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Analysis of midgut gene expression profiles from different silkworm varieties after exposure to high temperature

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ABSTRACT

The silkworm is a poikilothermic animal, whose growth and development is significantly influenced by environmental temperature. To identify genes and metabolic pathways involved in the heat-stress response, digital gene expression analysis was performed on the midgut of the thermotolerant silkworm variety '932' and thermosensitive variety 'HY' after exposure to high temperature (932T and HYT). Deep sequencing yielded 6,211,484, 5,898,028, 5,870,395 and 6,088,303 reads for the 932, 932T, HY and HYT samples, respectively. The annotated genes associated with these tags numbered 4357, 4378, 4296 and 4658 for the 932, 932T, HY and HYT samples, respecti'vely. In the HY-vs-932, 932-vs-932T, and HY-vs-HYT comparisons, 561, 316 and 281 differentially expressed genes were identified, which could be assigned to 179, 140 and 123 biological pathways, respectively. It was found that some of the biological pathways, which included oxidative phosphorylation, related to glucose and lipid metabolism, are greatly affected by high temperature and may lead to a decrease in the ingestion of fresh mulberry. When subjected to an early period of continuous heat stress, *HSP* genes, such as *HSP19.9*, *HSP23.7*, *HSP40-3*, *HSP70*, *HSP90* and *HSP70* binding protein, are up-regulated but then reduced after 24 h and the thermotolerant '932' strain has higher levels of mRNA of some *HSPs*, except *HSP70*, than the thermosensitive variety during continuous high temperature treatment. It is suggested that *HSPs* and the levels of their expression may play important roles in the resistance to high temperature stress among silkworm varieties. This study has generated important reference tools that can be used to further analyze the mechanisms that underlie thermotolerance differences among silkworm varieties.

1. Introduction

High temperatures can affect cellular structure and metabolism (Gibbs, 2002; Liu et al., 2006; Walter et al., 1990). For insects, environmental tolerance is an important factor for determining whether a population can survive within a particular ecological environment. Silkworms are poikilothermic, and thus environmental temperature influences their growth and development—especially in tropical and subtropical regions. We have observed that silkworm varieties associated with different regions, voltinisms, or systems exhibit extremely variable thermotolerances (Li QR, unpublished). Why these silkworm varieties have different thermotolerances, however, remains unclear. Stress-response systems, such as heat shock proteins (HSPs) and the antioxidase system, can improve organismal thermotolerance (Pirkkala et al., 2001; Sorensen and Loeschcke, 2007; Stanley and Fenton, 2000). HSPs that are induced by high temperature may be involved in maintaining the integrity of cellular proteins (Denlinger and Yocum, 1998). Based on molecular weight, HSPs can be divided into five families, which include HSP100, HSP90, HSP70, HSP60, and sHSP (only sHSP is not conserved) (Kim et al., 1998; Li et al., 2009; Waters and Rioflorido, 2007; Waters et al., 2008). Among poly-, bi-, and univoltine silkworm strains, heat shock responses vary considerably and levels of thermotolerance increase as larval development proceeds (Manjunatha et al., 2010). However, it is still unclear which genes are transcriptionally regulated by high temperature and what mechanisms underlie these regulatory processes.

Global transcriptome analysis could help characterize regulatory mechanisms that underlie high-temperature tolerance in the silkworm. Genome-wide transcriptional profiling is an important and powerful tool that has been used in silkworm to identify transcriptional programs underlying developmental processes (e.g., the silkworm life cycle) and defense responses to pathogens (Huang et al., 2009; Liu et al., 2009). Serial analysis of gene expression and microarray analysis have allowed us to visualize global changes in transcript abundance associated with spatial, temporal, or conditional differences (Huang et al., 2009; Liu et al.,

Abbreviations: DGE, digital gene expression; DEGs, differentially expressed genes; RIN, RNA integrity number; TPM, transcripts per million clean tags; qRT-PCR, quantitative real-time reverse transcription PCR; GO, gene ontology.

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2009). Li et al. subjected silkworms (both parental strains and hybrids) to high-temperature stress and measured changes to the proteome and changes to protein phosphorylation levels within the posterior silk gland (Li et al., 2012). These results indicated that high temperatures alter the expression of proteins related to both the heat response and silk synthesis in silkworm. Moreover, proteins that were differentially expressed between the hybrids and parental lines likely explain the observed heterosis. Recently developed RNA deep-sequencing (RNA-seq) technologies, such as digital gene expression (DGE) (Wang et al., 2009), have substantially altered our understanding of the eukaryotic transcriptome in terms of extent and complexity.

Here we analyzed two silkworm varieties, '932' and 'HY', which have been selected based on temperature-tolerance tests involving >200 available silkworm varieties. The thermotolerant variety, '932', is a bivoltine race that contains the China system. Its female parent is the polyvoltine race 'Jiubaihai', which can resist high temperature and high humidity. Its male parent is the bivoltine race '7302'. Its filial generation, named '932', was bred in high-temperature and high-humidity conditions, endowing it with the ability to resist and adapt to relatively high temperatures (35–40 °C) and relative humidity (95%). In contrast, the Sericultural Research Institute of the Chinese Agricultural Academy cultivated the 'HY' variety to exhibit exceptional cocoon-silk traits (e.g., high yields and good quality). 'HY' is a bivoltine race that contains the Japan system. Its female parent is the high-yield race '782', and its male parent is the high-yield and good-quality race '758'. Its filial generation, named 'HY', was bred in appropriate environmental conditions, so 'HY' has relatively weak resistance and adaptability to conditions of high temperature and humidity.

