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Universal and differential transcriptional regulatory pathways involved in the preparation of summer and winter diapauses in *Pieris melete*

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Research Paper

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Abstract

Much progress has been made in understanding the environmental and hormonal systems regulating winter diapause. However, transcriptional regulation of summer diapause is still largely unknown, making it difficult to understand an all-around regulation profile of seasonal adaptation. To bridge this gap, comparison RNA-seq to profile the transcriptome and to examine differential gene expression profiles between non-diapause, summer diapause, and winter diapause groups were performed. A total number of 113 million reads were generated and assembled into 79,117 unigenes, with 37,492 unigenes categorized into 58 functional gene ontology groups, 25 clusters of orthologous group categories, and 256 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. KEGG analysis mapped 2108 differentially expressed genes to 48 and 67 pathways for summer and winter diapauses, respectively. Enrichment statistics showed that 11 identical pathways similarly overlapped in the top 20 enriched functional groups both related to summer and winter diapauses. We also identified 35 key candidate genes for universal and differential functions related to summer and winter diapause preparation. Furthermore, we identified some genes involved in the signaling and metabolic pathways that may be the key drivers to integrate environmental signals into the summer and winter diapause preparation. The current study provided valuable insights into global molecular mechanisms underpinning diapause preparation.

Introduction

In adaptation to harsh environmental conditions, insects undergo a programed developmental arrest namely diapause. It is stimulated by photoperiod, regulated by endogenous hormones, and characterized by marked suppression at the metabolism level and enhanced stress tolerance to survive inimical seasons (Koštál and Denlinger, 2011; Koštál *et al.*, 2017). Many insects living in temperate climates undergo over-wintering diapause in response to seasonal dominate cues of shortening day lengths and declining temperatures in late summer and fall, whereas in others species, summer diapause occurs in response to extended photoperiod and increasing temperature. Interestingly, some species undergo both summer and winter diapauses, for example, the cabbage armyworm *Mamestra brassicae* (Goto *et al.*, 2001), the cabbage beetle *Colaphellus bowringi* (Wang *et al.*, 2004), the southern Iberian Peninsula population of large white butterfly *Pieris brassicae* (Spieth *et al.*, 2004), the onion maggot *Delia antiqua* (Hao *et al.*, 2016), the cabbage butterfly *Pieris melete* (Xiao *et al.*, 2012), etc. During over summering diapause, insects undergo a variety of physiological, biochemical, and molecular changes to pause development, reduce metabolism, tolerate high temperatures, and increase their ability to maintain water balance (Benoit, 2010). Therefore, summer diapause shares the same physiological and biochemical adjustments with winter diapause (Goto *et al.*, 2001, Koštál, 2006).

Limited effort has been dedicated to study diapause-specific gene expression using molecular biology or transcriptome techniques (Emerson *et al.*, 2010; Bao and Xu 2011; Poelchau *et al.*, 2011; Gong *et al.*, 2013; Poelchau *et al.*, 2013*a*; Hickner *et al.*, 2015; Sim *et al.*, 2015; Tu *et al.*, 2015; Yocum *et al.*, 2015; Hase *et al.*, 2017; Deng *et al.*, 2018; Zhai *et al.*, 2019). Even fewer studies have focused on physiological, biochemical, and molecular regulation of summer diapause. Therefore, more progress is needed to fully assess characteristics of key metabolic drivers involved in summer diapause (Hao *et al.*, 2016).

To date, the environmental and endocrine physiological regulation of winter diapause has been extensively reported and is well understood (Koštál, 2006). The past decade of research has yielded many insights into the metabolic pathways related to energy storage and utilization, signaling pathways, and molecular mechanisms that govern winter diapause (Hand *et al.*, 2016), such as transforming growth factor (TGF)-β and Wnt signaling pathways (Hickner *et al.*, 2015), tricarboxylic acid (TCA) cycle and hormone biosynthesis (Xu *et al.*, 2012), insulin and juvenile hormone signaling (Sim and Denlinger, 2013; Dong *et al.*, 2014),

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regulation of forkhead transcription factor (FOXO) (Sim *et al.*, 2015) and the target of rapamycin (TOR), mitogen-activated protein kinase (MAPK) signaling pathways (Ragland *et al.*, 2011), prothoracicotropic hormone (PTH) receptor signaling transduction (Dong *et al.*, 2014; Kučerová *et al.*, 2016), and circadian rhythm pathway (Hickner *et al.*, 2015; Kučerová *et al.*, 2016). However, little is known about the interactions between the physiological processes and related molecular initiation mechanisms that mediate the diapause pathway. Furthermore, there is scarcity of reports on global transcriptional analysis of photoperiod-regulated summer diapause. In particular, the mechanism by which components measure and interpret photoperiod remains unresolved, making it difficult to profile the overall mechanisms involved in diapause.

