.9**	**5.2**	2.5
Protein degradation						
Cathepsin B, a	ctsba	k86:458583	**2.2**	1.5	1.7	1.3
Dipeptidyl peptidase 1	ctsc	k86:838958	**2.7**	2.0	2.7	1.4
Cathepsin D	ctsd	k82:1070077	**2.9**	1.7	2.0	1.3
Cathepsin L, 1a	ctsl1a	k78:905860	**4.5**	**2.7**	**2.2**	**2.3**
Cathepsin Z	ctsz	k86:812568	**2.6**	1.9	**2.2**	−1.1
Legumain	lgmn	k82:251480	**2.8**	1.8	**2.2**	1.6
Sequestosome-1	sqstm1	k86:844100	**3.7**	1.7	**2.7**	1.2
Proteasome subunit alpha type-1	psma1	k82:1069340	**−2.1**	**−2.6**	−1.3	−1.5
Proteasome alpha 2 subunit	psma2	k78:1265298	**−3.2**	**−3.1**	**−2.3**	**−2.2**
Proteasome subunit alpha type-4	psma4	k86:802539	**−2.2**	**−2.5**	−1.5	−1.4
Proteasome subunit alpha type-5	psma5	k70:1168523	**−3.8**	**−3.9**	**−2.3**	**−2.5**
Proteasome alpha 6b subunit	psma6b	k86:817782	**−2.7**	**−2.4**	−1.9	**−2.6**
Proteasome subunit beta type-1	psmb1	k70:1376830	**−2.1**	**−2.2**	−1.6	−1.6
Proteasome subunit beta type-2	psmb2	k82:1042898	**−2.5**	**−2.4**	−1.5	−1.8
Proteasome subunit beta type-3	psmb3	k78:1293190	**−2.4**	**−3.2**	**−2.4**	**−2.4**
Proteasome subunit beta type-4	psmb4	k86:844329	**−2.6**	**−2.8**	**−2.3**	**−2.3**
Proteasome subunit beta type-8	psmb8	k86:829682	**−3.7**	**−2.4**	−2.1	**−3.6**
Proteasome subunit beta type-10	PSMB10	k94:1380637	**−3.7**	**−2.9**	**−2.8**	**−3.2**
Proteasome maturation protein	POMP	k86:822237	**−3.1**	**−3.5**	**−3.8**	**−3.4**
Ubiquitin-conjugating enzyme E2 G2	ube2 g2	k54:1968304	−1.2	−1.4	**−2.8**	−1.8
Ubiquitin-conjugating enzyme E2 L3	ube2L3	k58:1807238	**−2.4**	−2.0	−1.8	−1.9
Ubl carboxyl-terminal hydrolase 18	USP18	k74:1426845	**−25.3**	**−9.8**	−18.5	−7.2
Protein biosynthesis						
Brix domain containing 2	bxdc2	k70:1593689	**−2.3**	**−2.6**	−2.3	−1.2
Elongation factor 1-alpha	ef1a	k86:460537	**2.0**	1.7	1.6	1.3
Eukaryotic translation initiation factor 4A-III	eif4a3	k86:845339	−1.9	**−2.1**	**−2.5**	−1.9
Eukaryotic translation initiation factor 6	eif6	k78:1276539	**−3.5**	**−3.7**	**−3.5**	**−2.4**
High mobility group protein B1	hmgb1a	k86:816090	**−3.2**	**−4.7**	−1.5	**−4.6**
High-mobility group box 2a	hmgb2a	k94:113539	−2.0	**−2.9**	**−2.8**	**−2.6**
High-mobility group box 2b	hmgb2b	k58:1015178	**−2.1**	**−3.3**	**−3.1**	**−2.8**
U6 snRNA-associated Sm-like protein LSm5	lsm5	k86:817939	**−2.3**	**−2.9**	−2.4	−1.6
U6 snRNA-associated Sm-like protein LSm6	lsm6	k86:652739	−2.0	**−2.9**	**−2.6**	**−2.7**
Protein mago nashi homolog	magoh	k86:489024	**−2.3**	**−2.8**	**−2.6**	**−2.1**

