Identification of the acclimation genes in transcriptomic responses to heat stress of White Pekin duck

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Abstract White Pekin duck is an important meat resource in the livestock industries. However, the temperature increase due to global warming has become a serious environmental factor in duck production, because of hyperthermia. Therefore, identifying the gene regulations and understanding the molecular mechanism for adaptation to the warmer environment will provide insightful information on the acclimation system of ducks. This study examined transcriptomic responses to heat stress treatments (3 and 6 h at 35 °C) and control (C, 25 °C) using RNA-sequencing analysis of genes from the breast muscle tissue. Based on three distinct differentially expressed gene (DEG) sets (3H/C, 6H/C, and 6H/3H), the expression patterns of significant DEGs (absolute $\log_2 > 1.0$ and false discovery rate < 0.05) were clustered into three responsive gene groups divided into upregulated and downregulated genes. Next, we analyzed the clusters that showed relatively higher expression levels in 3H/C and lower levels in 6H/C with much lower or opposite levels in 6H/3H; we referred to these clusters as the adaptable responsive gene group. These genes were significantly enriched in the ErbB

signaling pathway, neuroactive ligand-receptor interaction and type II diabetes mellitus in the KEGG pathways ($P < 0.01$). From the functional enrichment analysis and significantly regulated genes observed in the enriched pathways, we think that the adaptable responsive genes are responsible for the acclimation mechanism of ducks and suggest that the regulation of phosphoinositide 3-kinase genes including *PIK3R6*, *PIK3R5*, and *PIK3C2B* has an important relationship with the mechanisms of adaptation to heat stress in ducks.

Keywords Peking duck · Heat stress · Acclimation · Transcriptome

Introduction

Global warming due to climate change has become a major environmental factor for production loss in various agricultural fields. Increasing temperatures due to global warming affects the development, growth, and productivity of animals and plants (Sohail et al. 2012; Li et al. 2013). These harmful influences are referred to as heat shock or heat stress (HS). Poultry have been generally known to be susceptible to HS, because of their limited ability to control heat release by evaporation (Stafford 2011). Thus, severe or prolonged HS in avian species can result in hyperthermia, inducing protein degradation in muscle tissues (Luo et al. 2000). Therefore, the productivity and sustainability of avian livestock such as meat production and egg laying are significantly affected by HS (Nardone et al. 2010).

Many domestic duck breeds are commonly used for meat, egg, and down production (Adzitey 2015). Moreover, duck meat in many Asian countries is one of the major meats consumed; hence, breeding of high-quality duck meat is important (Smith et al. 2015). Among the multiple duck breeds, the

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meat of White Pekin duck (*Anas platyrhynchos domesticus*) is the most famous in the world. It has better growth performance, such as body weight gain and feed efficiency, than the broiler chicken at a similar live weight, because of genetic improvements (Li 2011). Therefore, the decrease in meat productivity due to HS in ducks is a significant problem waiting to be solved. A comprehensive understanding of the acclimation system to HS is fundamental for solving this issue.

Although there are numerous papers on the function of molecular chaperones and response to HS in multiple organisms (Zeng et al. 2015), the accurate acclimation mechanism to HS has not been comprehensively defined, yet. A previous study has suggested different strategies for alleviating heat stress in fowl (Yahav 2009). The study suggested that heat tolerance could be improved by cyclic higher temperature incubation during the pre-natal period in eggs, depending on the length of exposure and the epigenetic adaptation of the thermoregulatory control system. A more recent study has tried to separate acute and chronic responses to HS and compared differentially expressed genes (DEGs) in the liver transcriptome using multiple distinct breed lines (Lan et al. 2016). Furthermore, tissue-specific responses to HS were characterized in the heart, liver, and muscle (Xie et al. 2014). However, it is still difficult to elucidate the acclimation mechanisms in complicated HS responses. Therefore, classification of various responsive aspects under HS conditions and identification of regulated genes are required to clarify the molecular mechanisms of the acclimation system.

