Expression analysis of global gene response to chronic heat exposure in broiler chickens (*Gallus gallus*) reveals new reactive genes

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ABSTRACT The process of heat regulation is complex and the exact molecular mechanism is not fully understood. To investigate the global gene response to chronic heat exposure, a breast muscle cDNA library and a liver tissue cDNA library from Silkie fowl were constructed and analyzed in bioinformatics. A total of 8,935 nonredundant EST were identified from and used for gene expression analysis. Microarray assay revealed that in breast muscle of broiler chickens (*Gallus gallus*), 110 genes changed expression levels after 3 wk of cycling heat stress. Ubiquitin B (*UBB*); ubiquitin C (*UBC*); tumor necrosis factor receptor-associated factor *3*-interacting Jun amino-terminal kinase activating modulator (*TRAF3IP3*); eukaryotic translation initiation factor 3, subunit 6 (*EIF3S6*); poly(A) binding protein, cytoplasmic 1 (*PABPC1*); and *F-box* only protein 11 (*FBXO11*) were the only genes that have been reported to be involved in heat regulation; the majority of the other genes were shown to be related for the first time. The finding of new heat-reactive genes [mitogen-activated protein kinase activating protein *PM20/PM21*; suppressors of cytokine signaling (*SOCS*) box-containing protein 2 (*ASB2*); ubiquitin-specific proteinase 45 (*USP45*); and *TRK*-fused gene (*TFG*)] suggests that the mitogen-activated protein kinase pathways as well as the ubiquitin-proteasome pathways and the nuclear factor κB pathways play important roles in heat regulation. This study provides new information on the regulation of heat stress, though the mechanism is far from being understood. Further in-depth research on the newly discovered heat-reactive genes is required to fully understand their molecular functions in thermoregulation.

Key words: broiler chicken, breast muscle, heat stress, microarray, gene expression

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INTRODUCTION

Heat shock proteins and heat shock factors have long been considered the most important regulation proteins and factors in response to heat stress (Morimoto, 1998; Sonna et al., 2002). Many other genes (e.g., tumor necrosis factor-α, *c-fos*, *c-myc*, and dual specificity phosphatase 1 and 5) that are not traditionally classified into heat shock proteins are also found to be associated with heat regulation (Bukh et al., 1990; Keyse and Emslie, 1992; Ishibashi et al., 1994; Chen et al., 1997; Singh et al., 2000). Recent research based on global gene expression analysis demonstrates that hundreds of the other genes are involved in the process as well (Sakaki et al., 2003; Gao et al., 2004; Buckley et al., 2006; Koide et al., 2006; Li et al., 2009).

The chicken (*Gallus gallus*), an important animal in both agricultural and biomedical research, is able to maintain its body temperature within a narrow range through physiological self-regulation. Nevertheless, excessive heat stress beyond the range of regulation may lead to serious damage or even death to the organism (Halevy et al., 2001). In the chicken, molecular research has shown that heat stress would change the metabolic process, affect the immune system, and consequently decrease growth and layer performance (Wallis and Balnave, 1984; Donkoh, 1989; Mashaly et al., 2004). However, heat regulation is a complicated process and the exact molecular mechanism is not fully understood.

Microarray has become the method of choice to monitor gene expression under various conditions (Schena et al., 1995). In this study, to investigate global gene response to chronic heat exposure, a breast muscle cDNA library and a liver tissue cDNA library from Silkie fowl were constructed and analyzed in bioinformatics, and the nonredundant EST contained in the libraries were

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identified from and used for gene expression analysis in breast muscle of broiler chickens.

MATERIALS AND METHODS

Animals and Treatments

Male commercial Arbor Acres broiler chickens were raised until they reached 24 d of age. Following an overnight fast, 36 healthy chickens were weighed and evenly allocated into 2 identical environment-controlled chambers (Institute of Animal Science of CAAS, Beijing, China). For the treatment group, ambient temperature was increased at the rate of 2°C/d until the temperature reached 27°C. To simulate the natural high temperature summer climate, a cycling heat stress procedure was induced on d 28 posthatch. Temperature was maintained at 27°C at night, increased from 0800 to 1400 h to 33°C, kept at the maximum temperature until 1800 h, and then decreased from 1800 to 2200 h to 27°C. Temperature for the controls was constantly maintained at 23°C. The RH for both groups was maintained at 50%. A 24-h photoperiod was provided by fluorescent lamps, and water and feed that met NRC (1994) requirements were supplied for ad libitum consumption throughout the experiment. All birds were weighed and killed by inhalation a gas of carbon dioxide and argon mixture on d 49 posthatch. Breast muscle samples were collected and frozen in liquid nitrogen for future use.