Continued

Table 5.—*Continued*

Genes and Pathways	Gene Name	Contig ID	Gill		Liver	
			T vs. C	I vs. C	T vs. C	I vs. C
Nonhistone chromosome protein 2-like 1	nhp2l1	k86:845565	**−3.8**	**−3.9**	**−3.5**	−1.9
60S ribosomal protein L12	rpl12	k70:1647174	**−462.3**	**−6.4**	−2.6	−2.9
60S ribosomal protein L13a	rpl13a	k54:2659627	**−1,585.1**	**−5.0**	−3.9	−18.0
60S ribosomal protein L15	rpl15	k90:1943685	**−4,546.1**	**−6.1**	−10.4	−17.8
60S ribosomal protein L18	RPL18	k70:1285008	**−152.8**	**−5.0**	−1.6	−3.9
60S ribosomal protein L23	rpl23	k54:2668119	**−1,851.9**	**−8.5**	−10.9	−3.5
60S ribosomal protein L27a	rpl27a	k66:1848087	**−189.9**	**−3.4**	−2.3	−2.4
40S ribosomal protein S15	rps15	k58:1845849	**−1,047.5**	**−6.2**	−1.7	−1.7
40S ribosomal protein S15a	rps15a	k50:2880055	**−183.2**	**−3.4**	−3.1	−1.3
40S ribosomal protein S23	rps23	k54:26742787u	**−272.5**	**−6.8**	−9.2	−3.7
Splicing factor, arginine/serine-rich 3b	sfrs3b	k94:134938	−1.9	**−2.4**	−1.7	**−3.8**
Small nuclear ribonucleoprotein polypeptides B	snrpb	k78:899812	**−2.1**	**−2.2**	**−2.3**	−1.4
Small nuclear ribonucleoprotein E	snrpe	k86:839318	**−2.8**	**−3.9**	−1.4	−1.2
Small nuclear ribonucleoprotein polypeptide F-like	snrpf	k74:246213	**−2.5**	**−3.7**	−1.8	−1.5
Single-stranded DNA-binding protein 1	ssbp1	k82:1063028	−1.6	**−2.4**	**−3.4**	**−5.9**
Signal transducer and activator of transcription 1b	stat1b	k86:839401	**−5.4**	**−2.3**	−4.2	−1.8
THO complex subunit 4	thoc4	k70:685388	**−2.5**	**−2.5**	−1.2	−1.3
Nucleolar protein of 40 kDa	zcchc17	k86:832796	−1.2	−1.4	**−2.6**	**−2.2**
Energy metabolism						
Glyceraldehyde 3-phosphate dehydrogenase, spermatogenic	gapdhS	k86:11563	**2.0**	1.6	**5.6**	2.8
Glucose phosphate isomerase a	gpia	k86:831094	**3.2**	1.9	**2.9**	**2.4**
Glucose phosphate isomerase b	gpib	k58:259573	**3.0**	1.8	**2.5**	**2.3**
L-lactate dehydrogenase A chain	ldha	k74:1396467	**3.1**	1.8	4.7	3.4
Brain creatine kinase	ckba	k86:813805	**3.9**	1.7	**4.5**	**2.3**
Inositol-3-phosphate synthase 1-A	isyna1-a	k82:1068707	**5.1**	**3.4**	5.1	2.3
Neutral alpha-glucosidase AB	GANAB	k54:2676704	2.3	1.8	**5.3**	**3.7**
Glucosidase 2 subunit beta	prkcsh	k86:840313	**2.5**	1.9	**4.1**	**3.4**
Cytochrome c oxidase subunit II	COX2	k86:783505	**−2.9**	**−3.6**	−1.5	−1.7
Cytochrome c oxidase, subunit VIIa 2	cox7a2	k82:44955	1.3	1.0	**2.1**	**2.6**
NADH dehydrogenase subunit 1	mt-nd1	k90:1883855	**−2.2**	**−3.5**	−2.0	**−7.0**
NADH dehydrogenase subunit 2	mt-nd2	k74:1374513	−1.3	**−2.0**	−1.2	**−2.0**
NADH dehydrogenase subunit 6	mt-nd6	k82:875412	−1.9	**−2.9**	**−3.4**	**−10.7**
Alpha-2-macroglobulin receptor-associated protein	lrpap1	k66:1808772	2.6	1.4	**4.5**	**4.1**
Apolipoprotein A-I precursor	apoa1	k78:1228908	**2.5**	1.1	−1.0	1.6
3 beta-hydroxysteroid dehydrogenase type 7	hsd3b7	k94:1379352	1.3	2.3	2.8	**4.6**
Lathosterol oxidase	sc5dl	k86:438358	1.4	−1.3	**2.0**	**−5.3**
Beta-hexosaminidase subunit beta	hexa	k86:840303	1.8	1.6	**3.8**	2.6
Cholesterol 7-alpha-monooxygenase	cyp7a1	k94:1397096	3.5	2.3	**12.2**	**29.5**
Sterol 12-alpha-hydroxylase	Cyp8b1	k94:1393302	2.5	2.8	**2.7**	**4.3**
Spermine oxidase	SMOX	k86:838382	**3.7**	**4.0**	3.5	3.6
L-threonine 3-dehydrogenase	tdh	k82:509646	1.2	**3.6**	3.6	**24.8**
Molecule and ion transport						
Fatty acid-binding protein 1, liver	FABP1	k82:44080	**6.0**	1.2	1.9	−2.1
Fatty acid-binding protein 3, heart	FABP3	k94:168024	**5.8**	1.0	1.7	−2.2
Fatty acid binding protein 11b	fabp11b	k70:1653527	**3.5**	1.1	4.5	2.2
Solute carrier family 27, member 6	slc27a6	k86:785730	1.1	1.7	**2.5**	**3.8**
Hemoglobin subunit alpha	hba	k62:785473	**36.6**	−2.0	**11.9**	−5.0
Hemoglobin subunit alpha	hba	k78:710863	**5.4**	−2.2	**2.4**	−2.4
Hemoglobin subunit beta	HBB	k82:991835	**5.2**	−1.0	2.0	−2.8
Hemoglobin subunit beta	hbb	k86:781905	**3.2**	−1.1	1.7	**−2.1**
Hemoglobin subunit beta	HBB	k86:758616	**2,091.6**	−1.7	**284.0**	−2.2
Hemoglobin subunit beta	HBB	k50:438230	**1,361.1**	**−6.3**	**713.7**	1.3
Hemoglobin subunit beta	hbb	k86:796083	**6.5**	−1.5	**3.5**	**−2.7**
Hemoglobin subunit beta	HBB	k86:818806	**4.2**	−1.1	**2.1**	**−2.3**
Rhesus blood group, C glycoprotein a	rhcga	k86:843910	**2.1**	**2.1**	15.9	9.8
Reticulocalbin-3	rcn3	k62:2057153	**9.4**	2.3	**4.5**	**2.6**
Protein S100-A11	S100A11	k86:94120	**2.2**	**2.8**	10.9	34.8
Corticosteroid-binding globulin	Serpina6	k82:1033615	10.4	15.7	**3.1**	**2.3**
Solute carrier family 2, facilitated glucose transporter member 1	Slc2a1	k66:1865805	**6.1**	4.6	6.4	3.0
Metalloreductase STEAP4	STEAP4	k82:213081	**2.5**	**6.2**	**2.1**	**2.1**
Ferritin heavy chain	FTH	k94:1382080	**2.3**	1.1	1.7	1.2
Ceruloplasmin	cp	k82:216553	7.4	3.3	**4.9**	**3.7**