Here, we used RNA sequencing (RNA-seq) to compare the genome-wide transcriptomic response to heat stress across three different time points (control, 3 h, and 6 h). We speculated that the transcriptomic response will be modulated according to the duration of HS, reflecting the adaptive response of the ducks, making it possible to identify the genes involved in the acclimation mechanism in ducks. Therefore, we aimed to investigate the DEG profiles at different time points to identify the critical genes that are modulated in response to HS as well as understand their molecular mechanisms in the muscle tissue of White Pekin duck. We believe that this study provides a novel insight into the acclimation mechanism to HS, as well as gene regulation and networks that can be used to guide future efforts to identify candidate genes or markers for heat tolerance for duck breeding strategies.

Materials and methods

Experimental animals and tissue collection

Nine 3-week-old male White Pekin ducks were used in this study. This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC no. NIAS2016-216). The drakes were maintained in a climate

chamber for an adaptation period of more than 2 weeks at 25 °C and 60% relative humidity (RH) before the acute HS treatment. Feed and water were supplied ad libitum during the experiment. After the adaptation period, the drakes were allocated into three groups ($n = 3$). The control group (C) was maintained at 25 °C and 60% RH throughout the experiment. The two treatment groups were subjected to acute HS at 35 °C without recovery for 3 h (3H) or 6 h (6H), as previously described (Lan et al. 2016). The body weight and rectal temperature of the fowls were recorded before and after the experiment to confirm the vital signs following the experiment. The drakes were sacrificed at the end of the treatment, and the breast muscle tissues were collected for RNA-seq analysis. The tissues were dissected into pieces (1 cm³), placed in RNA later, and stored at −80 °C.

RNA isolation and library construction for RNA-seq analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The quantity and purity of the extracted RNA were estimated using the Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Palo Alto, CA, USA) with the RNA integrity number >8.0. Approximately 10 µg of total RNA of each sample was used to isolate poly(A) messenger RNA (mRNA) with poly-T oligo-attached magnetic beads (Invitrogen). Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. Then, the cleaved RNA fragments were reverse transcribed to create the final complementary DNA (cDNA) library in accordance with the protocol for the mRNA-Seq Sample Preparation Kit (Illumina, San Diego, CA, USA). The average insert size for the paired-end libraries was 300 ± 50 bp. Subsequently, we performed the paired-end sequencing analysis using the Illumina HiSeq 2500 machine (Illumina).

RNA-seq data processing and DEG analysis

In total, 310 million paired-end reads were generated with averages of 34.5 million reads per sample and 51.42% GC content. We conducted quality filtering analysis to remove adapter sequences and low-quality reads from the produced raw paired-end sequence reads using FastQC and Trimmomatic (Bolger et al. 2014). After the filtering step, there were 302 million reads with an average of 33.6 million reads per sample. Following the Tuxedo protocol (Trapnell et al. 2012), the qualified reads were mapped and aligned against the duck reference genome (BGI_duck_1.0) by the tophat2 v2.0.13 using the default options. All possible transcripts were inferred using the Reference Guided Transcriptome Assembly (RABT) mode in Cufflinks v2.2.1 (Trapnell et al. 2010). The expression level of transcripts was

examined through the mapped reads of the previously annotated transcripts (BGI_duck_1.0) and calculated into the fragments per kilobase of transcript per million mapped reads (FPKM) value, considering the transcript length and reads' depth of coverage (Huang et al. 2013). The FPKM values were normalized to compare the expression profile between samples. To remove the false positive transcripts, we excluded genes that were silent (FPKM = 0) at least in two individuals within treatment groups (C, 3H, and 6H). Moreover, the transcripts were removed when they did not have a significant FDR ($q < 0.05$) in any of the treatment and control groups. Then, the log₂ fold change (FC) values were calculated to estimate the effect of the HS treatment. Finally, three kinds of log₂ FC values (3H/C, 6H/C, and 6H/3H) were used in the DEG analysis using Cuffdiff (Trapnell et al. 2010).