cDNA Library Construction and Analysis

Breast muscle and liver tissue samples were collected from a 6-mo-old Silkie fowl. Total RNA was isolated using TRIzol reagent (cat. no. 15596-026, Invitrogen, Carlsbad, CA), and the cDNA libraries were constructed using ZAP-cDNA Synthesis Kit (cat. no. 200400, Stratagene, Beijing, China) and ZAP-cDNA Gigapack III Gold Cloning Kit (cat. no. 200450, Stratagene) according to the manufacturer's protocol. The independent transformants were selected and subjected to 5′-end single-pass sequencing using BigDye Terminator Cycle Sequencing Kit (version 3.1, cat. no. 4337456, Applied Biosystems, Carlsbad, CA) and T3 universal sequencing primer 5′-AATTAACCCTCACTAAAGGG-3′.

Vector sequences and poly(A) tail were screened and trimmed, and sequences with inserts shorter than 20 bp and sequences having more than 5% ambiguous bases repeated were eliminated. Homologies were determined by comparing the high quality sequences against the nonredundant reference sequence database using Basic Local Alignment Search Tool (Altschul et al., 1997) with a minimum standard expect value of $1 \times e^{-10}$. Genomic sequences, mitochondrial-coded sequences, ribosomal RNA sequences, and repetitive sequences were eliminated. Finally, the uncontaminated sequences were assembled through Phrap (http://www.phrap.org) and evaluated by Consed (Gordon et al., 1998). The shared common genes were identified through Basic Local Alignment Search Tool procedure.

Microarray Fabrication

The nonredundant EST contained in the libraries were identified and amplified using T3 forward primer 5′-AATTAACCCTCACTAAAGGG-3′ and T7 reverse primer 5′-GTAATACGACTCACTATAGGGC-3′. For one 96-well plate with 100-μL reaction volume, a master mixture was prepared by mixing 7.5 mL of Milli-Q water (Millipore, Billerica, MA), 1 mL of 10× PCR buffer, 1 mL of $MgCl_2$ (25 m*M*), 100 μL of T3 forward 20-mer primer 5′-AATTAACCCTCACTAAAGGG-3′ (20 μ*M*), 100 μL of T7 22-mer reverse primer 5′-GTAATACGACTCACTATAGGGC-3′ (20 μ*M*), 20 μL of deoxynucleoside triphosphate (100 m*M*), and 20 μL of Platinum *Taq* (5 U/μL; Invitrogen, cat. no. 10966-034). For each reaction, 98 μL of master mixture was mixed with 2 μL of plasmid solution. Reactions were performed in a thermocycler (9700, PerkinElmer, Waltham, MA) using cycling conditions as follows: initial denaturation at 95°C for 5 min; 35 cycles of 95°C denaturing for 30 s, 55°C annealing for 30 s, and 72°C extension for 2 min; and 72°C elongation for 7 min after the last cycle.

Chicken housekeeping genes cytoplasmic *β-actin* (GenBank no. X00182) and glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***; GenBank no. AF047874) were used as positive controls. Three maize (*Zea mays*) gene fragments (GenBank no. AY105062, AY108832, and AY104930) without significant homology (<20 bp) to known animal nucleotide sequences and the printing solution 3× saline sodium citrate (**SSC**) +1.5 *M* betaine were used as negative controls. One 1.0-kb λ-DNA fragment (GenBank no. J02459) was amplified by forward primer 5′-GTACGGTCATCATCTGACACT-3′ and reverse primer 5′-GCAATCGGCATGTTAAACGGC-3′ and was used as internal control.