Continued

Table 5.—*Continued*

Genes and Pathways	Gene Name	Contig ID	Gill		Liver	
			T vs. C	I vs. C	T vs. C	I vs. C
Cytoskeletal reorganization						
Ras GTPase-activating protein-binding protein 2	G3BP2	k70:1642860	1.4	1.3	**3.3**	**2.6**
Serine/threonine-protein kinase Sgk1	sgk1	k58:1833023	2.8	1.2	3.1	**8.1**
Serine/threonine-protein kinase SIK2	SIK2	k94:1396796	2.0	5.9	2.7	**12.7**
Actin, aortic smooth muscle	acta2	k78:1206032	**2.4**	1.6	1.7	1.5
Actin, alpha cardiac	Actc1	k82:1048655	**2.1**	1.5	1.3	−1.1
Alpha-actinin-1	actn1	k82:1044661	**2.5**	1.2	2.2	1.5
Claudin-1	cldn1	k94:1394253	**2.6**	2.3	−4.7	−1.7
Claudin a	cldna	k82:1054157	**3.3**	2.3	1.3	−6.6
Claudin e	cldne	k86:825971	**3.7**	2.1	−1.1	−27.6
Claudin i	cldni	k82:1069563	1.9	**3.6**	−1.1	−1.4
Cytokeratin type I, enveloping layer	cyt1	k90:1891146	1.8	**2.9**	1.7	**3.0**
Cytokeratin-like	zgc:109868	k82:1013032	2.0	**4.7**	1.9	**3.9**
Tropomyosin alpha-4 chain	tpm4	k74:236151	**4.1**	1.5	**2.9**	**2.9**
Troponin C type 2 (fast)	tnnc2	k86:832884	**−2.1**	−1.5	−29.8	−240.8
Troponin I, cardiac muscle	tnni3	k94:1387663	**−22.7**	**−11.9**	−4.2	−1.8
Keratin, type I cytoskeletal 50 kDa	K1C1	k94:718429	**−6.9**	**−6.4**	−12.6	−10.0
Keratin 12	KRT12	k86:510928	**−3.4**	**−2.6**	−13.1	−8.0
Myosin regulatory light chain 2,	myl7	k50:2865364	7.1	**451.2**	1.6	−115.0
Myosin, light polypeptide 9,	myl9	k82:1064863	**2.5**	1.3	1.3	1.4
Tubulin, beta 2c	tubb2c	k82:1050477	2.0	**2.5**	4.4	−1.5
Tubulin, beta 5	tubb5	k78:1273875	**5.7**	1.1	4.5	−40.9
Gamma-aminobutyric acid receptor-associated protein-like	GABARAPL	k94:1381854	**5.1**	**14.8**	2.1	**5.6**
Matrix metalloproteinase-9	mmp9	k70:1632700	**5.9**	−1.2	6.7	1.4
Matrix metalloproteinase-13	mmp13	k86:843929	**2.5**	−1.6	1.1	−2.1
Matrix metalloproteinase-18	mmp18	k86:843930	**2.7**	−1.4	2.2	1.2
Collagen alpha-1(I) chain	col1a1a	k78:1250060	**2.0**	−1.2	2.2	−3.0
Collagen, type I, alpha 1b	col1a1b	k86:653563	**1.8**	−1.3	2.0	−4.3
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	Plod3	k86:836976	**5.3**	3.3	**5.3**	**5.7**
Disintegrin and metalloproteinase domain-containing protein 8	adam8	k82:1059795	**4.9**	1.6	36.5	5.6
A disintegrin and metalloproteinase with thrombospondin motifs 1	Adamts1	k82:1040356	2.0	**9.8**	2.2	5.4