DEG clustering of expression patterns and functional annotations

Gene expression pattern analysis was performed. We supposed that the separate DEG clusters could represent the different biological responses to HS over time. Therefore, we focused on identifying the various DEG clusters using the three log₂ FC values: 3H/C, 6H/C, and 6H/3H. For the gene expression pattern analysis, we used the *k*-means clustering algorithm in the MultiExperiment Viewer (MeV) software by narrowing down to the optimal number of clusters with 1 K iterations after median centering (Howe et al. 2011). We used the genes that had over 1 absolute value at least once in the three log₂ FC values.

The list of genes in the three categorized groups was used for functional enrichment analysis using the KEGG pathway database and for functional clustering of gene ontology (GO) using the DAVID Bioinformatics Resources 6.8 enrichment tool (Huang et al. 2007). The genes used in the enrichment analysis were identified by the official gene symbols for *Homo sapiens* in the HUGO gene nomenclature, and then we used the ENTREZ gene ID matched with the gene symbols to increase the functional definitions instead of using the relatively under-represented database of *A. platyrhynchos*. The significant levels of enriched pathways are represented by the log₁₀ *P* value with fold enrichment. The functional annotated GO clustering analysis was conducted with all three categories simultaneously, including biological process, cellular component, and molecular function. For further network analysis using the significantly enriched pathways in the adaptable genes, we carried out enrichment analysis by the ClueGO plugin in Cytoscape (Bindea et al. 2009). Then, we provided representative KEGG pathways to emphasize the expression levels of the responsible genes (proteins) using the Bioconductor, clusterProfiler (Yu et al. 2012).

Validation analysis by quantitative real-time PCR

The quantitative real-time PCR was conducted to validate the expression pattern of two significant DEGs (*UTS2R* and *PIK3R6*) chosen from clusters 1 and 2, respectively; 1.5 µg of total RNA was used in the synthesis of single-stranded cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR reaction was performed using an ABI 7500 Real Time PCR System (Applied Biosystems/Life Technologies, Carlsbad, CA, USA) with SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) and specific primer sets (Table S2). The *C_T* values of target genes were normalized to the values of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), which were similar among the groups (*C_t* value: control, 14.58 ± 0.39 ; 3H, 14.98 ± 0.47 ; 6H, 14.92 ± 0.69), and were quantified by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). The one-way analysis of variance (ANOVA) was used for comparing mRNA expression levels between the groups.

Results and discussion

Transcriptome analysis following heat stress treatment

In this study, the body weight (BW, g) and rectal temperature (RT, °C) of the experimented drakes were checked to examine the physiological responses to HS treatment (Table S1). The changes in BW and RT in the three groups (C, 3H, and 6H) were -67.58 , -76.24 , and -106.98 g, and -0.5 , $+1.48$, and $+1.68$ °C, respectively. The 6H group showed a significant decrease in BW compared with the 3H and C ($P < 0.05$). The RTs of the 3H and 6H groups were significantly higher than those of the C group ($P < 0.05$). These results indicated that our experimental conditions (HS of 35 °C) were sufficient to induce HS in ducks.

The HS effects on the gene expression levels in the transcriptome were compared among the treatment groups. The multidimensional scaling plot (Fig. 1a) demonstrated similarities in the expression patterns within the groups, but not among the groups. The generated transcriptome from the RNA-seq analysis had 12,458 transcripts after removing the genes expressed in at least two individuals within a group across the three groups. The 12,458 transcripts were used to identify the DEGs in a manner dependent on the HS duration relative to the control. First, we selected the significant DEGs from the entire transcripts, which showed a log₂ FC value > absolute 1 at least once among the 3H/C, 6H/C, and 6H/3H groups. Then, the selected DEGs were plotted in the volcano plot based on the whole transcripts of the different HS durations and control (Fig. 1b). We observed 2124 DEGs, with 946 upregulated and 1178 downregulated genes in all