The amplicons were purified by 96-well multiscreen filter plates (cat. no. MANU 030 50, Millipore) and then dissolved in printing solution to a final DNA concentration of 150 to 200 μg/mL. At 45 to 50% of relative environmental humidity and 22°C constant temperature, samples were arrayed onto Corning GAPS II Coated Slides (cat. no. 40005, Corning, Lowell, MA) in triplicate with 100 μm of spot diameter and 180 μm of spot central distance through SpotArray 72 MicroArrays Printing System (PerkinElmer). On one microarray, a total of 27,435 spots including 26,808 experiments and 672 controls were printed.

Probe Labeling and Hybridization

Total RNA from the breast muscle was extracted using TRIzol reagent (Invitrogen). To reduce the effects of individual variation and the number of arrays required,

equal amounts of total RNA from 6 chickens of the same group were pooled. Twenty micrograms of the RNA was labeled by incorporation of 5-(3-)-2′deoxyuridine-5′-triphosphate (cat. no. A0410, Sigma) during reverse transcription using Superscript II RT (200 U/μL; cat. no. 18064014, Invitrogen) and oligo dT(18) (2 μg/μL). The aminoallyl labeled cDNA probe was combined with Cy-dye mono NHS ester (cat. no. RPN5661, Amersham Pharmacia, Piscataway, NJ) and then purified through the protocol provided by FairPlay II Microarray Labeling Kit (cat. no. 252006, Stratagene). The Cy5- and Cy3-labled probes from both stress and control samples were dissolved in hybridization solution containing 50% formamide, 5× SSC, 0.2% SDS, 0.2 mg/mL of BSA (cat. no. TZC02042614, Sigma), and 30 μg of salmon sperm DNA (cat. no. 15632-011, Invitrogen).

Prior to hybridization, slides were rehydrated, UV cross-linked at energy of 150 to 300 mJ, washed in 0.1% SDS, and blocked for 45 to 60 min at 42°C in prewarmed prehybridization solution that contained 25% formamide, 5× SSC, 0.1% SDS, and 10 mg/mL of BSA (1%). Subsequently, the microarray was washed in Milli-Q water, rinsed with isopropanol, and dried by centrifugation. The probe preparation was denatured at 95°C for 5 min and then allowed to hybridize with microarray at 42°C for 12 to 16 h. Microarray was washed and dried according to the protocol provided by Corning GAPS II Coated Slides. To reduce the potential of high false positives and to minimize the dye-related bias, another 2 biological replicates and the dye-swap technical replicate for each were performed. In total, 6 microarrays were subjected to the following analysis.

Microarray Data Analysis

Microarrays were scanned using ScanArray 4000 confocal fluorescent scanner (GSI Group, Bedford, MA) with settings of 80% laser power, 80% photomultiplier tube, and 5 μm scanning resolution. Images from both Cy3 and Cy5 channels were analyzed in QuantArray 3.0 (Packard BioScience, Meriden, CT). The intensity was imported into and analyzed by GeneSpring 7.2 (Agilent Technologies, Santa Clara, CA). The average intensity of 3 spots for each gene was used. Data were transformed by logarithm, normalized by per spot and per chip, and filtered on gene expression level. The filtration threshold was defined based on the signal intensity of negative controls. Genes with raw signal intensity lower than the settings in any channels of the dye-swap paired microarrays were excluded from further analysis. By setting the cut-off values for downregulated genes at $\leq$0.6 and upregulated genes at $\geq$1.5, genes that met with or were higher than this standard were considered as differentially expressed. Functional definition and categorization were decided by searching the GenBank database and the gene ontology database (http://www.geneontology.org).

Quantitative PCR

To validate the identified genes, quantitative PCR (**Q-PCR**) was performed on 9 genes. Gene-specific primers were designed based on the EST using online software Primer3 (http://frodo.wi.mit.edu). The RNA used here was extracted independently, and as in microarray analysis pooled RNA from 6 individuals of the same group was used. In the presence of oligo dT(18) (2 μg/μL), 20 μg of pooled total RNA was reverse transcribed into cDNA using Superscript II. For LightCycler (Roche Diagnostics, Basel, Switzerland) reaction, a master mix of the following reaction components was prepared to the indicated final concentration: 13 μL of Milli-Q water, 2.4 μL of $MgCl_2$ (4 m*M*), 0.8 μL of forward primer (0.4 μ*M*), 0.8 μL of reverse primer (0.4 μ*M*), and 2.0 μL of Fast Start DNA Master SYBR Green I (cat. no. 12015099001, Roche Diagnostics). Nineteen microliters of LightCycler master mix and 1 μL of cDNA template were filled into the LightCycler glass capillaries. The quantification was performed in a LightCycler by running the following protocol: 95°C denature for 10 min; and 45 cycles of amplification and quantification program 95°C for 15 s, 60°C for 10 s, and 72°C for 30 s with a single fluorescence measurement. Housekeeping gene *β-actin* (GenBank no. X00182), amplified by primers GTCTCACTGGATTTCGAGCA and GACCTGACCATCAGGGAGTT, was used for standard curve construction and the relative expression ratios of target genes were normalized to *β-actin*. Similar to the microarray analysis, the quantification was also performed on the other 2 biological replicates.