The cabbage butterfly *P. melete* is a major insect pest of cultivated crucifers. In *P. melete*, both summer and winter diapauses occur at the pupal stage, and they can be induced by relatively long (>13 h) and short (<12 h) day lengths, respectively (Xiao *et al.*, 2012). Systematic investigation has been performed on the extraordinary flexibility of *P. melete* to local seasonal environmental challenges (Xiao *et al.*, 2006, 2008, 2009, 2012, 2013). However, little is known about the physiological, biochemical, and molecular mechanisms underlying such high adaptability (Wu *et al.*, 2018). As both summer and winter diapauses are induced by changes in relatively day-length, these characteristics make it a good model for comparative transcription analysis of key components involved in photoperiodic regulation mechanism by contrasting non-diapause, summer and winter diapause individuals.

Our study aimed to examine potential molecular and metabolic mechanisms of the preparation of summer and winter diapauses in *P. melete*. We performed transcriptome analysis to identify universal and differential transcription regulators involved in the preparation of summer and winter diapauses. Genes with differential expression are of particular interest, as they are known to affect important traits related to diapause, such as circadian clock, photoperiodism, stress tolerance, hormone metabolism, and defense response. Therefore, we analyzed differential expression genes between non-diapause, summer diapause, and winter diapause pupae and systematically compared genes related to key pathways and function groups which may reflect major physiological and biochemically shifts among non-diapause, summer and winter diapause states.

Materials and methods

Study insects

P. melete colony was derived from mature larvae collected in the suburbs of Nanchang (28°46′N, 115°50′E), Jiangxi Province, China in November 2006. Fully-grown *P. melete* larvae prior to pupation were collected from crucifers in late November and transferred to wooden cages (30 × 30 × 35 cm) for pupation and eclosion under natural conditions. In the spring of 2012, colony from a single female was established, from which three cohorts of a F7 *P. melete* strain were selected for this experiment. All of the newly hatched larvae were reared at 20°C and 75% relative humidity but grouped into three photoperiod treatments: LD12.5:11.5 was set for non-diapause (ND) control group, LD14:10 to induce summer diapause (SD), and LD10:14 to induce winter diapause (WD), respectively. Each of the three treatments had five biological replicates, with at least 60 individuals per sample. Pupae were collected every 4 h after pupation and were flash frozen in liquid nitrogen 3 days later (72–76 h) until RNA extraction (fig. S1). Additional individuals were reserved to determine the incidences of diapause.

RNA isolation, library preparation, and Illumina sequencing

To obtain an overview of background genetic information, a mixed sample from different developmental statuses (non-diapause, summer diapause, and winter diapause) was prepared. Specifically, pupae at prediapause stage (3 days prior to pupation) were sampled from non-diapause, summer and winter diapause groups with three replicates for each group ($n = 5$ individuals were pooled for RNA extractions in each replicate).

Total RNAs were purified with TRIzol® reagent (Invitrogen, Life Technologies, Grand Island, New York), treated with DNase I to eliminate any possible DNA contamination, and purified with RNeasy RNA Purification Kit (Qiagen, Hilden). RNA quality was assessed on an RNA chip (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA). mRNA was enriched using the oligo(dT) magnetic beads, followed by library construction and sequencing according to the manufacturer's instructions with Illumina HiSeq 2000 (BGI Tech, ShenZhen, China). Ten cDNA libraries (a mixed sample from different development status, three each for non-diapause, summer diapause, and winter diapause groups) were constructed using a TruSeq RNA kit (Illumina, San Diego, California). These cDNA libraries were then sequenced with HiSeq 2000 Illumina platform (BGI Tech, ShenZhen, China).

Assembly and gene prediction

Transcriptome *de novo* assembly was performed with the Trinity platform (Grabherr *et al.*, 2011). Clean reads were mapped to reference sequences or reference gene set using a short oligonucleotide alignment program to generate contiguous contigs (sequences).