Fig. 1 Transcriptome distribution under heat stress. **a** Multidimensional scaling (MDS) plot of the sample relationships based on individual transcriptomes before separation into differentially expressed genes (DEGs). The three treatment groups (C, 3H, and 6H) are represented by *different color dots*. **b** Volcano plot of significant DEGs. The three DEG sets (3H/C, 6H/C, and 6H/3H) were estimated from the entire transcriptome values of the three groups. Significant DEGs were compartmented by the range of cutoff thresholds [absolute log₂ fold change >1.0 and false discovery rate (FDR) <0.05] and emphasized by *different color dots*. **c** Venn diagram of significant DEGs. The *circles'* diameters were matched with the entire number of significant DEGs in each of the DEG sets. The *numbers marked in the diagram* indicate the significantly upregulated (*red upward arrows*) and downregulated (*blue downward arrows*) genes in the three DEG sets

three log₂ FC values, and the concordant DEGs among the three groups are represented by a Venn diagram (Fig. 1c).

DEG patterns and functional enrichment

For the gene expression pattern analysis, a stringent cutoff was applied, and 1743 genes were chosen for further clustering analysis. The chosen genes were clustered into six clusters based on their expression patterns including the 3H/C, 6H/C, and 6H/3H values (Fig. 1b). Seventeen genes, which were classified as outliers, were excluded from further analysis. The six clusters were categorized into *adaptable* genes, *chronic response* genes, and *maladaptive response* genes (Fig. 2) according to the time-dependent expression patterns observed in this study. The three categorized expression patterns included both upregulation and downregulation. Moreover, the log₂ FC values of 3H/C, 6H/C, and 6H/3H for all the respective clusters were significantly different in the ANOVA analysis ($P < 0.001$; data not shown).

The first two clusters included 349 and 482 genes, which were upregulated and downregulated, respectively. They were categorized as adaptable genes to the HS environment

(Fig. 2a). We found that the adaptable genes have a decreasing expression tendency with HS duration; i.e., these genes highly responded at 3 h and then went into a steady state. Hence, the 6H/3H value of the adaptable genes was opposite to the 3H/C and 6H/C levels. Our results showed that the adaptable gene group had the highest expression levels (average absolute log₂ FC > 1) at 3 h, while their expression levels at 6 h were lower than at 3 h, and consequently, the expression levels at 6H/3H flipped. Metabolic adaptation to heat stress in livestock animals is generally known to suppress feed intake under hot conditions, which can be associated with acclimation to reduce metabolic heat production (Renaudeau et al. 2012). A histogram of significant KEGG pathways enriched with the

Fig. 2 Identification of diverse responses to heat stress by expression pattern analysis. Gene clustering analysis revealed six distinct expression profiles that represent clearly diverse responses. The clusters were classified into three major responses, including both upregulation and downregulation to heat stress treatment: **a** the pattern of adaptable response genes, **b** the pattern of chronic response genes, and **c** the pattern of maladaptive response genes. Histograms of the enriched KEGG pathways in the classified responses are shown under the cluster patterns

adaptable genes is given in Fig. 2a (bottom square). In the list of the significant pathways, several pathways involved in metabolic adaptation were enriched. For example, the Rap1 signaling pathway is closely associated with the energy balance regulation including glucose homeostasis with leptin (Kaneko et al. 2016). Moreover, it has been reported that lipid metabolism is important for maintaining the energy homeostasis in thermoregulatory responses (Renaudeau et al. 2012) and is a fundamental biological process in the immune system under heat stress (Das et al. 2016). In the current study, several meaningful KEGG pathways that are associated with energy homeostasis and lipid metabolism were significantly enriched, such as adipocytokine signaling, cAMP signaling, and fatty acid biosynthesis. Interestingly, the neuroactive ligand-receptor interaction is initiated in chickens to stimulate the central nervous system for feed intake under cold stress

conditions (Chen et al. 2014). From the significant KEGG pathways, we inferred that the adaptable genes induced the energy homeostasis to respond to the relatively high energy demand under heat stress conditions.

