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Transcriptome sequencing reveals potential mechanisms of diapause preparation in bivoltine silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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ABSTRACT

In the bivoltine strain of the silkworm, *Bombyx mori*, embryonic diapause is induced transgenerationally as a maternal effect. Progeny diapause ability is determined by the environmental condition such as temperature and lightness that mothers experience during their own embryonic development. Diapause preparation is a crucial phase of this process; diapause-destined individuals undergo a series of preparatory events before the entry into developmental arrest. However, the molecular regulatory mechanisms of diapause preparation have largely remained unknown. In the present study, we sequenced the transcriptome of bivoltine silkworm Qiufeng's ovaries resulted in laying of diapause destined or non-diapause eggs, using high-throughput RNA-Seq technology. Differential expression analyses identified 183 genes with higher expression, and 106 with lower expression under diapause-inducing conditions. GO and KEGG analysis revealed that the enrichment of several functional terms related to peroxisome, glycerolipid metabolism, steroid biosynthesis, longevity regulating pathway - multiple species, three signaling transductions, insect hormone biosynthesis, and cytoskeleton components. We conducted a detailed comparison of transcript profile data of ovaries from diapause-inducing and non-diapause conditions, the results imply up-regulation of peroxisomal metabolism, triacylglycerides accumulation, cryoprotectant production, and ecdysteroid biosynthesis in diapause-inducing group. Differential expression of genes related to actin cytoskeleton implies the occurrence of shifts in cellular structure and composition between diapause-inducing and non-diapause-inducing groups. The Hippo and FOXO signaling pathways may play an important role in preparing for entering diapause. This study provides an insight into the molecular events of insect diapause, in particular for the preparatory phase.

1. Introduction

Many species of insects have developed a special strategy called diapause to ensure survival under unfavorable environmental conditions such as low temperature, drought or food shortage and to synchronize their life cycles to these changes. Diapause can occur at any stage of the life cycle, i.e., adult, pupa, larva or egg, and each species enters diapause at a fixed stage (Chippendale, 1982). Diapause is triggered by a particular set of environmental signals such as photoperiod, temperature and humidity; these signals are transduced into endogenous chemical messengers viz., hormones, altering from development to diapause through structural and functional changes in the target organs (Yamashita, 1985).

Before the entry into developmental arrest, diapause-destined insects go through a series of preparatory events; the phase containing these events is called diapause preparation, or pre-diapause (Kostal, 2006). Diapause-inducing stimuli lead to specific alteration in gene transcription, neuroendocrine milieu and metabolic pathways and that individual is destined for later entry into diapause or not. The information about developmental destiny is “stored” during the preparation phase (Kostal, 2006). The preparation phase may be also characterized by different behavioral activities or physiological processes such as migration, location of suitable micro-habitats, aggregation, or the building-up of energy reserves before the final transition into the diapause stage (Kostal, 2006). Some events observed during the preparation phase are surely reflected in diapause-related expression patterns of select genes, but the molecular events governing most of these pre-diapause characteristics remain unidentified (Denlinger,

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2002).

The preparation phase is best documented in those organisms where the mother exerts control over the developmental fate of her progeny (Kostal, 2006). Diapause of *Bombyx* bivoltine strain (two generations per year) is under maternal control and is induced transgenerationally as a maternal effect. The environmental temperature that mothers experience during their own embryonic development determines whether they produce diapause or nondiapause eggs (Yamashita and Hasegawa, 1966). When eggs (parental generation) are incubated at 25 °C in lightness, the moths lay diapause destined eggs. In contrast, when eggs are incubated at 15 °C in darkness, the moths lay non-diapause eggs. The character is closely related to diapause hormone (DH) synthesized and secreted by subesophageal ganglion (SG) in the pupal stage of the mother moth (Yamashita, 1996). The signals of high temperature (25 °C) and lightness during maternal embryonic development stage are received by the central nervous system and transduced into endogenous chemical messengers, which are maintained for a long time period until the early pupal stage, and is transduced into neuroendocrine signals (DH release) to acts on developing oocytes. Then there are a series of physiological metabolic reaction caused by DH in the oocytes, especially the changes of lipids, hormones, carbohydrate and related enzymes. As silkworm ovary continue to develop, these changes were stored in the oocyte and transferred later to the offspring, which bring about diapause destined eggs (Yamashita et al., 2001; Emerson et al., 2009; Sato et al., 2014). Either entry into diapause or direct development results in profound physiological and morphological changes that are reflected by shift of gene expression. When DH acts on ovary in large quantities during early pupal stage, many genes involved in metabolic pathways related to diapause are either expressed or prevented from being expressed, forming a complicated molecular regulatory network (Kubota et al., 2014).

High-throughput sequencing technology permits genome-wide transcriptomic analysis at high resolution. In this study, we sequenced and assembled the transcriptome of silkworm oocytes on day 3 of the pupal stage from females reared under non-diapause (ND) and diapause-inducing (DI) condition. Our goals were to identify and verify candidate transcripts involved in the transcriptional bases of diapause preparation phase. It is worth analyzing the differential gene expression at this crucial period to find out the significant up as well as down regulation network of diapause related genes in maternal pupae.

2. Material and methods

2.1. Insect rearing and tissue collecting

The bivoltine silkworm strain Qiufeng used in this study has been maintained by the key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, JiangSu University of Science and Technology. Six single batches of activated hibernating eggs (each batch laid by one female moth) prepared last fall were stored at 5 °C for at least 60 days to terminate diapause. The activated eggs were divided into two groups. One group was incubated for hatching at 25 °C and 80% relative humidity, with a photoperiod of 12 h L:12 h D (diapause egg producers, DEPs), while the other was incubated at 17 °C in darkness (non-diapause egg producers, NDEPs). After hatching, larvae of each batch were collected respectively and were fed with fresh mulberry leaves under 25 °C. According to silkworm oocytes on day 3 after pupation are most sensitive to the exogenous DH action (Yamashita, 1966, 1970), during which ovaries of three individuals from the same batch were collected together as one sample on ice and totally 12 samples (two replicates for each batch) were prepared, then stored at − 80 °C until RNA extraction.

2.2. RNA extraction and RNA-Seq

Total RNA was extracted from the ovarian samples separately using TRIzol reagent (Takara, China) following the manufacturer's protocol. The total RNA templates were quantified using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The integrity of the total RNA from each sample was determined using gel electrophoresis. Based on the concentration and quality values, best three RNA samples of each group were used for the library preparation and Sequencing. mRNA was isolated from total RNA using Oligo-dT magnetic beads (Dynal Biotech, Oslo, Norway), and then interrupted into short fragments (200 bp–250 bp) with a zinc chloride solution, the double stranded cDNA was synthesized from the fragmented mRNA using random hexamer primers. Sequencing adaptors were ligated to cDNA fragments from each cDNA library, the ligated products were purified by agarose gel electrophoresis and enriched by PCR amplification. The cDNA library was sequenced on the Illumina HiSeq™ 2000 platform using paired-end technology in a single run (2 × 75 bp). The raw reads from the images were generated by Illumina GA Pipeline V.1.6. Low quality reads of over 50% bases with quality scores of 5 or lower and/or over 10% bases unknown (N bases) were removed from each dataset to gain more reliable results.