Values in boldface indicate groups where the gene was significantly changed relative to the control ($P < 0.05$). C, control; I, intolerant; T, tolerant.

stress-denatured proteins to prevent their aggregation and malfolding (66). The most strongly inducible genes were usually those of molecular chaperones such as heat shock proteins (12).

Heat shock proteins are a class of functionally related proteins involved in the folding and unfolding of other proteins (25, 58). The expression was reported to increase when cells are exposed to elevated temperature or other stress (21). In this study, numerous heat shock proteins were upregulated after exposure to heat stress (Table 5). These included three members of the HSP40 family: DNAJA1, DNAJA4, and DNAJB1B. The HSP40 family plays a role in regulating the ATPase activity of HSP70 by interacting HSP70 with J domain. As expected, three HSP70 proteins were also upregulated: GRP78 (HSPA5), HSPA1A, and HSC73L. These proteins have been reported to be involved in protein folding and unfolding, providing thermo-tolerance to cells on exposure to heat stress (21). Four members of the HSP90 family, HSP90AA1, HSP90AA2, HSP90AB1, and HSP90B1, and two cofactors, CDC37 and AHAS1, were upregulated in both gill and liver (Table 5). HSP90 assists in protein folding and stabilization, and AHSA1 has been shown to interact with HSP90AA1 as an activator (64). In addition, the expressions of two small heat shock proteins (HSPB1 and HSPB11) were highly induced in gill of both tolerant and intolerant catfish. Notably, small heat shock protein beta 11 (HSPB11) and HSP90 were reported to associate with upper temperature tolerance in Arctic charr (71).

Protein folding is mediated by an array of proteins that act as molecular chaperones and foldases. The foldases catalyze protein folding by rearrangements of disulfide bonds by the protein disulfide isomerase (PDI) or isomerization of peptide bonds by peptidyl-prolyl isomerases (27, 28). PDI encodes the beta subunit of prolyl 4-hydroxylase, catalyzing the formation, breakage, and rearrangement of disulfide bonds (86). Peptidyl-prolyl isomerases were reported to be heat-stress inducible in plants (44). In addition, CALRs function as quality-control chaperones that bind to misfolded and unfolded proteins to prevent them from being exported from the endoplasmic reticulum to the Golgi apparatus (41).

The expressions of genes involved in protein degradation were coordinately modulated. Despite the protein rescue and folding process, heat stress can cause irreversible misfolded and damaged proteins that molecular chaperones cannot remedy (66). Such damaged proteins need to be removed through proteolysis to avoid forming cytotoxic aggregates (43). There are increasing evidences that the function of chaperones in preventing or reversing protein aggregation is linked to their roles in presenting misfolded intermediates to the cellular machinery for proteolytic degradation (33, 52). The majority of protein degradations are achieved in two ways: proteolysis in lysosome by the autophagy-lysosomal pathway and transport of unwanted proteins to the proteasome by the ubiquitin-proteasome pathway (UPP). The autophagy-lysosomal pathway is normally a nonselective process where the lysosome