We also performed enrichment analyses on the other categorized clusters that are listed as the chronic and maladaptive response clusters. Clusters 3 and 4 (215 and 253 genes, respectively) represented the genes in the chronic response. Their expression patterns displayed high expression levels at both 3 and 6 h of heat stress (average absolute log2 FC > 1), whereas the levels of 6H/3H were relatively low (Fig. 2b). The genes of clusters 3 and 4 were regulated in an inverse pattern to each other. The enriched KEGG pathways revealed important physiological parameters in the thermoregulation system of the heart. For examples, primary bile acid biosynthesis can be associated with circulating metabolites supporting cardiomyocyte

Fig. 3 Functional associations of the adaptable responsive genes with KEGG pathways by the enrichment analysis. **a** Network of the enriched KEGG pathways. The node size was increased by the term's *P* value corrected by the Bonferroni step down, and the node color was determined by KEGG terms. The widths of the edge between nodes were mapped by a kappa score of connectivity among terms. **b** Pie chart of the enriched KEGG pathways. The color and size of the pieces were matched with the categorized nodes in the network. Asterisks represent significant pathways: **P* < 0.05 and ***P* < 0.01

degeneration (Stallings and Ippolito 2015), and the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway induced by heat stress can be a central mediator of thermal ablation in hepatocellular carcinoma (Thompson et al. 2016). Therefore, we speculated that the chronic responding genes are responsible for thermoregulation and heart damage, according to the list of enriched pathways and the related literature.

The genes of the maladaptive response are represented in clusters 5 and 6. In cluster 5, there were 167 upregulated genes, and in cluster 6, there were 260 downregulated genes (Fig. 2c). The genes in cluster 5 were highly expressed at 6 h of HS, whereas they were inactive at 3 h. We supposed that these genes may be involved in severe malfunction under prolonged heat stress conditions. This supposition is supported by a significantly enriched KEGG pathway, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, which is involved in irreversible aggregation under the febrile temperature (Nespital and Strous 2012).

In summary, using DEG patterns and functional enrichment analysis, we clearly identified differential expression patterns based on the increasing HS duration and characterized three groups of gene bundles based on the expression

patterns. Moreover, the three gene categories represented specific functions according to the different HS durations, though they also shared common functional thermoregulation pathways. However, further studies such as longer time course experiments and gene functional validations at the molecular level are required to clarify the different responses to increasing HS durations.

Gene modulations in the mechanism of acclimation to heat stress conditions

Recent studies have reported that there are functional genes that have roles in epigenetic thermoregulatory mechanisms during the induction of the acclimation response, which is closely associated with the heat acclimation-mediated cytoprotective memory (Horowitz 2014; Akerman et al. 2016). Therefore, we attempted to identify the adaptable genes among the DEGs under heat stress conditions and characterized their functional annotations in the biological roles.

Using the list of adaptable genes from the enrichment analysis, the enriched KEGG pathways were organized into a network to elucidate their biological relationships (Fig. 3a). Moreover, a pie chart is displayed to show the proportion of

Table 1 Top 5 functional GO annotation clusters enriched in categorized GO terms for the adaptation responsible genes