Statistical Analysis

Chicken BW data were analyzed using Duncan's multiple range test procedure of SAS (SAS Institute, Cary, NC). Statements of statistical significance were based on $P \leq 0.05$. The variation between samples is expressed as mean ± SEM. Fold change in Q-PCR and microarray analysis was presented as the arithmetic mean of the replicates.

RESULTS

Effects of Heat Stress on Chicken Performance

The BW of chickens and the feeders was individually measured starting on trial d 1(d 28) and finishing on trial d 21(d 49). Data were subjected to variance analysis through SAS software using Duncan's multiple range test. Analysis results show that the daily BW gain of the heat stress-treated group was significantly lower ($P < 0.01$) than that of the control group, and the feed conversion efficiency of the heat stress group was also lower than that of the control group (Table 1).

Table 1. Mean daily feed intake, weight gain, and feed:gain ratio[1]

Item	Heat stress	Control
ADFI (g/d)	110.27	150.61
ADG (g/d)	37.97 ± 1.59[b]	61.43 ± 4.83[a]
Feed:gain ratio (g:g)	2.91	2.45

[a,b]Means within a row with no common superscript differ significantly ($P < 0.01$).

[1]Eighteen birds/group.

cDNA Library Analysis

A breast muscle cDNA library and a liver tissue cDNA library from a Silkie fowl were constructed and analyzed in bioinformatics (Table 2). Sequence assembly showed that the breast muscle cDNA library contained 6,023 clusters, which included 4,087 singletons and 1,936 contigs, and the liver tissue cDNA library contained 4,839 clusters, which included 3,019 singletons and 1,820 contigs. Comparison of the nonredundant clusters from both libraries revealed that the 2 libraries shared 1,413 common genes. By eliminating the common genes, a total of 9,449 nonredundant EST were obtained, amplified, and purified. After removal of the weak or failed and multiband amplifications, 8,935 high quality amplicons were finally obtained for expression analysis.

Identification of the Upregulated Genes

In GeneSpring, by setting the cut-off values at 1.5, 67 genes were shown to have increased expression in heat stress samples when compared with the controls. Homology analysis showed that 42 of these genes were functionally annotated; homologous sequences were found for 12 genes but without functional definitions, and the remaining 13 genes were shown to be novel.

Based on the definition in molecular function, the 42 annotated genes were categorized into 8 categories: cell structure and motility, signal transduction and transport, protein metabolism and modification, nucleoside and nucleic acid metabolism, lipid metabolism, carbohydrate metabolism, cell cycle, and functionally unclassified genes. These EST were submitted to the GenBank database and assigned accession numbers. The detailed information on these genes is listed in Table 3.

Identification of the Downregulated Genes

By setting the cut-off values for downregulated genes at 0.6, the expression levels of 43 genes were observed to be decreased in the stress group. In the GenBank database, 17 of these downregulated genes were previously reported with functional annotations, 17 other genes could find homologous sequences but their functions were unknown, and the remaining 9 genes were newly discovered.

The 17 annotated genes were classified into 6 categories based on their molecular functions: cell structure and motility, signal transduction and transport, protein metabolism and modification, nucleoside and nucleic acid metabolism, carbohydrate metabolism, and functionally unclassified genes. All EST sequences of these genes were submitted to the GenBank database. The detailed information on these genes is listed in Table 4.