2.3. Normalization of gene expression levels and DEGs screening

The reference genome and annotation data of *B. mori* were downloaded from the SilkDB web site (http://www.silkdb.org/silkdb). All clean reads were well mapped to the silkworm genome and gene reference sequences using TopHat software (version 2.1.1) (Trapnell et al., 2009). The expression level of each gene was measured by fragments per kilobase of exon per million fragments mapped (FPKM) based on the number of uniquely mapped reads, and the following equation was used:

$$\mathrm{FPKM} = 10^{9}\,(\mathrm{C})/\mathrm{NL}$$

Here the given FPKM(A) is the expression of gene A, C is the number of reads uniquely aligned to gene A, N is the total number of reads uniquely aligned to all genes, and L is the number of bases in gene A. For genes with more than one alternative transcript, the longest transcript was selected to calculate the FPKM (Langmead and Salzberg, 2012).

After obtaining the normalized gene expression level, Poisson distribution analysis was performed to screen the differentially expressed genes (DEGs) between the DEPs and NDEPs. To correct for multiple testing, the false discovery rate (FDR) was calculated to adjust the threshold of P value. The genes with FDR ≤ 0.05 and the absolute value of $|\log2\ \mathrm{ratio}| \geq 1$ (minimal 2-fold difference in expression) were defined as DEGs (Anders and Huber, 2013).

2.4. GO and KEGG functional enrichment for DEGs

Gene ontology (GO) classification was performed to predict the possible functions of all DEGs by the WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye et al., 2006). P value ≤ 0.05 was selected as the threshold. GO terms fulfilling this condition are defined as significantly enriched GO terms in DEGs. The GO categorization results were expressed as 3 independent hierarchies for molecular function, biological process and cellular component. A pathway analysis for DEGs was performed based on the KEGG database (Kanehisa et al., 2008). This analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DEGs relative to the whole genome background, the pathway with a P value ≤ 0.05 was considered as an enriched pathway. Principal component analysis (PCA) and Pearson correlation coefficient analysis on all samples were performed by R package (version 3.1.3) using the log transformed FPKM values of all genes.

2.5. Quantitative real-time reverse transcription PCR

The expression levels of 20 genes were determined by qRT-PCR using SYBR Premix Ex Taq kit (Takara, China) according to the manufacturer's instructions in a LightCycler® 96 Real-Time PCR System (Roche, USA). The RNA samples were the same as those used for RNA-Seq. First strand cDNA was synthesized from total RNA with oligo $(dT)_{18}$ using M-MLV reverse transcriptase (Takara, China) according to the manufacturer's instructions, and 2 μL of cDNA product was amplified by RT-PCR. The silkworm actin 3 (forward primers 5′-CGGCTACTCGTTCACTACC-3′, antisense of 5′-CCGTCGGGAA GTTCGTAAG-3′) and Glyceraldehyde-3-phosphate dehydrogenase (forward primers 5′-TGTTGAGGGCTTGATGAC-3′, antisense of 5′-ACCTTACCCACAGCTG-3′) were selected as reference genes. All primers for qRT-PCR designed with Oligo Primer Analysis Software V.7 were shown in Supplementary Table 1. All reactions were performed in a 20 μL volume containing 10 μL of AceQ®qPCR SYBR®Green Master MIX (Vazyme, China), 400 nM of each forward and reverse primer, and 2 μL of cDNA (50 ng/μL) template. The qPCR cycling program was run as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. All reactions were done in triplicate. Results were analyzed with the integrated LightCycler® 96 service software. The relative expression ratio of the target genes was determined by Cq and calculated using $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. Transcriptomic sequences analysis and detection of DEGs

The ovary transcriptomes of two groups were sequenced individually, six mRNA libraries were generated from DEPs and NDEPs (three repeats for each group). We obtained approximately 21.5–46.8 million reads with an average length of 75 bp in each library. After quality filtering, the remaining 21.4–46.4 million clean reads were mapped to the *B. mori* genome. 65.58%–67.72% of the total clean reads uniquely mapped to the silkworm genome, covering 14,624 of the 15,260 genes predicted in the Silkworm Genome Database (SilkDB). Average 81.2% of the tags of cross-intron reads were mapped to known exons and 19.8% were located in predicted intergenic or intronic regions. The major characteristics of these libraries were summarized in Supplementary Table 2. The raw reads of RNA-Seq were deposited in the Sequence Read Archive (SRA) database of NCBI with the accession numbers of **SRR5713878**, **SRR5723283**, **SRR5723278**, **SRR5723273**, **SRR5723683**, **SRR5738673**.

Principal component analysis (PCA) showed that 99.1% of the transcriptional changes associated with the different treatments were explained by the two first PCA components. The first and second component clustered the samples under same treatment, the transcriptional response to the conditions of DI and ND could be separated by PCA analyses (Fig. 1).

To investigate expression patterns of pre-diapause related genes, comparative analysis of the normalized data in DEPs versus NDEPs was applied to screen the DEGs. Under the criteria of false discovery rate (FDR) ≤ 0.05 and absolute log2 ratio ≥ 1, 289 DEGs in total were detected between the two groups, including 183 up-regulated and 106 down-regulated genes. These results revealed that visible expression changes occurred in the early-middle pupal stage (Fig. 2).

3.2. Validation of RNA-Seq data by qPCR

To confirm the reliability of the RNA-Seq data in the present study, 20 DEGs with different expression patterns were selected to perform qRT-PCR. The results showed a good consistency between the data of RNA-Seq and qRT-PCR (Pearson's $r = 0.836$, Fig. 3). For each gene, the expression level of RNA-Seq data exhibited similar expression profile comparing with the results of qRT-PCR. It suggested a reliable expression results generated by RNA-Seq experiment of the present study.

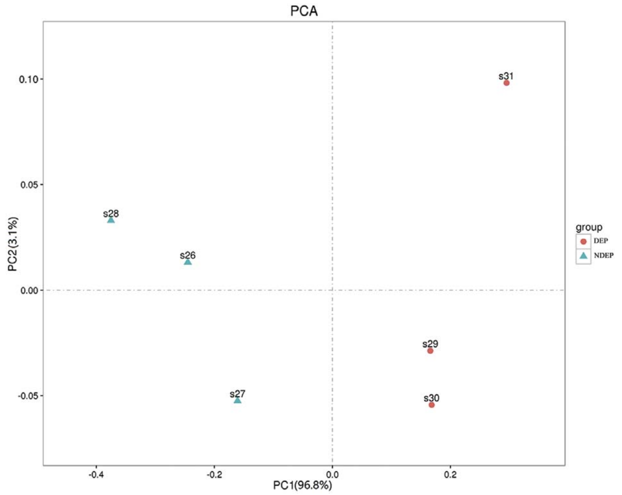

Fig. 1. Principal component analysis (PCA) for all RNA-Seq samples. The samples of DEPs treatment (25 °C) were represented by the blue triangles, the samples of NDEPs treatment (17 °C) by the red dots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. GO classification of DEGs

To gain further insight into the biological processes of DEGs involved in diapause preparation of pupal stage, all of the DEGs were mapped to the whole transcriptome background and given GO/KO annotations. GO enrichment analysis showed that Go terms were generated for 283 DEGs in DEPs versus NDEPs (Fig. 4). Among these, 81 genes were mapped to biological processes, 87 genes were mapped to cellular components, and 122 genes were mapped to molecular functions. Of the biological process related genes, most were involved in single-organism process, single-organism metabolic process, single-organism localization and transport. Most of the cellular component related genes were involved in integral component of membrane, intrinsic component of membrane, membrane part and membrane. While most of the molecular function related genes were involved in oxidoreductase activity, fatty-acyl-CoA reductase (alcohol-forming) activity, oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor and transporter activity (Table 1).