Cluster	Enrichment score	Category ^a	Term	Count ^b	<i>P</i> value	Genes ^c
1	2.21	MF	Very long-chain fatty acid-CoA ligase activity	4	2.22E-03	<i>SLC27A6, ACSL4, SLC27A3,</i>
		MF	Long-chain fatty acid-CoA ligase activity	4	4.97E-03	<i>ACSBG1</i>
		BP	Long-chain fatty acid metabolic process	4	2.07E-02	
2	1.03	MF	Ionotropic glutamate receptor activity	3	6.39E-02	<i>GRIA2, GRIA1, GRIN3B</i>
		MF	Extracellular glutamate-gated ion channel activity	3	8.82E-02	
		BP	Ionotropic glutamate receptor signaling pathway	3	1.48E-01	
3	0.88	CC	Phosphatidylinositol 3-kinase complex	3	5.47E-02	<i>PIK3C2B, PIK3R5, PIK3R6</i>
		BP	Phosphatidylinositol biosynthetic process	5	8.24E-02	
		BP	Phosphatidylinositol phosphorylation	4	5.02E-01	
4	0.86	CC	Extrinsic component of cytoplasmic side of plasma membrane	6	3.83E-02	<i>HCK, ZAP70, TGM3, ERRF1, SRC, MATK</i>
		BP	Peptidyl-tyrosine autophosphorylation	4	1.04E-01	
		MF	Non-membrane spanning protein tyrosine kinase activity	4	1.36E-01	
		MF	Protein tyrosine kinase activity	7	1.67E-01	
		BP	Peptidyl-tyrosine phosphorylation	7	2.74E-01	
		BP	Transmembrane receptor protein tyrosine kinase signaling pathway	5	2.91E-01	
		BP	Transmembrane receptor protein tyrosine kinase signaling pathway	5	2.91E-01	
5	0.76	MF	Microtubule motor activity	6	6.91E-02	<i>DYNC1H1, KIF25, KIF1A, KIF5C,</i>
		CC	Microtubule	13	1.61E-01	<i>DYNLRB2, DNAH7</i>
		BP	Microtubule-based movement	5	1.99E-01	
		CC	Kinesin complex	3	4.28E-01	

^a Category of GO terms: biological process (BP), molecular function (MF), and cellular component (CC)

^b Count on significant terms ($P < 0.1$) were represented in italics

^c Genes in the most significant term in the clusters are listed

Fig. 4 Modulations of adaptable responsive genes in acclimation. Diagrams represent the target genes' regulation in the significantly enriched KEGG pathways: neuroactive ligand-receptor interaction (a), type II diabetes mellitus (b), and the ErbB signaling pathway (c)

the most significant KEGG pathways (Fig. 3b). The network revealed that the ErbB signaling pathway and type II diabetes mellitus were located in the core of the network ($P < 0.01$). The ErbB signaling pathway was directly connected to glycolysis and the Rap1 signaling pathway, while type II diabetes mellitus was widely connected to the other pathways in the network. Moreover, the neuroactive ligand-receptor interaction was significantly enriched ($P < 0.05$), though it was hardly associated with the other pathways in the network. This network analysis revealed that type II diabetes mellitus and neuroactive ligand-receptor interaction were still important in the relationships with other pathways, which is consistent with the DAVID enrichment results. The effects of diabetes on the physiological response to thermal stress have recently been intensively reviewed (Kenny et al. 2016). This study reported the effect of diabetes on heat or cold exposure with respect to the core temperature regulation, cardiovascular adjustments, and glycemic control. Moreover, it has been suggested in a previous study that the ErbB signaling pathway including *Neuregulin-1* (*NRG1*) is involved in stress-induced cardiac dysfunction, indicating the adaptive responses of the *NRG1*/ErbB system in the stressed heart (Dang et al. 2016).

Additionally, we performed functional GO clustering analysis including three categories: biological process (BP), molecular function (MF), and cellular component (CC), on the list of adaptable genes (Table 1). The first ranked cluster demonstrated that four genes (*SLC27A6*, *ACSL4*, *SLC27A3*, and *ACSBG1*) in the list were significantly expressed in long-chain fatty acid metabolism. Regulation of fatty acid metabolism and accumulation of fatty acids are closely associated with obesity and type II diabetes mellitus (Blaak 2003). To sum up the results, we think that the significantly enriched terms revealed meaningful functional mechanisms of thermoregulation and acclimation.