Validation by Q-PCR

The fold change of target genes was normalized to the expression level of housekeeping gene *β-actin*. The Q-PCR confirmation results are displayed in Table 5. The upregulated genes *EH380121* (PDZ and LIM domain protein 3; *PDLIM3*), *EH380152* (*TRK*-fused gene; ***TFG***), *EH380181*, and *EH380183* (ribosomal protein S 24; *RPS24*), which were observed to be 2.24-, 2.17-, 2.42-, and 1.98-fold changed, respectively, in microarray analysis, were confirmed to be 6.18-, 4.48-, 6.96-, and 2.23-fold changed, respectively, in Q-PCR. The downregulated genes *EH380201* (α-actinin-1; *ACTN1*), *EH380225*, *EH380226*, *EH380229*, and *EH380232*, which were observed to be 0.49-, 0.5-1, 0.48-, 0.50-, and 0.46-fold changed, respectively, in microarray analysis, were confirmed to be 0.35-, 0.57-, 0.29-, 0.69-, and 0.35-fold changed, respectively, in Q-PCR.

DISCUSSION

The Silkie fowl is physically characterized by its grayish-black meat, black bones, blue earlobes, and 5 toes on each foot. In this study, a breast muscle cDNA library and a liver tissue cDNA library from this animal were constructed and analyzed in bioinformatics, and a total of 8,935 nonredundant EST representing

Table 2. Results of bioinformatics analysis of the breast muscle and the liver tissue cDNA libraries[1]

cDNA library	Sequencing reaction	Cleaned EST	Cluster	
			Singleton	Contig
Breast muscle	25,467	16,600	4,087	1,936
Liver tissue	28,276	17,733	3,019	1,820

[1]Values are number of genes. There were 1,413 common genes and 9,449 unique genes.

Table 3. Information on the upregulated genes

Accession no.	Gene symbol	Gene description	Fold change
Cell structure and motility			
EH380121	PDLIM3	PDZ and LIM domain protein 3	2.24
EH380129	PHACTR1	Phosphatase and actin regulator 1	1.80
EH380186	SPTBN1	Spectrin β chain, brain 1	2.31
Signal transduction/transport			
EH380122	FGG	Fibrinogen gamma chain	1.97
EH380126	LOC425113	Immunoglobulin-like receptor CHIR-B2 precursor	2.44
EH380135	VTN	Vitronectin	2.18
EH380145	ADRA1B	Adrenergic, α-1B-, receptor	1.80
EH380160	CD82	CD82 molecule	2.07
EH380125	HPX	Hemopexin	2.03
EH380175	ABCF2	ATP-binding cassette, subfamily F (GCN20), member 2	2.11
EH380128	FTMT	Ferritin H chain protein	2.02
Protein metabolism and modification			
EH380127	EIF3S6	Eukaryotic translation initiation factor 3, subunit 6	2.35
EH380142	AGXT2L1	Alanine-glyoxylate aminotransferase 2-like 1	2.14
EH380143	SERPINA1	α-1-Antitrypsin	1.82
EH380144	FBXO11	F-box only protein 11	1.70
EH380171	UBB, UBC	Ubiquitin B, ubiquitin C	2.40
EH380183	RPS24	Ribosomal protein S24	1.98
EH380134	RPL17	Ribosomal protein L17	2.11
Nucleoside and nucleic acid metabolism			
EH380133	PABPC1	Poly (A) binding protein, cytoplasmic 1	1.87
EH380136	DHX15	DEAH (Asp-Glu-Ala-His) box polypeptide 15	1.71
EH380137	Lhx6	LIM homeobox protein 6 isoform 1	2.62
EH380147	SMYD2	SET and MYND domain containing 2	2.38
EH380172	EFTUD2	Elongation factor Tu GTP binding domain containing 2	1.74
EH380179	SPEN	Msx-2 interacting nuclear target protein	1.62
EH380185	TCF3	Transcription factor 3	1.96
Lipid metabolism			
EH380130	ACAT2	Acetyl-coenzyme A acetyltransferase 2	1.96
EH380139	Elovl2	Elongation of very long chain fatty acids	1.74
Carbohydrate metabolism			
EH380138	ALG2	Asparagine-linked glycosylation 2 homolog	1.65
EH380148	GALM	Aldose 1-epimerase	1.84
EH380149	UGT1A9	UDP-glucuronosyltransferase	2.09
EH380174	IDH2	Isocitrate dehydrogenase 2 (*NADP+*), mitochondrial	2.27
EH380180	FBP1	Fructose-1, 6-bisphosphatase	1.82
Cell cycle			
EH380176	MAD2L1	Mitotic spindle assembly checkpoint protein MAD2A	1.75
Unclassified genes			
EH380131	ETFA	Electron-transfer-flavoprotein, α polypeptide	1.94
EH380140	FADS2	Delta-6 fatty acid desaturase	1.70
EH380150	FNDC3B	Fibronectin type III domain containing 3B	2.25
EH380152	TFG	TRK-fused gene	2.17
EH380153	LOC421151	RIKEN cDNA 2610318G18	1.63
EH380156	DOHH	Deoxyhypusine hydroxylase/monooxygenase	1.91
EH380177	TMEM59	Transmembrane protein 59	2.24
EH380178	ASB2	Ankyrin repeat and SOCS box-containing protein 2	1.74
EH380182	LOC419462	MAPK activating protein *PM20/PM21*	2.22
Genes with homologous sequence but without functional definition			
EH380124		Hypothetical protein	2.34
EH380146		*Gallus gallus* BAC clone	1.94
EH380151		*Gallus gallus* finished cDNA	1.56
EH380154		Hypothetical protein	2.04
EH380155		*Gallus gallus* finished cDNA	1.61
EH380157	LOC395778	Hypothetical protein	1.81
EH380158	LOC420731	Hypothetical protein	1.55
EH380159		*Gallus gallus* finished cDNA	1.51
EH380184		*Gallus gallus* finished cDNA	1.64
EH380187	LOC418756	Hypothetical gene supported by CR390784	2.08
EH380188		*Gallus gallus* finished cDNA	1.56
EH380191		*Gallus gallus* finished cDNA	1.62
Novel genes			
EH380141			1.72
EH380161			2.90
EH380162			2.08
EH380163			2.83
EH380164			1.98