contains a large number of proteases such as cathepsins, while the ubiquitin-mediated process is selective. Proteins marked for degradation are covalently linked to ubiquitin. The polyubiquinated proteins are targeted to an ATP-dependent protease complex (proteasome) for degradation. Cathepsins are lysosomal proteases that have been reported to be involved in fish muscle degradation (5). Cathepsin L was induced in both gill and muscle tissues in the goby fish exposed to heat stress (12). Legumain encodes a cysteine protease that may be involved in the processing of endogenous proteins for MHC class II presentation in the lysosomal/endosomal systems (18). Legumain has been reported as a stress-responsive protein induced under certain conditions including heat shock and hypoxia (17, 89). SQSTM1 was reported in the cellular response to unfolded proteins, serving a pivotal role in forming protein aggregates, protein turnover and autophagy (29). In contrast, an array of genes involved in UPP proteolysis were downregulated in catfish (Table 5), indicating that the ATP-dependent proteolysis way was repressed. Inhibition of the proteasome has been reported to confer thermotolerance in canine kidney cells (16). Treatment of cells with proteasome inhibitors can stimulate the expression of cytosolic heat shock proteins and/or endoplasmic reticulum molecular chaperones and markedly increase the survival of cells subjected to high temperatures up to 46°C (16). Therefore, it is reasonable to speculate that the repressed expression of proteasome genes relates to heat response. In addition, the repression of ATP-dependent proteolysis probably also relates to ATP-saving for survival during exposure to long-time lethal high temperatures.

Genes involved in forming transcription and translation machineries were downregulated in response to heat stress. It has been widely observed that heat stress preferentially induces specific stress-responsive proteins while repressing general protein synthesis (14, 46). The effects of acute heat stress on the transcriptional regulation of genes involved in protein synthesis have been reported in several studies (12, 13, 15, 49). Heat stress-associated expression changes of numerous components of the protein synthetic machinery were observed in both gill and muscle of goby fish (12, 49). In the present study, the ribosomal protein genes were significantly repressed in gills of catfish exposed to high temperature. The repression of these genes in catfish rendered slightly different observations compared with previous studies. In a recent study, ribosomal genes including RS4, RL28, RL6, RS30, RS19, RL34, and RLAO were upregulated in response to thermal acclimation in goby fish (50). In another study, ribosomal protein genes RL10, RL32, RS11, and RS23 were repressed, whereas RL19 and RS8 were induced in the gill (12). The ribosomal proteins can stabilize various subregions of the ribosomes, and perhaps their expression or repression during heat stress protects ribosomal structure and function through replacement or substitution of these key structural components (12). However, under lethal heat stress such as in the present study, such general protein synthesis process may be repressed, while specific stress-responsive proteins were preferentially produced to enhance cell survival on exposure to heat stress.

Similarly as ribosomal protein genes, several other genes involved in transcription and translation were repressed as well. Two translation initiation factors (EIF4A3 and EIF6) were repressed in both gill and liver in catfish (Table 5). EIF4A3 was implicated in a number of cellular processes involving alteration of RNA secondary structure, such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. EIF6 functions as a translation initiation factor and catalyzes the association of the 40S and 60S ribosomal subunits along with EIF5 bound to GTP. Of these, the snRNA-associated protein and mago nashi protein homolog were reported to be downregulated in *Penicillium glabrum* subjected to thermal stress (59).

Genes involved in ATP-derived energy production were induced by heat stress. Stress response and repair mechanisms are energy-costing processes. Therefore, the expression of genes involved in metabolisms may be in response to heat stress. Several genes involved in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, and the electron transport chain were induced or repressed in both gill and liver (Table 5).

Heat stress resulted in significant induction of several ATP-generating enzymes. Genes encoding enzymes involved in glycolysis and ATP production included GPIA, GPIB, LDHA, and GAPDHS. The GPI and LDHA were reported to be induced in response to heat stress in goby fish (12). GAPDHS was upregulated in both gill and liver in catfish. In mammals, GAPDHS is a sperm-specific glycolytic enzyme that may regulate glycolysis and energy production required for sperm motility and male fertility (57). However, the GAPDHS gene identified in this work and its counterparts identified from other fishes are not necessarily sperm-specifically functional in fish. The induced expression of GAPDH was reported in bovine endothelial cells on exposure to hypoxia (30). In contrast, several genes encoding enzymes involved in respiratory chain were repressed (mt-ND1, mt-ND2, mt-ND6, and COX2). The repression of these genes indicates that glycolysis might act as the main means of generating ATPs rather than by aerobic metabolic processes, which require a high level of oxygen consumption. Under conditions with low-oxygen levels caused by high water temperature, processes consuming less oxygen are used to increase survival chances. The transition to anaerobic metabolisms as a result of thermally induced hypoxia has been observed in other aquatic animals (4, 78, 79). Animals show reduced aerobic activity due to the limited capacity of the oxygen supply (56, 68, 70).