3.4. KEGG pathway classification of DEGs

The pathway enrichment analysis revealed that, of all genes with a Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, 100 DEGs with pathway annotation were assigned to 53 KEGG pathways. We filtered the pathways that were significantly enriched in DEGs with P value ≤ 0.05. 9 KEGG pathways were significantly enriched (Supplementary Table 4). We generated a scatter plot for these enriched results, as shown in Fig. 5. The enriched pathways could be grossly divided into several functional aspects, covering transport and catabolism, lipid metabolism, as well as biosynthesis of other secondary metabolites. Pathways related to transport and catabolism included peroxisome and lysosome. Lipid metabolism related pathways included glycerolipid metabolism, glycerolipid metabolism and fatty acid elongation. Caffeine metabolism was mainly linked to biosynthesis of other secondary metabolites. The pathway of longevity regulating pathway - multiple species also enriched significantly. These annotations provided a valuable resource for studying specific processes, functions and pathways in silkworm diapause research.

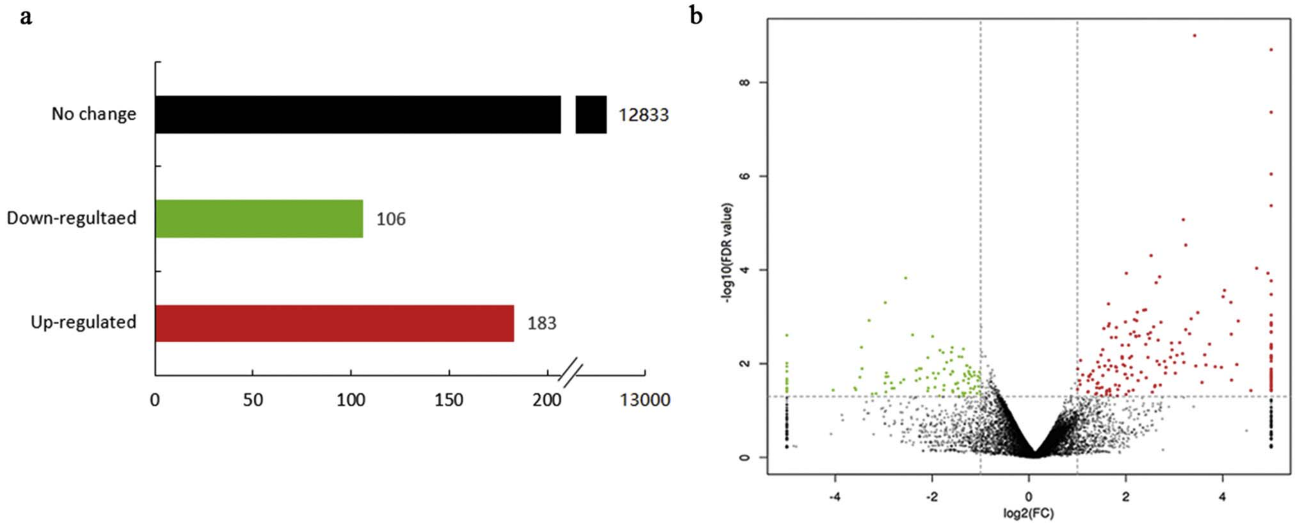

Fig. 2. Expression levels in DEPs vs. NDEPs. The number of DEGs and non-regulated genes between DEPs compared with NDEPs (a). Volcano plot of logarithmic fold change values (DEPs vs. NDEPs) against negative log10 of FDR value (b). Red denotes upregulated genes, green denotes downregulated genes, and black denotes non-regulated genes in DEPs compared with NDEPs under the criteria of FDR ≤ 0.05 and absolute log2 ratio ≥ 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

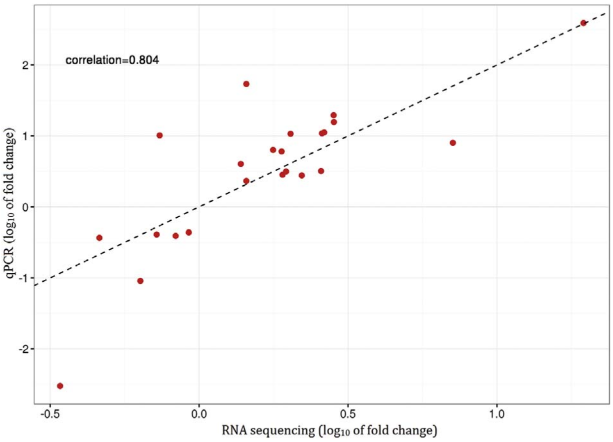

Fig. 3. The Pearson correlation analysis of qRT-PCR results and RNA sequencing data for the 20 selected DEGs between DEPs and NDEPs. Each point represents a value of fold change of expression level in NDEPs comparing with that in DEPs. Fold-change values were log10 transformed.

3.4.1. DEGs related to peroxisome pathway

KEGG pathway analysis showed that peroxisome was the only pathway highly significantly enriched. Peroxisomes are membrane-bound subcellular organelles found in most eukaryotic cells, they perform a number of critical functions, including antioxidant system formation, ether phospholipid biosynthesis, peroxisomal fatty-acid oxidation, and glyoxylate detoxification (Goodman, 2006). A list of 10 DEGs related to peroxisome was provided in Supplementary Table 3, and a heat map of these DEGs was presented (Fig. 6A). The functions involved DEGs have three aspects: Matrix protein import, plasmalogen biosynthesis and antioxidant system formation. For plasmalogen biosynthesis, 5 DEGs encoding fatty acyl-CoA reductase 1 (FAR-1), BGIBMGA011147, BGIBMGA011120, BGIBMGA011207, BGIBMGA006569, and BGIBMGA011129, showed up-regulation in DEPs, FAR-1 catalyzes the reduction of saturated fatty acyl-CoA to fatty alcohols. For antioxidant system, aldehyde oxidase (AOX) is one of the important peroxisomal oxidoreductases, *Bombyx mori* has numerous potential AOX like genes. Of these examined, 2 genes, BGIBMGA008244 and BGIBMGA008268, were clearly up-regulated in DEPs; while two homologous genes encoding superoxide dismutase (SOD), anther oxidoreductase for antioxidant system, BGIBMGA002798 and BGIBMGA002907, were down-regulated in DEPs. Peroxisomal matrix proteins (PMP) were imported into peroxisomes after they were synthesized on free polysomes, such as PMP34, PMP22 and so on (Goodman, 2006). PMP34 is an integral membrane protein, two genes have been identified to encode PMP34 in silkworm. One of two, BGIBMGA0011180, was up-regulated in DEPs. After all, most of the peroxisome-related DEGs showed increased expression. By contrast, eight of these genes examined were down-regulated in NDEPs, and only two were up-regulated. Overall, these results implied an increased peroxisomal activity in DEPs but not NDEPs. The expression of 10 DEGs related to peroxisome was validated using qRT-PCR (Fig. 6B).

3.4.2. DEGs related to glycerolipid metabolism pathway

Glycerolipid (GL) metabolism mainly refers to the process of esterification of fatty acyl-coenzyme A (FACoA) onto a glycerol backbone to synthesize triacylglycerol (TAG), followed by its hydrolysis with the release of the free fatty acid (FFA) that can be reesterified (Prentki and Madiraju, 2008). As the most important storage fuel of eukaryotes, TAG synthesis occurs on the mitochondrial outer membrane and in the endoplasmic reticulum (ER). The anabolic phase of this process utilizes glycerol and acyl-coenzyme A as substrates. First, glycerol is converted into glycerol 3-phosphate by glycerol kinase, then glycerol 3-phosphate obtained is coupled with 2 acyl-CoA by the catalytic action of glycerol phosphate acyltransferase and lipid phosphate phosphohydrolase to yield diacylglycerol, the final triacylglycerol is obtained by coupling of fatty acyl-CoA with diacylglycerol through the action of diacylglycerol transferase. The catabolic phase occurs with the hydrolysis of the ester bonds by lipase enzymes such as esterase FE4, acylglycerol kinase, and diacylglycerol kinase etc. (Fig. 7A) (Henry et al., 2014).