To uncover the molecular function underlying the transcriptome profile, unigene sequences were aligned to nucleotide database NT (*e*-value <0.00001) using BLASTx and protein databases of NR, clusters of orthologous group (COG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (*e*-value <0.00001) using BLASTN in NCBI, respectively. Gene annotations of unigenes (contigs) were carried out by BLASTx alignment, followed by running the Blast2GO program (http://www.blast2go.com/b2ghome) (gene ontology (GO), COG, and KEGG annotation; http://www.kegg.jp/). All transcriptome datasets generated from the current study were deposited in Sequence Read Archive of NCBI with accession number of PRJNA625900.

Differential expression analysis

Gene expression levels were calculated using the RPKM method. Pearson correlation coefficient between replications was all >0.85, indicating consistency among replicates. NOIseq method, a strict algorithm referring to the significance of digital gene expression profiles, was applied to screen differentially expressed genes (DEGs) between two samples. Zero RPKM values were replaced by 0.001 to calculate the fold change. DEGs were identified under the filtering conditions of the mean fold change across three replicates ≥2 ($|\log 2\ \text{ratio}| \geq 1.0$, probability ≥0.8 and adjusted $P \leq 0.001$). Genes with similar expression patterns are usually functionally related. With Nr annotation, the Blast2GO program was used for GO enrichment analysis of digital DEGs.

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Pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in DEGs compared with the whole transcriptome background. We generated a scatter plot for KEGG enrichment result, with RichFactor (the ratio of numbers of DEG to all gene annotated in the same pathway terms) and *Q*-value (corrected *P* value). The top 20 pathway terms enriched in each treatment are showed in the scatter plot.

Results

Diapause incidence

For winter diapause group at 20°C and LD10:14, all pupae entered diapause. For summer diapause group at 20°C and LD14:10, the mean incidence of diapause was 89.78%. At 20°C and intermediates day-length of LD 12.5: 11.5, the diapause incidence with an average of 7.73%, which was significantly lower than that under LD10:14 and LD14:10 (fig. S2).

Transcriptome analyses

Transcriptome sequencing of mixed mRNA samples from different developmental stages generated a total number of 113,409,662 raw reads (table 1). After filtering, a total number of 109,339,284 clean reads were assembled into 133,893 contigs with an average length of 355 nt and a N50 of 812 nt (table 1). Among the 79,117 unigenes with an average length of 913 nt and N50 of 1888 nt. These unigenes included 27,706 that were classified as distinct clusters while 51,411 were singletons (table 1). The length distributions of unigenes are shown in fig. S3, with 39,948 unigenes were longer than 500 nt, 24,270 unigenes were longer than 1000 nt, and 11,059 unigenes were longer than 2000 nt (fig. S3).

Proteins with the highest sequence similarity to the given unigenes were retrieved along with their functional annotations. A total number of 34,534 (43.65% of all unigenes) sequences had a BLASTX result (table 1). The *e*-value, similarity, and species distribution of the best retrieves in Nr database are shown (fig. S4). Using GO and COG analysis, 19.73 and 15.23% of the unigenes were categorized into 58 functional GO groups (table 1 and fig. S5) and 25 COG categories (table 1 and fig. S6), respectively. To gain further insights into gene functions in relative biological pathways, KEGG pathway analysis was also conducted (table S1). A total number of 22,769 unigenes were successfully assembled into 256 KEGG pathways, including 3356 unigenes in metabolic pathways, 981 unigenes in the regulation of actin cytoskeleton, and 840 unigenes in purine metabolism (table S1).

Unigenes annotated as MAPK signaling pathway, insulin signaling pathway, insect hormone, and steroid hormone biosynthesis transduction pathways were also assembled to related KEGG pathways (table 2). These candidate unigenes classified belonged to MAPK/insulin signaling pathway and insect hormone biosynthesis provided a meaningful perspective on signal transduction for preparation of diapause (table 2).