Continued

Table 3 (Continued). Information on the upregulated genes

Accession no.	Gene symbol	Gene description	Fold change
EH380165			1.74
EH380166			1.56
EH380167			1.77
EH380169			1.84
EH380170			2.10
EH380181			2.42
EH380192			1.80
EH380195			1.69

Table 4. Information on the downregulated genes

Accession no.	Gene symbol	Gene description	Fold change
Cell structure and motility			
EH380201	ACTN1	α-Actinin-1	0.49
EH380209	MYBPC1	Myosin-binding protein C, slow type	0.51
Signal transduction and transport			
EH380190	KITLG	KIT ligand	0.47
EH380198	LEPROT	Leptin receptor overlapping transcript	0.45
EH380123	SNX27	Sorting nexin family member 27	0.36
Protein metabolism and modification			
EH380197	TPP-II	Tripeptidyl peptidase II	0.29
EH380200	USP45	Ubiquitin specific proteinase 45	0.44
EH380206	Dpp9	Dipeptidyl peptidase 9	0.48
EH380207	PDK4	Pyruvate dehydrogenase kinase-like protein	0.49
Nucleoside, nucleotide, and nucleic acid metabolism			
EH380173	SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	0.59
Carbohydrate metabolism			
EH380193	PFKM	6-phosphofructokinase, muscle type	0.43
Unclassified genes			
EH380196	ZDHHC13	Zinc finger, DHHC-type containing 13	0.59
EH380189	TRAF3IP3	TRAF3-interacting JNK-activating modulator	0.51
EH380202	COL4A1	Collagen IV a1 chain	0.52
EH380203	CACNG1	Calcium channel, voltage-dependent, gamma subunit 1	0.49
EH380205	CLEC12A	C-Type lectin domain family 12, member A	0.54
EH380208	CHIR-B3	Immunoglobulin-like receptor CHIR-B3 precursor	0.50
Genes with homologous sequence but without functional definition			
EH380199	LOC424617	Protein KIAA0494	0.46
EH380204	LOC421816	ACY1L2 protein	0.38
EH380210		*Gallus gallus* finished cDNA	0.37
EH380211		Hypothetical protein	0.45
EH380212	LOC421477	PRO1853 protein isoform 1	0.46
EH380213	LOC419404	*Gallus gallus* LOC419404	0.50
EH380214	LOC422735	Hypothetical protein LOC286097	0.38
EH380215		*Gallus gallus* finished cDNA	0.49
EH380216		*Gallus gallus* finished cDNA	0.52
EH380217		*Gallus gallus* finished cDNA	0.49
EH380218		Hypothetical protein	0.43
EH380219	LOC418604	Hypothetical protein FLJ34960	0.53
EH380220		*Gallus gallus* finished cDNA	0.56
EH380221		*Gallus gallus* finished cDNA	0.48
EH380222		*Gallus gallus* finished cDNA	0.59
EH380223		*Gallus gallus* finished cDNA	0.56
EH380224		*Gallus gallus* BAC clone	0.56
Novel genes			
EH380225			0.51
EH380226			0.48
EH380227			0.48
EH380228			0.56
EH380229			0.50
EH380230			0.59
EH380231			0.56
EH380232			0.46
EH380233			0.58