We examined the following DGEs encoding enzymes involved in synthesis and breakdown of TAG: aldo-keto reductases (AKRs) (geneID: BGIBMGA009801), lipid phosphate phosphohydrolase 1 (LPPs1) (geneID: BGIBMGA010468, BGIBMGA003967), diacylglycerol acyltransferase 1 (DGATs-1) (geneID: BGIBMGA013553) and esterase FE4 (geneID: BGIBMGA000777) (Supplementary Table 3). Of these, BGIBMGA000777 encoding esterase FE4-like was the only significant down-regulated gene in DEPs. An esterase FE4 is a hydrolase enzyme that splits a TAG into a FFA and a diacylglycerol (DAG) in a chemical reaction called hydrolysis, this enzyme of the endoplasmic reticulum is rate limiting in the biosynthesis of TAG. Low expression levels of *fe4-like* genes may lead to increased levels of TAG accumulation (Field and Devonshire, 1998); LPPs are integral membrane proteins believed to

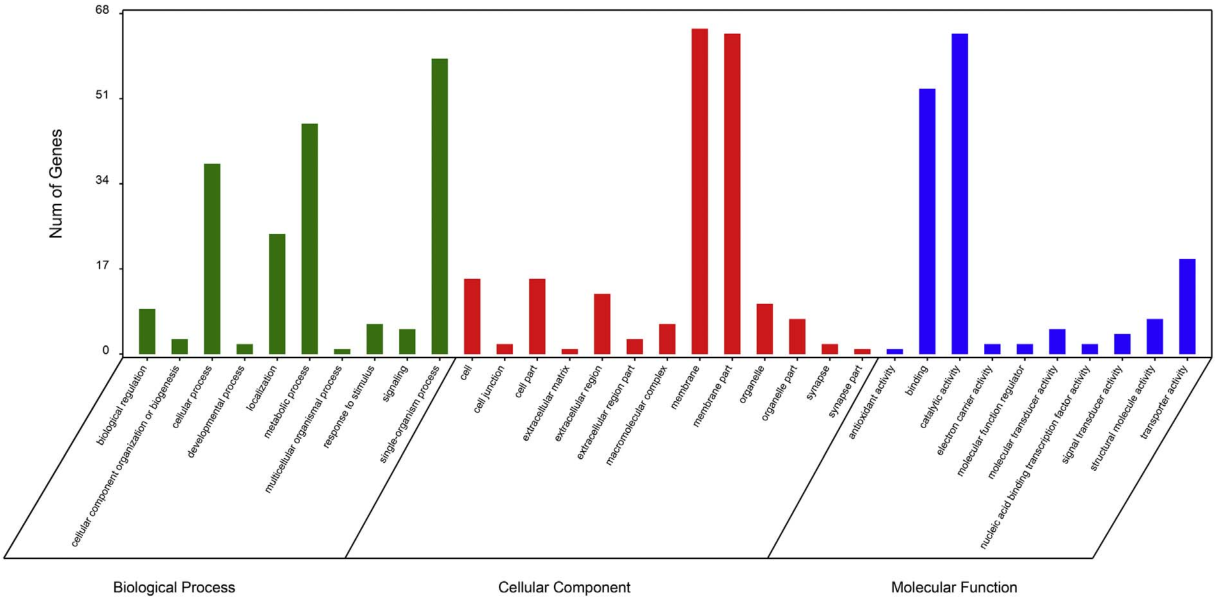

Fig. 4. Gene ontology (GO) classification of DEGs. The x-axis is gene functional classification of GO and the y-axis indicates the number and percent of unigenes mapped to the indicated GO term.

dephosphorylate diacyl-glycerol phosphate to DAGs. In mammals, LPPs might participate in controlling the concentrations of DAGs relative to phosphatidic acid (PAs), overexpression of LPPs in mice endothelial cells increased their DAG concentrations by about 50% (Jasinska et al., 1999). The *B. mori* genome contained five genes similar to *lpp*. Of these, the two DEGs, BGIBMGA010468 and BGIBMGA003967, were up-regulated in DEPs, and the remaining three were no changes; DGAT acylates DAG to TAG and plays an important role in the regulation of

Table 1
GO functional enrichment analysis of DEGs between DEPs and NDEPs (the top ten items).

GO ID	Gene ontology term	GeneRatio	BgRatio	*P* value	FDR
	Terms from the process ontology				
GO:0044699	Single-organism process	59/81	2556/4840	0.16×10^{-3}	0.043
GO:0044765	Single-organism transport	21/81	712/4840	0.55×10^{-2}	0.35
GO:1,902,578	Single-organism localization	21/81	724/4840	0.67×10^{-2}	0.35
GO:0006801	Superoxide metabolic process	2/81	8/4840	0.73×10^{-2}	0.35
GO:0055085	Transmembrane transport	15/81	455/4840	0.76×10^{-2}	0.35
GO:0044710	Single-organism metabolic process	28/81	1084/4840	0.79×10^{-2}	0.35
GO:0055114	Oxidation-reduction process	15/81	483/4840	0.013	0.38
GO:0072593	Reactive oxygen species metabolic process	2/81	11/4840	0.014	0.38
GO:0006810	Transport	24/81	925/4840	0.014	0.38
GO:0051234	Establishment of localization	24/81	929/4840	0.015	0.38
	Terms from the function ontology				
GO:0080019	Fatty-acyl-CoA reductase (alcohol-forming) activity	5/122	26/7292	0.6×10^{-4}	0.011
GO:0016491	Oxidoreductase activity	21/122	569/7292	0.43×10^{-3}	0.027
GO:0016620	Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	5/122	39/7292	0.44×10^{-3}	0.027
GO:0016903	Oxidoreductase activity, acting on the aldehyde or oxo group of donors	5/122	46/7292	0.95×10^{-3}	0.044
GO:0003774	Motor activity	6/122	79/7292	0.20×10^{-2}	0.067
GO:0051537	2 iron, 2 sulfur cluster binding	3/122	16/7292	0.22×10^{-2}	0.067
GO:0005215	Transporter activity	19/122	581/7292	0.34×10^{-2}	0.089
GO:0003824	Catalytic activity	64/122	3020/7292	0.85×10^{-2}	0.19
GO:0004252	Serine-type endopeptidase activity	8/122	176/7292	0.92×10^{-2}	0.19
GO:0042302	Structural constituent of cuticle	7/122	148/7292	0.012	0.22
	Terms from the component ontology				
GO:0016021	Integral component of membrane	64/87	2430/4490	0.13×10^{-3}	0.0041
GO:0031224	Intrinsic component of membrane	64/87	2436/4490	0.14×10^{-3}	0.0041
GO:0044425	Membrane part	64/87	2521/4490	0.53×10^{-3}	0.010
GO:0016459	Myosin complex	4/87	22/4490	0.73×10^{-3}	0.010
GO:0016020	Membrane	65/87	2650/4490	0.15×10^{-2}	0.017
GO:0005576	Extracellular region	12/87	265/4490	0.46×10^{-2}	0.043
GO:0015629	Actin cytoskeleton	4/87	42/4490	0.84×10^{-2}	0.068
GO:0031513	Nonmotile primary cilium	1/87	1/4490	0.019	0.10
GO:0005856	Cytoskeleton	6/87	1/4490	0.019	0.10
GO:0036038	TCTN-B9D complex	1/87	1/4490	0.019	0.10

GeneRatio: the denominator represents the total number of DEGs with GO annotation, and the numerator represents the number of DEGs mapped to each GO term. BgRatio: the denominator represents the total number of reference genes with GO annotation, and the numerator represents the number of reference genes annotated in each GO term. FDR: *P*-value in hypergeometric test after correction.