Quantification of DEGs

The total clean reads for quantification of DEGs in each sample ranged from 22.94 to 24.66 million (fig. S7 and table S2). The total mapped reads to unigenes ranged from 21.22 (92.46% of clean reads) to 22.92 million (93.22% of clean reads) across the nine libraries (table S2). The distribution of mapped read counts was also evaluated (fig. S8). Among the detected transcripts, a total number of 2108 genes were differentially regulated in three pairwise comparisons of summer diapause vs. non-diapause (table S3), winter diapause vs. non-diapause (table S4), and summer diapause vs. winter diapause (table S5). Among genes with significant differential expression across samples, 209 genes were up-regulated and 634 genes were down-regulated between non-diapause vs. summer diapause, 276 genes were up-regulated and 927 genes were down-regulated between non-diapause vs. winter diapause, and 52 genes were up-regulated and ten genes were down-regulated between summer and winter diapause (fig. 1). Log_2 fold change of differential gene expression ranged from −16 to 11 (fig. 1 and tables S3–S5). Differential gene expression results showed little global change between summer and winter diapause groups, with only 62 DEGs.

Table 1. Overview of statistics of transcriptome sequencing, assembling, and functional annotation in *P. melete*

Overview of statistics and annotation		
RNA-seq results	Total raw reads	113,409,662
	Total clean reads	109,339,284
	Total clean nucleotides (nt)	9,840,535,560
	Q20%	97.27%
	N %	0.01%
	GC%	41.77%
Assembling results	Total no. of contigs	133,893
	Mean length of contigs	355
	Total no. of unigenes	79,117
	Mean length of unigenes	913
	N50 of contigs/unigenes	812/1888
	Distinct clusters/ singletons	27,706/51,411
Annotation	Unigenes with Nr database	34,534 (43.65%)
e-value <0.00001	NT	21,194 (26.79%)
	Swiss-Prot	25,679 (32.46%)
	KEGG	22,769 (23.78%), 258 pathways
	COG	12,053 (15.23%) 25 functional categories
	GO	15,609 (19.73%) 58 subcategories
	All	37,492 (47.38%)

DEGs functional enrichment of summer and winter diapauses

Results from GO enrichment annotations suggested that DEGs between SD and ND were enriched in metabolic and cellular processes, single-organism process, regulation of biological regulation, responses to stimulus, and the molecular function of binding and catalytic activity (fig. S9). For DEGs between winter diapause and non-diapause, enrichment module showed the same trait with summer diapause in the molecular function term of catalytic

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Table 2. Unigenes annotated to steroid hormone biosynthesis and prothoracicotropic hormone (PTTH) receptor signaling transduction pathways.

Pathway ID	Pathway	Unigenes with pathway annotation	Ratio[a] (%)
Ko04010	MAPK signaling pathway	599	2.63
Ko04910	Insulin signaling pathway	541	2.38
Ko00140	Steroid hormone biosynthesis	126	0.55
Ko04020	Calcium signaling pathway	422	1.85
Ko04310	Wnt signaling pathway	348	1.53
Ko04150	mTOR signaling pathway	219	0.96
Ko00981	Insect hormone biosynthesis	105	0.46
Ko00100	Steroid biosynthesis	88	0.39
Ko04013	MAPK signaling pathway – fly	66	0.29
Ko00900	Terpenoid backbone biosynthesis	41	0.18

[a]Ratio represent the given pathway all genes with pathway annotation (22,769).

activity, cellular process, metabolic process, single-organism process, biological regulation, regulation of biological process, and response to stimulus (fig. S9). GO term analysis of DEGs between summer and winter diapauses revealed enrichment of genes associated with responses to stimulus, multi-organism process, extracellular region, metabolic process, immune system process, and single-organism process (fig. S9).

KEGG pathway enrichment between summer and winter diapauses

KEGG analysis mapped 1908 DEGs to 48 and 67 pathways for summer and winter diapauses, respectively. Pathway enrichment analysis using the KEGG database indicated that 11 primary diapause-regulated pathways were related to both summer and winter diapauses (based on the enrichment of differentially-regulated transcripts) (figs S10 and S11). The primary pathways in summer diapause regulation were metabolic pathway (18 transcripts), pyruvate metabolism (18 transcripts), and purine metabolism pathway (eight transcripts) (table 3). Similarly, these three pathways were also the top three primary pathways in winter diapause regulation, with 33 transcripts in metabolic pathway, 19 transcripts in pyruvate metabolism, and 15 transcripts in biosynthesis of secondary metabolites. There was an overlap in enriched functional groups between summer and winter diapauses, suggesting a similar regulating mechanism in the processes of summer and winter diapause preparation. Differentially regulated transcripts only enriched in winter diapause preparation included amino sugar and nucleotide sugar metabolism (ten transcripts), pyrimidine metabolism (nine transcripts), six transcripts each for galactose metabolism, pentose and glucuronate interconversions, and ribosome biogenesis (table 3).