The expression of genes involved in lipid metabolism is always found to change during heat stress because of changes in membrane composition and in fuel preferences for ATP production (34, 36). High temperature can affect cell membrane integrity. The maintenance of membrane fluidity at high temperature is a common acclimatizing response involving major alterations to membrane composition (36). Therefore, expression of genes related to lipid synthesis were expected to change in response to heat shock. In catfish, we identified several genes with expression changes involved in lipid synthesis (Table 5). For instance, beta-hexosaminidase subunit beta (HEXA), involved in lipid membrane metabolic processes, was induced in liver of tolerant catfish. HEXA is responsible for degrading glycosphingolipids and other molecules with terminal N-acetyl hexosamines. The upregulation of this gene was observed in goby fish in response to thermal acclimation as well (50). Alpha-2-macroglobulin receptor-associated protein (RAP or LRPAP1), implicated in lipid metabolism (63), was significantly induced in catfish liver.

Heat stress causes increased expression of genes encoding proteins involved in transporting various molecules and ions. Genes encoding proteins involved in transporting various molecules and ions throughout the cell were identified and grouped according to this shared functional property (Table 5), but these genes can function in different cellular processes. Some of the gene products are for transporting specific molecules such as glucose, lipids, proteins, oxygen, iron, or calcium throughout the cell, while others mediate transport though the Golgi.

Corresponding to the upregulation of genes involved in ATP-derived energy production, genes associated with transporting molecules that are involved in these metabolic processes were modulated as well. SLC2A1 (GLUT1) was significantly upregulated in the gill of the tolerant fish. SLC2A1 was reported to facilitate the transport of glucose across the plasma membranes of mammalian cells (61). Genes involved in lipid transport include several fatty acid binding proteins and a solute carrier transporter. The fatty acid binding proteins (FABP1, FABP3, and FABP11B), recognized as intracellular lipid transporters, were highly induced in gill of tolerant fish. The solute carrier family 27 member 6 (SLC27A6), known as a fatty acid transporter, was also upregulated in liver of both tolerant and intolerant fish. Corticosteroid-binding globulin (SERPINA6 or CBG), encoding an alpha-globulin protein with corticosteroid-binding properties, was upregulated. SERPINA6 is the major transport protein for glucocorticoids and progestins in the blood of most vertebrates and was reported to change in response to stress including heat (3, 35, 80).

Genes involved in oxygen transport, including several hemoglobin subunits, were significantly upregulated in both gill and liver. As mentioned, elevated water temperature is closely associated with decreased concentration of dissolved oxygen. However, we do not believe such a change in dissolved oxygen led to hypoxic conditions, as dissolved oxygen levels never went below 6.8 ppm during heat stress treatment. We do believe that high temperature could reduce the capacity of oxygen delivery system (ventilation and circulation) and probably the affinity of oxygen molecule with hemoglobin, thus decreasing transport efficiency. In addition, under heat stress conditions, the requirement for oxygen in delivering an elevated metabolic rate could account for the dramatically induced expression of hemoglobins. The observation that insufficient internal oxygen supply can limit thermal tolerance in a variety of aquatic organisms at temperature extremes has led to the concept of oxygen limitation of thermal tolerance in animals (4, 26, 56, 68–70). This concept suggests that animals at the borderline of their thermal tolerance show a reduction in aerobic capacity because of the limited capacity of the oxygen supply, rather than falling levels of ambient oxygen (4). It is of interest to mention that increased gill perfusion has been reported as one of the compensations for decreasing oxygen (73). However, we do not believe that increased perfusion was the major reason for the observed increase in hemoglobin expression. Expression of various hemoglobin genes was upregulated hundreds- to thousandsfold (Table 5), and apparently increased red blood cell perfusion into the gill could not possibly account for such large changes alone without transcriptional regulation. As discussed in Quinn et al. (71), these results warrant future investigations on expression profiling of individual hemoglobin genes in blood-only and gill-only tissues.

A number of ion transporters were identified as differentially expressed (Table 1). Ion transport is critical for regulation of osmotic regulation and acid-base balance, as well as excretion of nitrogenous wastes (24). One ammonia transporter (RHCGA), likely responding to the increased secretion of ammonia at high temperature, was upregulated. Several genes involved in iron transport and metabolism were upregulated, including the metalloreductase (STEAP4), ceruloplasmin (CP), and ferritin heavy chain (FTH). STEAP4 is involved in iron transport using NAD^+ as acceptor. CP plays a role in iron metabolism and exhibits a copper-dependent oxidase activity to oxidize ferrous iron into ferric iron, assisting its transport in the plasma in association with transferrin. Appropriate iron level is essential for hemoglobin to function; however, excess iron can contribute to the formation of reactive oxygen species, leading to protein, lipid, and DNA damage (20). Once the iron level is too high, transferrin receptor synthesis is repressed, while ferritin synthesis is expected to be induced (47). The protein S100-A11 (S100A11), reticulocalbin-3 (RCN3), and calumenin-B precursor (CALUB), involved in calcium transport, were upregulated. Proteins involved in Ca^{2+} handling including troponins (TNNC2 and TNNI3) and parvalbumin (PVALB3) were downregulated. These proteins control the release and reuptake of Ca^{2+}, playing the central role in intracellular transport (1) and participating in the secretory process and signal transduction (37).