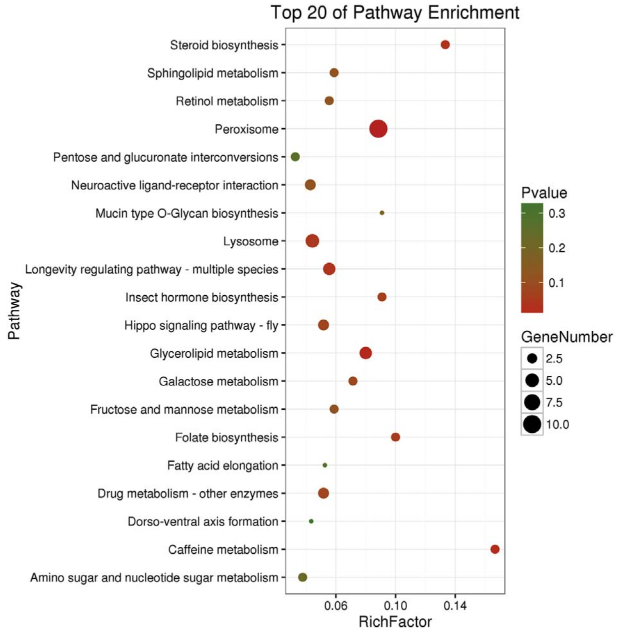

Fig. 5. Scatter plot for KEGG enrichment results. The Rich factor is the ratio of DEG numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the Rich factor, the greater the degree of pathway enrichment. A lower *P* value indicates greater pathway enrichment.

energy storage. Two separate genes code for the DGAT-1 and -2 isoforms. DGAT-1 is present on the cytosolic aspect of the ER membrane, where it is responsible for the synthesis of cytosolic TAG (Turchettozolet et al., 2011). BGIBMGA013553, only a gene encoding DGAT-1 in silkworm genome, was up-regulated. In short, with respect to TAG synthesis, *lpp* and *dagt-1* were clearly up-regulated in DEPs; BGIBMGA009801 encodes Aldo-keto reductases (AKRs) that catalyze the reduced NAD (P) H-dependent reduction of carbonyl groups to yield primary and secondary alcohols on a wide range of substrates, including aliphatic and aromatic aldehydes etc. In glycerolipid metabolism pathway, AKRs catalyze D-glyceraldehyde to yield glycerol. Moreover, they are able to reduce excess glucose to sorbitol, an important cryoprotectant in diapause egg (Bell and Coleman, 1980). BGIBMGA009801 was up-regulated in DEPs. In general, this upregulation of DGEs related to GL synthesis likely reflects the uptrend of TAG accumulation, the concurrent suppression of its catabolism during diapause preparation. The expression of 5 DEGs related to TAG metabolism was validated using qRT-PCR (Fig. 7B).

3.4.3. *DEGs related to the FOXO signaling pathways and the longevity regulating*

Increased longevity is an important feature of the diapause program, while the FOXO (forkhead box protein O) signaling pathway may play a crucial role in regulation of longevity (McElwee et al., 2006). We found that three DEGs participate in the longevity regulating and the FOXO signaling pathways simultaneously: BGIBMGA012305, BGIBMGA006212 and BGIBMGA004277 (Supplementary Table 3). In the two pathways, IGF-like peptides (IGFLPs) can activate IGF receptor. Activated receptor tyrosine kinases (RTKs) activate phosphatidylinositol 3-kinase (PI3K), PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). In the end, PI3K phosphorylates Akt protein at serine and threonine residues. Activated Akt phosphate phosphorylates FOXO, the phosphorylation of FOXO by Akt subsequently decreases the production of antioxidant enzymes extended lifespan, reduces glucose and pyruvate production through a decrease in transcription of mitochondrial phosphoenolpyruvate carboxykinase (PEPCK), and inhibits fat accumulation in the cell by a decrease of adenylate cyclase 2 (AC-2) transcription, but in its un-phosphorylated state, FOXO has the opposite effect (Sim et al., 2015). In *Bombyx mori*, BGIBMGA004277 encoding PEPCK and BGIBMGA006212 encoding AC-2 were up-regulated in DEPs; while BGIBMGA012305 encoding *Bombyx* IGFLP (BIGFLP) was down-regulated in DEPs. These results implied that BIGFLP may be an inactive state and FOXO is activated (phosphorylated state) during diapause preparation of silkworm. The expression of the three DEGs related to the two pathways was validated using qRT-PCR (Fig. 8a).

3.4.4. *DEGs related to molting hormone biosynthesis*

The steroid hormone ecdysone (E) is also involved in the regulation of insect diapause. It is thought that circulating ecdysone is converted to the active form, 20-hydroxyecdysone (20E), at the target tissues, where it binds its nuclear receptor to elicit specific changes in gene

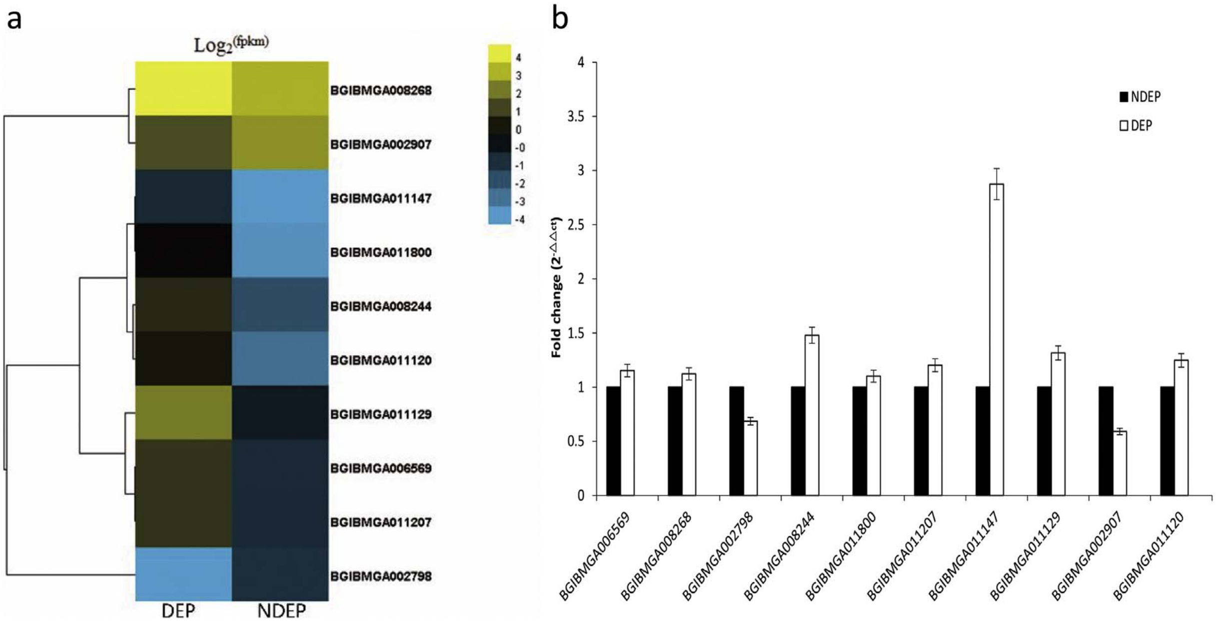

Fig. 6. Cluster analysis of differential gene expression pattern. The heatmaps show the expression levels of 10 peroxisome-related DEGs (a). Real-time PCR analysis for peroxisome-related DEGs between DEPs and NDEPs (b).