Potential gene differential expression in summer and winter diapause preparation

Thirty five candidate genes were selected with differentially expressed (fold change ≥2) in pairwise comparisons among non-diapause, summer and winter diapause (fig. 2). Up-regulation of

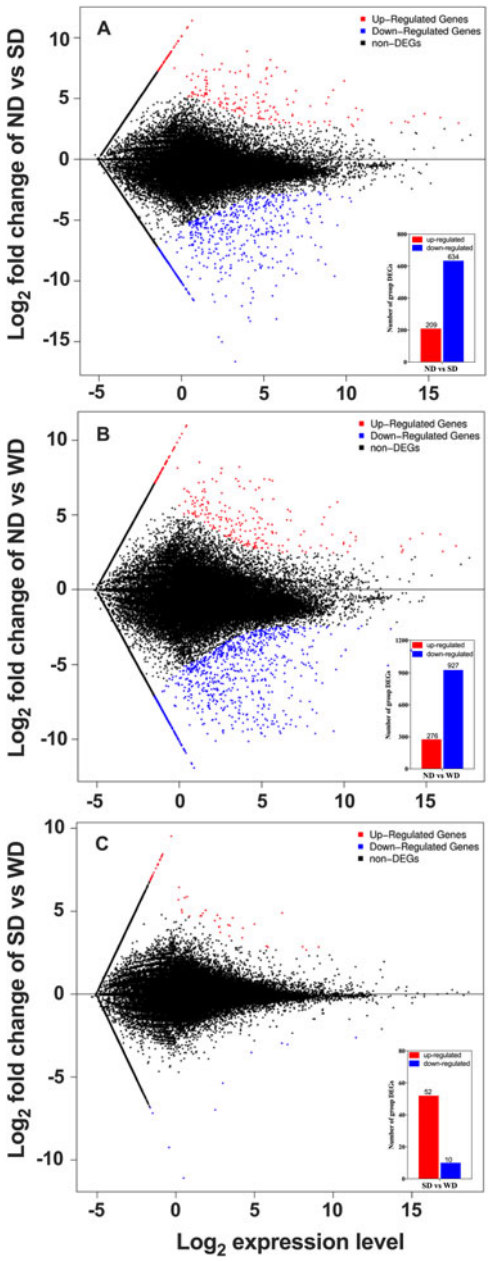

Figure 1. Changes of up- and down-regulated genes in differential expression profiles in pairwise comparison among non-diapause (ND), summer diapause (SD), and winter diapause (WD). Log 2 fold-change of ND vs. SD to log abundance of TMM-normalized gene expression levels (A). ND vs. WD (B), and SD vs. WD (C). Each point represents an individual gene. Up-regulated genes with higher expression and positive fold-change values are given in red, and genes with lower expression and negative fold-change values are given in blue, and genes that are not differentially expressed are given in black. Genes that are significantly differentially expressed are define as having probability ≥0.8 and absolute log 2 fold-change >2.0.

the cytochrome P450 (CYP15A1, methyl farnesoate epoxidase to JH) and neither inactivation nor after potential B (*nina B*, phototransduction), compared to non-diapause, were differential up-regulation expression both in summer and winter diapauses, whereas cryptochrome2 (*Cry2*), dopa decarboxylase (*Ddc*), and peptidoglycan-recognition protein (*Pgrp*) were specific to winter diapause state (only up-regulation in the comparison between winter diapause and non-diapause). A total of 28 genes (such as *Cry1*, *cpo*, *dCLK*, *dy*, *Fmr*, *Jheh*, *Jhe*, etc.) showed down-regulation between

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Table 3. Pathway categories that were significantly enriched for DEGs in the paired comparison between summer diapause (SD) and winter diapause (WD).