Exposure to heat results in the increased expression of genes encoding cytoskeletal organization and extracellular matrix factors. In addition to the effects on internal cellular processes, heat stress can affect the cell ultrastructure. Ras GTPase-activating protein-binding protein 2 (G3BP2) was induced significantly in liver of both tolerant and intolerant fish. G3BP2 contributes to the formation of stress granule in response to heat stress (54), serving as putative heat and/or pressure sensors. Genes encoding multiple structural components of the cytoskeleton were induced including several actin, claudin, myosin, and tubulin (Table 5). In addition, several cytoskeleton-associated proteins including the contractile protein tropomyosin (TPM4) was upregulated, while troponin (TNNC2 and TNNI3) and keratin (KRT12 and K1C1) were downregulated. These proteins play roles in adjusting cell volume through manipulation of transporters and cytoskeletal reorganization, respectively (85). Exposure to heat stress also increase expression of genes involved in extracellular matrix synthesis and remodeling. These genes include matrix metalloproteinase genes (MMP9, MMP13, and MMP18) and collagen genes (COLLA1A and COLLA1B).

The induction of multiple structural components of the cytoskeleton was mainly observed in gill, consistent with the observation in Buckley et al. (2006) (12). There was no induction of the major cytoskeletal genes such as those encoding claudin, myosin, and tubulin in liver (Table 5). Heat stress can cause a secondary osmotic shock in the gill perhaps by temperature effects on the membrane. Osmotically stressed cells modulating cell volume and therefore the cytoplasm's osmotic strength by reorganizing the cytoskeleton structure may reflect an attempt to regain osmotic balance (12, 22).

Different responses to heat stress exist between tolerant and intolerant catfish. We fully acknowledge the limitations of this study when comparing the "tolerant" fish with the "intolerant" fish because we have not established lines with different heat tolerance. Although it is true that some fish were quite tolerant of the heat stress in this study compared with those who lost equilibrium at the earliest times, a direct comparison of molecular responses to heat stress is difficult because the tolerant fish were treated at 36°C for a much longer period of time than the intolerant fish. However, it is too intriguing not to compare

these fish, as one wonders what made these tolerant fish tolerant. With this curiosity, we compared the differences in gene expression between the tolerant and intolerant fish (Table 6). Although many more genes were differentially expressed between the tolerant and intolerant fish, 39 genes were specifically presented because these 39 genes belong to specific gene pathways after enrichment analysis. Interestingly, of the 39 differentially expressed genes, three heat shock proteins, HSP47, HSP70 protein 1, and HSC70-like, were expressed significantly more highly in tolerant fish in both tissues than in

Table 6. *Enriched differentially expressed genes between intolerant and tolerant catfish*