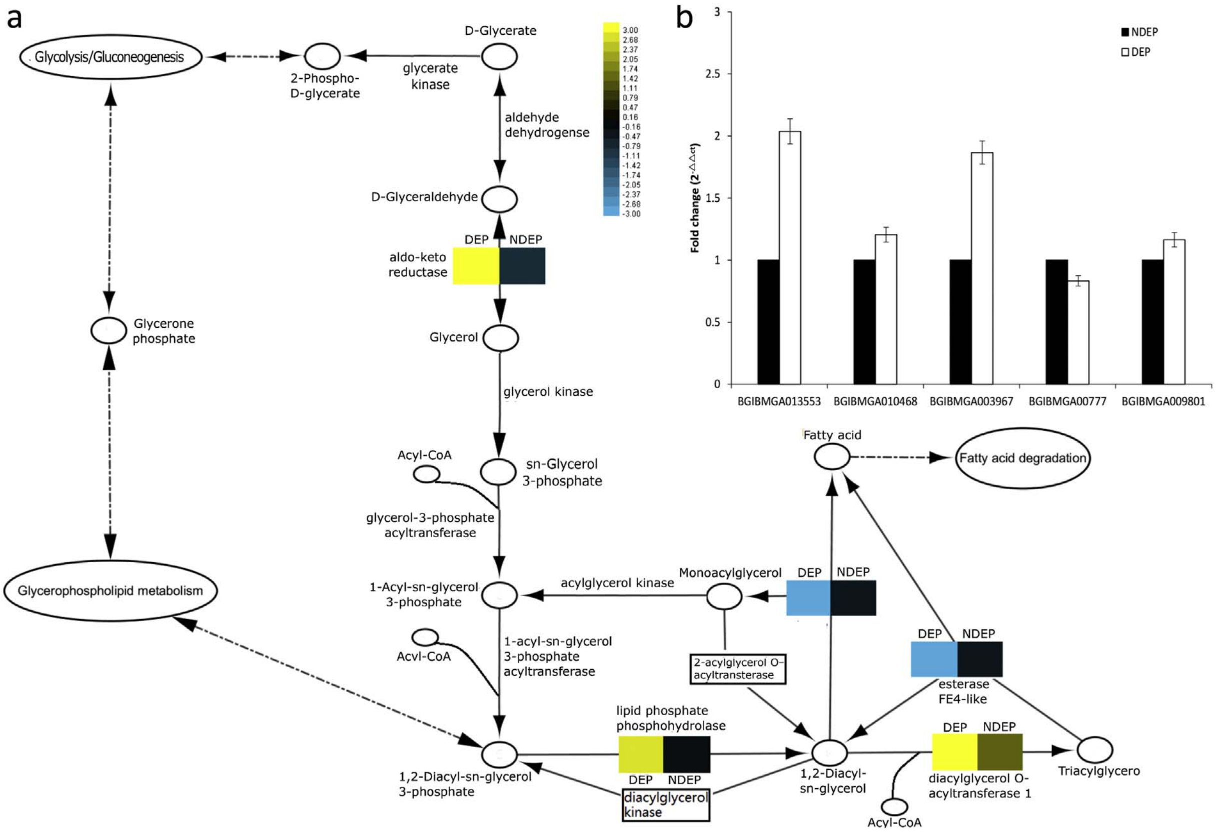

Fig. 7. Expression levels of DEGs involved in glycerolipid metabolism pathway between DEPs and NDEPs. Transcripts differentially expressed between DEPs and NDEPs were mapped to TAG metabolism pathway (a). The pathway diagrams were modified from the portions of KEGG maps for glycerolipid metabolism (bmor00561). The data for DEG expression level is taken log2 of it. For enzyme reactions, the arrows between two metabolites represented the directions of catalytic reactions. The solid lines indicate direct reaction; the dotted lines indicate indirect reaction. Real-time PCR analysis for DEGs related to TAG metabolism between DEPs and NDEPs (b).

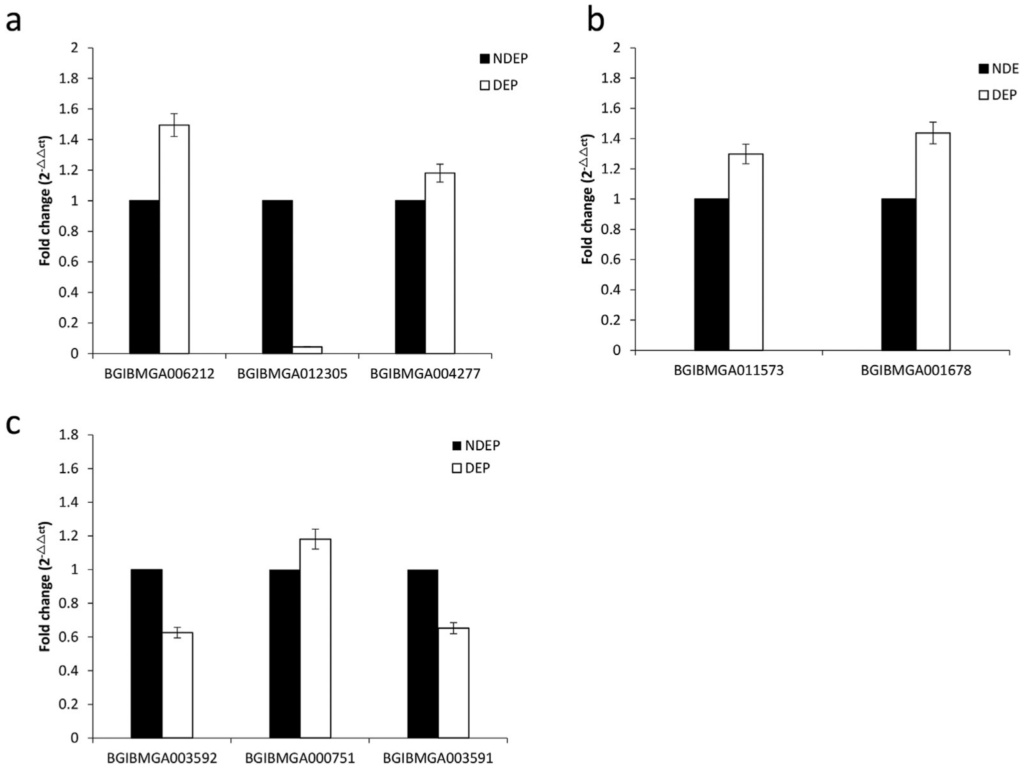

Fig. 8. Real-time PCR validation for DEGs involved in several pathways between DEPs and NDEPs. Real-time PCR analysis for DEGs related to longevity regulating pathway between DEPs and NDEPs (a). Real-time PCR analysis for DEGs related to 20E biosynthesis between DEPs and NDEPs (b). Real-time PCR analysis for DEGs related to the Hippo signaling pathway between DEPs and NDEPs (c).

transcription (Ohnishi et al., 1971). The biosynthesis of 20E from cholesterol is mediated by the P450 cytochrome enzymes (CYPs) encoded by members of the Halloween gene family. In the biosynthetic pathway, Cholesterol is first converted to 7-dehydro-cholesterol (7dC) by neverland (an oxygenase-like protein), the conversion from dehydrocholesterol to diketol is catalyzed by CYP307A1/2, cytochrome P450, family 307, subfamily A, numbers 1/2, subsequently (Sonobe and Yamada, 2004). Here, two DEGs involved in 20E biosynthesis were differentially regulated. BGIBMGA001678, an ortholog of *cyp307a1*, and BGIBMGA0011573 encoding neverland (nvd) protein were clearly up-regulated in DEPs. The expression of the two DEGs related to 20E biosynthesis was validated using qRT-PCR (Fig. 8b).The increased activity of CYP307A1 and nvd may raise the titre of 20E in the cell, but the principal regulatory role for the diapausing preparation needs to be further investigated.