		No. DEGs up-regulation		No. DEGs down-regulation	
Category name	Category ID	SD	WD	SD	WD
Biosynthesis of secondary metabolites	Ko01110	1	1	4	14
Biosynthesis of unsaturated fatty acids	Ko01040	4	4	1	1
Cutin, suberine, and wax biosynthesis	Ko00073	2	2	2	2
Fructose and mannose metabolism	Ko00051	3	3	1	3
Metabolic pathways	Ko01110	6	8	12	25
Peroxisome	Ko04146	4	4	3	5
Purine metabolism	Ko00230	0	0	8	10
Pyruvate metabolism	Ko00620	2	2	15	17
RNA polymerase	Ko03020	0	0	5	6
RNA transport	Ko03013	2	3	4	5
Spliceosome	Ko03040	0	0	6	7
Galactose metabolism	Ko00052		3		3
Amino sugar and nucleotide sugar metabolism	Ko00520		0		10
Pentose and glucuronate interconversions	Ko00040		2		4
Pyrimidine metabolism	Ko00240		0		9
Ribosome biogenesis in eukaryotes	Ko03008		3		3

Functional groups are KEGG pathways and GO-slim categories. Groups with a Benjamini–Hochberg corrected $P < 0.05$ and six or more differential expression genes were considered significantly enriched. The direction of gene expression refers to up- and down-expression under summer diapause (SD) or winter diapause (WD) conditions.

summer diapause and non-diapause. Similarly, 18 genes (i.e. *Cry1*, *cpo*, *dCLK*, *dy*, *Fmr*, *Jheh*, *Jhe*, *disco*, *Gbeta76C*, etc.) were down-regulated between winter diapause and non-diapause (fig. 2). Specific genes showed differential status between summer and winter diapauses were down-regulation genes of cryptochrome2 (*Cry2*) and dopa decarboxylase (*Ddc*), up-regulation genes of spook (*spo/spok*), peptidoglycan-recognition protein (*Pgrp*), *Hinnavin II* (*Hin II*), and triacylglycerol lipase (*Tgl*) (fig. 2).

Discussion

In this study, we performed comparative transcriptome analysis to characterize the gene expression patterns in summer and winter diapause preparation compared to non-diapause state in *P. melete*. We also identified potential regulatory mechanisms both shared by and differed between summer and winter diapause preparation. DEGs between summer and winter diapause related to key pathways identified and compared diapause-enriched pathways that showed a similar overlap in enriched functional groups. Furthermore, through pairwise comparisons between non-diapause, summer and winter diapause, 35 genes from 2108 candidate DEGs showed similar differential status in summer and winter diapauses, and thus, they were considered to be potential genes with universal functions related to diapause preparation. Our results highlighted shared and differential regulation mechanisms of diapause preparation between summer and winter diapause, and we revealed that seasonal adaptation regulation pathways, metabolism and key groups of some key genes were shared between summer and winter diapause preparation.

Previously, the onion maggot *D. antiqua* was used as a model to investigate the molecular mechanisms of summer and winter diapauses (Ishikawa *et al.*, 2000; Hao *et al.*, 2016). However, for this species the duration of SD (within 2 weeks) was too short, compared to WD, and SD was sensitive to temperature (Ishikawa *et al.*, 2000; Hao *et al.*, 2016), making it not an ideal model for comparing molecular mechanisms between summer and winter diapauses that are regulated by photoperiod. To the best of our knowledge, the current example should be the first report to compare global transcriptional components on photoperiod regulated both in summer and winter diapauses under the same temperature.

Biological and metabolic pathways related to prediapause

Summer and winter diapauses induced by signals such as photoperiod and temperature are important adaptations that allow insects to escape harsh seasonal environments. As a physiological condition of developmental delay, series of physiological changes are known to be the key characteristics of diapause, such as developmental arrest with a significant decline in respiration and metabolism, enhanced stress tolerance, cell cycle regulation, circadian rhythms, energy storage, and lifespan extension. Therefore, targeted genes related to these physiological changes are expected to be differentially expressed in diapausing individuals (Koštál *et al.*, 2017).

Pathway enrichment analysis of the biological and metabolic pathways revealed some potential regulatory mechanisms that could drive the preparation of summer and winter diapauses. Complex switch both in biological processes and metabolic mechanisms was involved in the preparation of summer and winter diapauses. We found that in the cabbage butterfly *P. melete* unique transcripts and genes related to metabolic process, catalytic activity, regulation of biological process, and response to the stimulus, and