Based on the significantly enriched pathways, further study was narrowed down into the genes that are responsible for the pathways and their roles. We used the clusterProfiler package from Bioconductor to examine the expression levels of the responsible genes (Yu et al. 2012). According to the expression levels, the responsible genes in each of the significantly enriched pathways are marked on the KEGG pathway (Fig. 4). Firstly, in the neuroactive ligand-receptor interaction pathway (Fig. 4a) from the list of adaptable genes, *SSTR* and *GRI* were upregulated and they are coded as *SSTR1* and *GRIP2* (glutamate receptor-interacting protein 2), respectively. In particular, *GRIP2* functions in a neuroactive role with a previous report showing that chronic or acute stress induces glucocorticoid secretion which results in changes in glutamate transmission (Popoli et al. 2012). In contrast, *GPR7/8*, *UTS2R*, *EDGL*, *GABR*, and growth hormone receptor (*GHR*) were downregulated with relatively high differential expression levels. A previous report claimed that the physiological significance of reduced *GHR* expression during heat stress in dairy cattle is unclear (Rhoads et al. 2010), and the downregulation of *GHR* in our study makes us concur with this claim.

In the type II diabetes mellitus pathway, *PI3K* and *VDCC* were upregulated, while *MafA* was downregulated (Fig. 4b). *PI3Ks* are known to function in intracellular signal transduction pathways and are responsible for many biological functions including metabolic control, immunity, and cardiovascular homeostasis (Vanhaesebroeck et al. 2010). In the current study, *PIK3R6*, *PIK3R5*, and *PIK3C2B* were grouped in the adaptable gene cluster in association with the *PI3K* isoforms. Moreover, the *PIK3R5* upregulation under heat stress in our study may be a counter response, which is consistent with a previous study that reported that *PIK3R5* was significantly downregulated under chronic excess of the neurotransmitter glutamate in the mouse brain (Wang et al. 2010). The counter response was closely associated with the *GRIP2* upregulation in the current study, suggesting it has a role in the changes in glutamate transmission as mentioned above. Upregulated *PI3K* appeared in the ErbB signaling pathway (Fig. 4c). In this pathway, *PI3K* responds to ErbB-2/3, ErbB-4/4, and

Fig. 5 Validations of DEGs by the quantitative real-time PCR for *UTS2R* (a) and *PIK3R6* (b). Each group for C, 3H, and 6H was represented as bar charts with the mean and standard deviation. Significant differences are indicated by letters *a* and *b* in the chart, as determined by one-way analysis of variance

ErbB-2/4 heterodimer complexes, which respond to four NRG isoforms. As mentioned in the previous paragraph, the signaling between NRG1 and ErbB may have a role in the adaptive responses to stress-induced cardiac dysfunction (Dang et al. 2016).

We validated mRNA expression levels of *UTS2R* and *PIK3R6* which were included in the acclimation gene set using quantitative real-time PCR (qPCR) and tested their differential expression patterns between the treatments (Fig. 5). We observed similar differences between control and treatments in the qPCR analysis, compared with the expression levels in RNA-sequencing analysis ($P < 0.05$). The results revealed that those genes were significantly downregulated (*UTS2R*) or upregulated (*PIK3R6*) in the 3H treatment group and came back to a similar level of control in expressions of the 6H group.

In conclusion, the differentially expressed transcriptome can be categorized by the response patterns depending upon the HS duration. Furthermore, the list of adaptable genes represents the genes involved in thermoregulation mechanism and acclimation. Finally, we suggest that upregulation of *PI3K* in the type II diabetes mellitus and ErbB signaling pathways is closely associated with acclimation to heat stress. Therefore, further studies to clarify the molecular functions of the *PIK3R6*, *PIK3R5*, and *PIK3C2B* genes, which are responsible for *PI3K* expression, are required to more comprehensively understand the mechanisms of adaptation to heat stress.

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