3.4.5. DEGs related to the Hippo signaling pathway

A number of genes related to the Hippo signaling pathway showed significant alterations between DEPs and N DEPs. Lowfat (Lft), a conserved cytoplasmic protein, and Homothorax (Hth), a DNA-binding transcription factor, are involved in the Hippo signaling pathway as an upstream modulator and a downstream regulator, respectively (Mao et al., 2009). Two *lft* homologs (BGIBMGA003592, BGIBMGA003591) were significantly down-regulated in the DEPs when compared to NDEPs, while *hth* (BGIBMGA000751) was in the opposite. The expression of the three DEGs related to Hippo signaling pathway was validated using qRT-PCR (Fig. 8c).

3.4.6. Several KEGG pathways related to the production of cryoprotectants

In this study, several amino acid and carbohydrate metabolism pathways were enriched with no significant *P* value, which include glycine, serine and threonine metabolism, biosynthesis of amino acids, starch and sucrose metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, amino sugar and nucleotide sugar metabolism, and fructose and mannose metabolisms(Supplementary Table 4). The majority of DEGs in these categories were up-regulated in DEPs oocytes. Enhancing gluconeogenic pathways and amino acid concentrations can raise synthesis of glycerol and sorbitol that are the most common cryoprotectant molecules in insects. Moreover, some other polyols could be synthesized such as erythritol, mannitol, ribitol, and threitol, as well as sugars with a cryoprotective role (Hahn and Denlinger, 2011). These results led to a better understanding on cold and desiccation resistance of silkworm.

4. Discussion

During diapause preparation phase, insects must complete developmental processes while at the same time retaining the programmed information specifying developmental arrest later in ontogeny or next generation. In addition to the rearing condition during larvae developing, the diapause determination of the progeny eggs is almost maternal in silkworm bivoltine strain. Mother's experience of the environment during their own embryonic development is transduced to neuroendocrine signals to induce progeny eggs' diapause or non-diapause. In this process, DH plays a key regulatory role to prepare the biochemical condition leading to diapause destined eggs (Yamashita, 1985, 1996). DH acts on a G protein-coupled receptor in the developing ovaries, triggers some key genes activation, and leads to changes in energy metabolism or storage to improve cold/stress tolerance, which can be transmitted to the progeny through factors in the egg cytoplasm. These factors influence the development of the progeny, and finally result in diapause in the gastrula-embryo. Some key mechanistic processes displayed by maternal oocytes, where genes are expressed to produce properties different from those laid non-diapause eggs (Yamashita et al., 2001; Homma et al., 2006; RÄSÄNen and Kruuk, 2007). In this study, we compared gene expression changes between pre-diapause and non-diapause, our results presented here provided some clues about the molecular events that characterize diapause preparation of *Bombyx mori*.

4.1. More active peroxisomal metabolism in DEPs

The peroxisomes have remarkable metabolic plasticity, as protein composition, and biochemical functions vary depending on physiological conditions and metabolic needs. Our studies showed that the expression of genes related peroxisomes was different between the prediapausing and non-diapausing oocytes of *B. mori*.

We observed markedly increased expression of five *B. mori* genes encoding the peroxisomal enzyme fatty acyl-CoA reductase 1 (Far1) in DEPs oocytes (Table 1 and Supplementary Table 3). Far1 is essential for the formation of long-chain fatty alcohols, which catalyzes the transformation of fatty acids into fatty alcohols with NADPH (Yang et al., 2012). Fatty alcohols are the precursors of wax-esters and ethers lipids in insects (Teerawanichpan et al., 2010; Yang et al., 2012; Carotsans et al., 2015). Wax-ester plays an important role in reducing water evaporation and enhancing defense against micro-organisms and environmental suitability (Jackson and Baker, 1970; Russell, 2004); while ethers lipids are essential membrane components that have many diverse roles, including in protection against reactive oxygen species, in membrane-fusion-mediated events, and in establishing a structural permeability barrier for cells, and so on. 1-alkenyl (Vinyl ether) lipids are known as plasmalogens, which are thought to act as cellular antioxidants (Nagan and Zoeller, 2001). The high amount expression of the *far1* homolog in DEPs oocytes is very likely to have arisen from the rapid accumulation of fatty alcohols, wax-esters and ether lipids in the cells, which in turn lead to the difference of the composition, structure, and physical properties of cellular membranes between the two groups being compared. It is speculated that such changes contribute to the membrane to maintain the liquid crystalline state at lower temperatures, and to the high tolerance to all kinds of stress during diapause.

Two stress response genes identified in DEPs oocytes are putative aldehyde oxidase, which encodes a multifunctional molybdo-flavoenzyme with broad substrate specificity involved in the oxidation of aromatic N-heterocycles and aldehydes (Garattini et al., 2003). Several functions have been proposed for this peroxisomal enzyme including its involvement in catalyzing metabolic pathways, vitamin degradation, and detoxification of environmental pollutants (Garattini et al., 2003). In addition, aldehyde oxidase plays an important role in insecticide resistance in the common house mosquito, *Culex quinquefasciatus* (Coleman et al., 2002). Although the function of aldehyde oxidase in pre-diapausing silkworm is yet unknown, it is possibly a component of an elevated stress–response system operating during diapause.

Peroxisomes are the main site for the break-down of very-long and long-chain fatty acids through β-oxidation. As a lot of research shows that genes involved in β-oxidation were strongly suppressed in early diapause (Poelchau et al., 2013; Hao et al., 2016), but in this study, we did not detect the differential expression genes related to β-oxidation between pre-diapause and non-diapause oocytes. The peroxisomal β-oxidation generates H_2O_2, NADH and shortened acyl-CoAs as the major end products. Since H_2O_2 can potentially damage cellular molecules, such as proteins, nucleic acids and lipids, its elimination by antioxidant enzymes is an important process (Kobayashi et al., 2014). However, two genes encoding superoxide dismutase (SOD), a typical antioxidant enzymes, are less abundant in pre-diapause oocytes. Similar results were also observed in diapausing flesh fly (Ragland et al., 2010). We hypothesize that the down-regulation of some anti-oxidative genes is most likely due to a slow accumulation of H_2O_2 that may be resulted from a decrease in β-oxidation during the diapause preparing process.

At present, peroxisomes are increasingly recognized as the participation factors of diapause program. It is speculated that peroxisomal roles associated with diapause may be more important than we think.