Table 5. Primers used for quantitative real-time PCR and the analysis results

Accession no.	Forward gene-specific primers 5′-3′	Reverse gene-specific primers 5′-3′	Product size (bp)	Fold change[1]
EH380121	TGACAAATGTGGGAGTGGAA	TTTATCACGTGCCTTCACCA	52	6.18
EH380152	GAGCCGTTCATGCTTTGAAT	TATCCTTCGAACGAGGCAAG	126	4.48
EH380181	GGAACCGAAGCTACACGTCTT	ATAGCTGCATCGGTTCGAGTA	98	6.96
EH380183	GGAACCGAAGCTACACGTCTT	AGATCATGCCAAAGCCTGTT	149	2.23
EH380201	TCACCCTGAAGTACCCCATT	TGCCAGATCTTCTCCATGTC	67	0.35
EH380225	CCCTGCTCTTTAGAGGCTTG	GGATGCAGAATGGACAGAGA	91	0.57
EH380226	TTGAATGGACATTGGAAAGC	TGGGATTCAGCATGGTCTAA	150	0.29
EH380229	CAGTTTGTGAGGGCCTGTTA	GGATGTCACCCCTTTACCTG	50	0.69
EH380232	GAACAGCGATTTTACCAGCA	TTGCTGAAACAGCCTCAAAC	65	0.35

[1]Fold change was calculated by averaging the value of 3 replicates.

unique genes were identified. These EST can be further used for specific traits as well as general species-related research.

Housekeeping genes cytoplasmic *β-actin* and *GAPDH* are frequently used as positive controls in microarray analysis and Q-PCR confirmation. In this experiment, chicken cytoplasmic *β-actin* (GenBank no. X00182) and *GAPDH* (GenBank no. AF047874) were amplified and printed onto slides together with other elements. Microarray data analysis showed that the 2 genes were 1.046- and 0.429-fold changed, respectively, after the cycling heat stress, indicating that *β-actin* is more suitable as an internal control than *GAPDH* in stress-related research.

In general, the broiler chicken has the most rapid growth between 4 and 7 wk. We presumed that the related genes might have highest activity as well, and introduced heat stress during this period is more likely to affect their expression levels. Data analysis showed that the group treated with heat stress had remarkably decreased feed consumption and weight gain ($P < 0.01$; Table 1). However, all chickens were raised in 2 identical chambers and no significant difference was detected at the beginning, suggesting that heat stress had a significant effect on chicken growth.

Accompanied with the phenotypic changes, the following microarray analysis revealed that more than 100 genes have altered expression in breast muscle. The validation of Q-PCR on 9 genes indicates that the microarray analysis is reliable even though it tends to underestimate the extent of upregulated genes (Table 5), and also indicates that in microarray analysis the pooled RNA strategy and the dye-swap analysis method can improve the efficiency of gene discovery. Furthermore, the confirmation demonstrates that the phenotypic variations are the consequence of gene expression differences.