Functional Categories	Gene Name	Contig ID	Tolerant-Intolerant	
			In Gill	In Liver
Protein folding				
Heat shock protein 47	hsp47	k86:263021	**3.2**	2.0
Heat shock 70 kDa protein 1	HSPA1	k78:1282572	**2.7**	**8.5**
Heat shock cognate 70 kDa protein, like	HSC73L	k86:91142	**2.8**	**8.3**
Calreticulin	crt	k86:641264	**2.3**	1.2
T-complex protein 1 subunit delta	cct4	k82:1065713	−1.2	**−2.0**
Peptidyl-prolyl *cis*-trans isomerase FKBP5	fkbp5	k82:82465	**−5.8**	**−2.1**
Protein degradation				
Cathepsin Z	ctsz	k86:812568	1.4	**2.4**
Sequestosome-1	sqstm1	k86:844100	**2.1**	**2.2**
Protein biosynthesis				
60S ribosomal protein L13a	rpl13a	k54:2659627	**−319.3**	4.6
60S ribosomal protein L15	rpl15	k90:1943685	**−745.6**	1.7
60S ribosomal protein L18	RPL18	k70:1285008	**−30.6**	2.4
60S ribosomal protein L27a	rpl27a	k66:1848087	**−55.3**	1.1
Energy metabolism				
Glyceraldehyde 3-phosphate dehydrogenase, spermatogenic	gapdhS	k86:11563	1.3	**2.0**
Brain creatine kinase	ckba	k86:813805	**2.3**	2.0
NADH dehydrogenase subunit 1	mt-nd1	k90:1883855	1.6	**3.5**
NADH dehydrogenase subunit 6	mt-nd6	k82:875412	1.5	**3.1**
Lathosterol oxidase	sc5dl	k86:438358	1.9	**10.7**
L-threonine 3-dehydrogenase	tdh	k82:509646	**−3.1**	**−6.9**
Molecule and ion transport				
Fatty acid binding protein 11b	fabp11b	k70:1653527	**3.2**	2.1
Fatty acid-binding protein 1, liver	FABP1	k82:44080	**4.9**	**4.2**
Fatty acid-binding protein 3, heart	FABP3	k94:168024	**5.7**	**3.6**
Hemoglobin subunit alpha	hba	k62:785473	**73.2**	**59.4**
Hemoglobin subunit alpha	hba	k78:710863	**11.7**	**5.6**
Hemoglobin subunit beta	HBB	k86:758616	**3,501.8**	**615.**
Hemoglobin subunit beta	HBB	k50:438230	**8,626.4**	**545.5**
Hemoglobin subunit beta	hbb	k86:796083	**9.8**	**9.3**
Hemoglobin subunit beta	HBB	k86:818806	**4.7**	**4.8**
Reticulocalbin-3	rcn3	k62:2057153	**4.1**	1.7
Ferritin heavy chain	FTH	k94:1382080	**2.1**	1.5
Cytoskeletal reorganization				
Alpha-actinin-1	actn1	k82:1044661	**2.0**	1.5
Tropomyosin alpha-4 chain isoform 2	tpm4	k74:236151	**2.8**	1.0
Matrix metalloproteinase-9	mmp9	k70:1632700	**7.0**	5.0
Matrix metalloproteinase-13	mmp13	k86:843929	**4.1**	2.4
Matrix metalloproteinase-18	mmp18	k86:843930	**3.8**	1.8
Collagen alpha-1(I) chain	col1a1a	k78:1250060	**2.4**	**6.6**
Collagen, type I, alpha 1b	col1a1b	k86:653563	**2.4**	**8.4**
Serine/threonine-protein kinase SIK2	SIK2	k94:1396796	**−3.0**	**4.8**
Cytokeratin-like	zgc:109868	k82:1013032	**−2.3**	**−2.0**
Gamma-aminobutyric acid receptor-associated protein-like	GABARAPl	k94:1381854	**−2.9**	**−2.6**

Numbers are fold of difference in gene expression in tolerant fish as compared expression of the same gene in the intolerant fish. Boldfaced values indicate statistically significant differences between the tolerant group and the intolerant group ($P < 0.05$). Negative signs indicate low expression in tolerant fish but high expression in intolerant fish.

intolerant fish; similarly, six hemoglobin genes were dramatically higher in tolerant fish than in intolerant fish in both tissues as well. However, a number of ribosomal protein genes were dramatically less expressed in tolerant fish than in intolerant fish (Table 6). Such differences could apparently make sense because higher levels of heat shock proteins in tolerant fish could have facilitated more vigorous protein folding, thereby maintaining the functionality of proteins under heat stress. Similarly, dramatically higher levels of hemoglobin could have been the consequences of prolonged exposure to heat stress, but alternatively, the dramatically higher levels of hemoglobin could also have provided an oxygen advantage to tolerant fish. To the contrary, the drastic reduction in protein synthesis in tolerant fish could also have helped in the survival of tolerant fish. With such interesting observations, we need to caution once again that the treatment periods of the two groups of fish were quite different, with the tolerant fish being under lethal temperature for 3 additional days, and such observed differences could be entirely consequences, not causes.

Studies have shown that the level of hemoglobin directly correlates with the thermal tolerance in Antarctic notothenioid fishes (8), whose high hematocrit levels exhibit a high critical thermal maximum. The hematocrit closely correlates to expression of hemoglobin in these species (7). However, the opposite was observed in Arctic charr, with hemoglobin expressed more highly in intolerant fish than in tolerant fish (71), even though Antarctic notothenioid fishes and Arctic charr are both cold water species. It is unknown at present why such opposite phenomena exist with different species. Future investigations to both determine the hematocrit and measure hemoglobin gene expression change in blood cells are of interest.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.L., X.W., and Z.L. conception and design of research; S.L., X.W., F.S., J.Z., J.F., H.L., K.R., L.S., Y.Z., Y.J., E.P., L.K., and H.K. performed experiments; S.L., X.W., F.S., and J.Z. analyzed data; S.L. interpreted results of experiments; S.L. prepared figures; S.L. drafted manuscript; S.L. and Z.L. edited and revised manuscript; S.L., X.W., F.S., J.Z., J.F., H.L., K.R., L.S., Y.Z., Y.J., E.P., L.K., H.K., and Z.L. approved final version of manuscript.

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