4.2. Increased TAG synthesis for diapause initiation

This energy storage is critical not only for surviving prolonged periods of developmental arrest but also for maximizing reproductive success once development resumes (Hahn and Denlinger, 2007). Insects store the energy reserves in the fat body to meet energy demands during the diapause. Lipids are the dominant reserve stored in complex organisms and animals. Given energy stores in form of triacylglycerides (TAG) are common (Ohtsu et al., 1992), identification of genes involved in TAG synthesis and metabolism is significant during diapause preparation. In this pathway, the up-regulated genes of DEPs oocytes are mainly that contribute to accumulation of TAG in the fat body such as aldo-keto reductase (*akr*), lipid phosphate phosphohydrolase 1 (*lpp-1*), and diacylglycerol acyltransferase 1 (*dagt-1*); while transcript of esterase FE4 involved in catabolism of TAG is significantly down-regulated by comparison to NDEPs oocytes of the same stage (Fig. 6). This suggests the possibility that the transcript profiles of fat-related genes contribute to the elevated TAG levels in DEPs but not N DEPs. TAG stores are the most important energy reserve in most diapausing insects, often accounting for as much as 80–95% of total lipid content. In fact, Diapause-destined individuals of many insects accumulate greater TAG stores than nondiapausing individuals, and increased TAG storage is thought to be an extremely important factor mitigating the metabolic demands of diapause. Failure to adequately prepare for diapause will reduce the likelihood of survival to the end of diapause, interactions between energy storage and metabolism can even influence the decision to enter diapause and determine how long to remain in diapause (Hahn and Denlinger, 2011).

BGIBMGA010163 encoding an Acyl-CoA Δ11 Desaturase-like (ACD) from Fatty-Acyl-CoA desaturases family was more abundant in DEPs oocytes (Supplementary Table 4). Acyl-CoA desaturase catalyze the introduction of unsaturations into long-chain fatty acids to synthesize unsaturated fatty acids. Up-regulation of *acd* may result in an increase of unsaturated fatty acids production. The fatty acid composition of TAG accumulated prior to diapause can differ qualitatively and quantitatively between non-diapausing and diapause-destined individuals within a species. Despite feeding on the same diet, the TAG stores of diapausing individuals from several species contain more unsaturated fatty acids, whereas the TAG stores of nondiapausing individuals contain more saturated fatty acids. Greater unsaturation in diapause-destined individuals may be important for lipid mobilization at low temperatures (Hahn and Denlinger, 2011).

BGIBMGA004157 involved in steroid biosynthesis was more abundant in DEPs oocytes (Supplementary Table 4). In silkworm, it encodes an egg-specific protein that catalyzes the hydrolysis of cholesteryl esters to cholester. Cholester serves as a precursor for the biosynthesis of molting hormone in many insects, such as 20-hydroxyecdysone. Besides, it is an essential structural component of all animal cell membranes that is required to maintain both membrane structural integrity and fluidity (Ikekawa et al., 2016).

4.3. Important signaling pathways during diapause preparation

Signaling pathways play critical roles in regulating the initiation and progression of insect diapause. The Hippo signaling pathway controls organ size in animals through the regulation of cell proliferation and apoptosis. In Drosophila, Lft binds to the cytoplasmic domains of Fat (Ft) and Dachsous (Ds) protocadherin, and initiates the Hippo pathway signal as an upstream modulator. Ubiquitous expression of the *lft* RNAi transgene resulted in flies with slightly shorter wings (Mao et al., 2009); Hth is required for cell survival and proliferation anterior to the morphogenetic furrow in the eye imaginal disc as a downstream regulator of the Hippo signaling pathway (Peng et al., 2009). In this study, *lft* was significantly down-regulated and *hth* was up-regulated in the DEPs when compared to NDEPs. We speculate that this transcript profile in DEPs oocytes may play a role in a heavier body of diapausing insects than that of nondiapausing insect. However, its real function related diapause of silkworm remains to be investigated.

IGFLPs, a new class of insulin family peptides, are produced by the ovariole sheaths during the early pupal stages, whose functions as a growth hormone to regulate the early follicular development in a paracrine manner in *B. mori* (Okamoto et al., 2009, 2011). IGFLPs exert their biological effects by binding to and activating cell surface transmembrane receptors with intrinsic tyrosine kinase activity (Ullrich et al., 1986). Activated IGFLPL receptors are involved in the regulation of components of downstream cascades such as FoxO pathway, and mTOR pathways (Taniguchi et al., 2006). These signaling pathways have been linked to diverse features of the diapause phenotype including arrested development, extended lifespan, suppressed metabolism, fat hypertrophy and enhanced stress tolerance (Lant and Storey, 2010; MacRae, 2010; Meller et al., 2012). In this study, the decreased expression of *bigflp* (BGIBMGA012305) was observed in DEPs oocytes of silkworm. In the absence of BIGFLP, FOXO is activated, and this activation is essential for increasing transcript levels of the genes in fatty acid synthesis, heat shock proteins, catalase, glutathione S-transferase and metallothionein production in pre-diapausing females (Sim et al., 2015). Among them, fat accumulation is an important component of the diapause syndrome in many insects. Activated FOXO facilitates fat accumulation by the increased expression of PEPCK (Sim and Denlinger, 2013). *Pepck* (BGIBMGA004277) was distinctly enriched in in DEPs oocytes. In addition, PEPCK is also part of the gluconeogenesis pathway. It was hypothesized that the higher expression of this transcript in DEPs oocytes may either represent a maternally provisioned regulatory cue, or initiation of the gluconeogenic pathway in advance of the onset of developmental arrest (Poelchau et al., 2011).

The mTOR pathway enriched with no significant *P* value plays an important role in regulation of actin cytoskeleton and cell survival in most insects. Our enriched GO terms (GO:0015629, GO:0005856, Table 1) also revealed that the expression of 13 genes related to actin cytoskeleton and structural constituent of cuticle are differentially alerted between DEPs and NDEPs. This result is consistent with the previous reports on the modification of actin expression occurring in the early diapausing stage of *Culex pipiens* (Kim et al., 2005), *Nasonia vitripennis* (Wolschin and Gadau, 2008) and *Praon volucre* (Colinet et al., 2012). Actin participates in such important cellular processes as muscle contraction and cell motility during cell division. Once the concentration of actin cytoskeleton was changed, cell migration will be affected (Orth and McNiven, 2003). It is speculated that the composition of the cuticle of diapausing eggs may is different from that of non-diapausing eggs.

4.4. Up-regulation of genes related to ecdysteroid biosynthesis in DEPs

Maternal ecdysteroids appears to be required at different titres for fertilization, embryogenesis and hatching in insect eggs. The low titre of ecdysteroids may be involved in the induction of embryonic diapause (Kadono Okuda and Yamashita, 1994). Eggs of most insects contain various molecular species of ecdysteroids in free (activation) and conjugated (inactivation) forms, 20E is a physiologically active molecule in *B. mori* eggs. In this study, two homologs of genes (BGIBMGA001678, BGIBMGA0011573) potentially involved in 20E biosynthesis are expressed most heavily in DEPs oocytes, which may raise the titre of 20E in ovarian cells. The results are consistent with those reported for *Locusta migratoria* (Lagueux et al., 1977) and for *Schistocerca gregaria* (Greenwood et al., 1984). In diapause eggs of silkworm, the levels of free ecdysteroids, such as 20E, hardly increase during early diapause stage, maintain in a low level during diapause, and then increase drastically after diapause termination (Ohnishi et al., 1971). In fact, free ecdysteroids does not produced by de novo biosynthesis in diapause eggs, but ecdysteroids are synthesized in maternal follicle cells during pre-diapause, accumulated in mature ovaries and are transferred to eggs. Most of maternal ecdysteroids stored in the ovaries exist as conjugated forms, the conjugates are bound to the yolk protein, vitellin, and this binding apparently prevents their leakage into the haemolymph. After diapause termination, ecdysteroid conjugates are hydrolyzed to release free ecdysteroids for the developmental resume of diapause eggs (Tawfik et al., 2002; Sonobe and Yamada, 2004; Fujiwara et al., 2006).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbd.2017.07.003.

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