Gene analysis showed that 59 of the differentially expressed genes have been functionally annotated and the other 51 have not yet been defined (Table 3 and 4). Within the annotated genes, the only genes that have been described to be associated with stress response are the following: ubiquitin B (***UBB***); ubiquitin C (***UBC***); tumor necrosis factor receptor-associated factor (**TRAF**) 3-interacting Jun amino-terminal kinase-activating modulator (*TRAF3IP3*); eukaryotic translation initiation factor 3, subunit 6 (*EIF3S6*); poly(A) binding protein, cytoplasmic 1 (***PABPC1***); and F-box only protein 11 (***FBXO11***; Bond and Schlesinger, 1985; Laroia et al., 1999; Sonna et al., 2002; Tsan and Gao, 2004; Gorostizaga et al., 2005; Lee et al., 2005; Buckley et al., 2006; Wheeler and Wong, 2007). However, most of the other genes are for the first time shown to be related, including the following: mitogen-activated protein kinase (***MAPK***) activating protein *PM20/PM21*, the ankyrin repeat and suppressors of cytokine signaling (*SOCS*) box-containing protein 2 (***ASB2***), the ubiquitin-specific proteinase 45 (***USP45***), and *TFG*.

The MAPK activating protein *PM20/PM21* is upregulated and is associated with the regulation of *MAPK* pathways. The *MAPK* cascade is a highly conserved module involved in various cellular functions. Three subfamily members [extracellular signal-related kinase-1/2/5, Jun amino-terminal kinase-1/2/3, and *p38* mitogen-activated protein kinase (α, β, γ, δ)] have been discovered in the *MAPK* family, and the component genes act as the integration point of multiple biochemical signals in cellular processes. Research has shown that the *MAPK* pathways are modulated by the heat-responsive genes dual specificity phosphatase-1, dual specificity phosphatase-5 and *MAPK* phosphatase-1 (*MKP-1*; Keyse and Emslie, 1992; Ishibashi et al., 1994; Gorostizaga et al., 2004). The discovery of new heat-reactive gene *PM20/PM21* suggests that it may also play a role in heat regulation in the *MAPK* pathways.

Gene classification found that 5 genes (*UBB/UBC*, *PABPC1*, *FBXO11*, *ASB2*, and *USP45*) are ubiquitin related. Ubiquitin is a highly conserved and heat-stable regulatory protein that plays a wide range of functions in the cell (Hershko and Ciechanover, 1998). The main role of the ubiquitin-proteasome pathways is to control the concentration of some key proteins by selectively conjugating and marking, so as to be recognized and shuttled to the proteasome for degradation (Burger and Seth, 2004). The ubiquitin-related genes *UBB/UBC*, *PABPC1*, and *FBXO11* have been shown to react sensitively to heat stress (Bond and Schlesinger, 1985; Laroia et al., 1999; Sonna et al., 2002; Buckley et al., 2006). However, genes *ASB2* and *USP45* are for the first time shown to be related. These findings further

confirm that the ubiquitin-proteasome pathways are involved in heat regulation.

As a newfound heat stress-associated gene, *TFG* was first known to be a partner of neurotrophic tyrosine kinase, receptor, type 1 in generating the thyroid *TRK-T3* oncogene and was presumed to be a novel member of the nuclear factor κB (**NF-κB**) pathways (Greco et al., 1995). In NF-κB pathways, the activation of NF-κB is activated by a variety of stimuli, such as growth factors, cytokines, and UV irradiation and stress. The *TFG* has functions in modulating the NF-κB pathways in inducing the activity of NF-κB by interacting with tumor necrosis factor-α, *TRAF* family member-associated NF-κB activator, *TRAF2*, and *TRAF6* (Miranda et al., 2006).

Research has shown that the NF-κB pathways and *MAPK* pathways shared approximately 30 common genes (Matsuda et al., 2003). For instance, gene *TRAF6*, one of the genes that have certain functions in NF-κB pathways, also plays a role in activation of the *MAPK* pathways (Kishida et al., 2005; Miranda et al., 2006). On the other hand, the function of the NF-κB pathways and the ubiquitin-proteasome pathways is associated as well (Chen, 2005), evidencing that the NF-κB pathways play an important role in thermoregulation.

In this study, a total of 110 genes were detected to be differentially expressed by investigating the expression of 8,935 genes in breast muscle. Further research on the encoded proteins should provide better information on the mechanism of heat regulation. It is estimated that the chicken genome contains 20,000 to 23,000 genes (International Chicken Genome Sequencing Consortium, 2004). This means that many other genes might have been involved. To comprehensively illuminate the molecular mechanism of heat regulation, more extra genes and different tissues should be investigated, and an additional time-course experiment might be helpful in displaying gene expression patterns and their regulation models.

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