To analyze transcriptional differences between thermotolerant and thermosensitive races, RNA-seq was used to analyze gene expression within the midgut of '932' and 'HY' following exposure to high temperature. Bioinformatic analyses were then used to identify differentially expressed genes (DEGs) and differences in metabolic or function pathways. Through this study we hoped to identify genes and biological pathways that underlie differences in high-temperature tolerance among silkworm varieties and establish a theoretical platform for further investigating transgenic, high-resistance breeding.

2. Materials and methods

2.1. Experimental animals

Two silkworm varieties were selected for analysis, namely '932' (thermotolerant) and 'HY' (thermosensitive). Both are bivoltine.

2.2. High-temperature treatment and sampling

2.2.1. Sampling for digital gene expression profile sequencing

For both '932' and 'HY', 50 healthy fifth-instar larvae were placed into a cultivation cabinet at 35 ± 0.5 °C and 75 ± 2% relative humidity. Control '932' and 'HY' animals were placed at 27 ± 0.5 °C and 75 ± 2% relative humidity. After ~18 h the midgut was dissected from each silkworm, mulberry leaf digestate was removed from the enteric cavity, and samples were frozen in liquid nitrogen for storage. Samples were designated 932T ('932' after exposure to high temperature), 932 ('932' after control treatment), HYT ('HY' after exposure to high temperature), and HY ('HY' after control treatment).

2.2.2. Sampling to determine the mRNA expression levels of several HSPs, after heat treatment

One hundred healthy fifth-instar larvae were placed into the same heat and control cultivation conditions as described in Section 2.2.1, for both the '932' and 'HY' varieties. After 6, 12, 18, 24, 30 and 36 h, the midgut was dissected from each silkworm and frozen in liquid nitrogen for storage.

2.3. Isolation of total RNA

Total RNA was isolated using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Each sample consisted of larval midgut from at least three silkworms. To remove residual genomic DNA, RNA samples were incubated with 10 U DNase (Roche Applied Science) at 37 °C for 20 min. RNA was then purified using RNeasy columns (Qiagen). RNA integrity was then confirmed using an Agilent 2100 Bioanalyzer. Samples had an average RIN (RNA integrity number) value of 9.3 (minimum RIN value was 8.9). A minimum of 6 μg total RNA was used for Illumina sequencing (Illumina Inc., San Diego, CA, USA).

2.4. Construction and evaluation of DGE-tag profiles

DGE libraries for four samples (from 932T, 932, HYT, and HY) were constructed using Illumina gene expression sample-preparation kits. Briefly, oligo (dT)-coated magnetic beads were used to capture mRNAs from the four total-RNA samples. First- and second-strand cDNAs were synthesized, and bead-bound cDNA was digested with NlaIII. The 3′-cDNA fragments attached to the oligo (dT)-beads were then ligated to the Illumina GEX NlaIII adapter 1, which contains an MmeI recognition site. Samples were incubated with MmeI, which cuts 17 bp downstream of the recognition site, thereby leaving an adapter-1 tag. The 3′ fragments were then released from the beads via magnetic-bead capture, and an Illumina GEX adapter 2 was introduced at the MmeI site. The resulting adapter-ligated cDNA tags were then amplified using 15 cycles of PCR. The 105-bp PCR fragments were purified via 6% polyacrylamide Tris–borate–EDTA gel and subjected to Illumina/Solexa sequencing. The four tag libraries were constructed using in situ amplification and were subsequently deep-sequenced using an Illumina Genome Analyzer. Sequencing quality was evaluated and the data were summarized using the Illumina/Solexa pipeline software. Saturation of the four libraries was also analyzed. For the raw data, adaptor sequences were filtered, and the types of clean tags were represented as distinct clean tags. Subsequently, we classified clean tags and distinct clean tags according to their copy number within the library and calculated their percentage of the total clean and distinct tags. The raw data were deposited in the GEO database under submission number GSE55990.

2.5. Annotation and analysis of DGE profiles

For annotation, all tags were mapped to the reference sequence, which included silkworm unigenes from the silkworm Genomics Database (http://silkworm.genomics.org.cn/). Expression of each gene was estimated from the frequency of clean tags and then normalized to the number of transcripts per million clean tags (TPM) ('t Hoen et al., 2008), which is the standard method used for DGE analysis (Morrissy et al., 2009). TPM indicates reads per kilobase of transcript per million sequenced reads. For differential expression analysis, fold changes were assessed using the log2 ratio (TPM-T/TPM-932, TPM-HYT/TPM-HY) after expression abundances were normalized to TPM. After assignment, gene ontology (GO) and pathway annotation and enrichment were analyzed using the NCBI COG (http://www.ncbi.nlm.nih.gov/COG) (Tatusov et al., 2003), the GO Database (http://www.geneontology.org/) (Gene Ontology Consortium, 2008), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/) (Kanehisa, 2002). The DGE analysis data (Gene ID and counts in four samples) were submitted to the GEO database under submission number GSE55990.

2.6. Real-time PCR

Initially, to validate the DGEs obtained from Solexa sequencing, 12 genes: including *chemosensory protein 11* (*CSP*, BGIBMGA004041-TA), *juvenile hormone diol kinase* (*JHK*, BGIBMGA008813-TA), *glucan binding*