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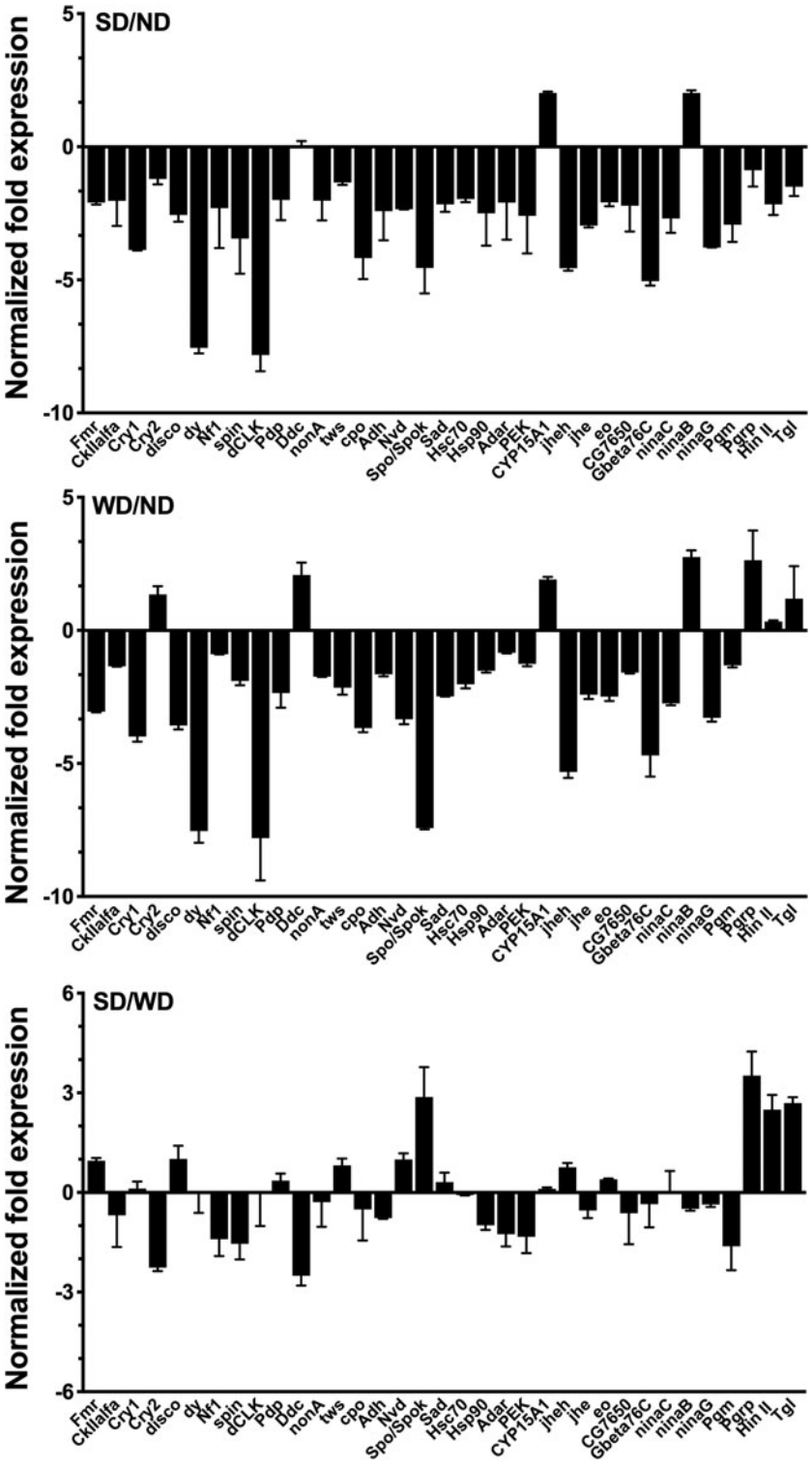

Figure 2. Normalized differential expression in some of the selected candidate genes in RNA-Seq analysis. Normalized fold expression of the candidate genes in SD vs. ND, WD vs. ND and in SD vs. WD in RNA-Seq analysis. Positive values on the *Y*-axis indicate up-regulation whereas negative values indicate down-regulation.

cellular process are enriched in the same way as and other species including *Drosophila melanogaster* (Zhang *et al.*, 2011), the small carpenter bee *Ceratina calcarata* (Durant *et al.*, 2016), the alfalfa leaf-cutting bee *Megachile rotundata* (Yocum *et al.*, 2015), the migratory locust *Locusta migratoria* (Yocum *et al.*, 2015), and the spotted wing drosophila *Drosophila suzukii* (Zhai *et al.*, 2019). Except for these gross similarities, quantitative comparisons of gene expression patterns between summer and winter diapauses at GO levels, although transcripts of DEGs for summer and winter diapauses were enriched largely in similar function categories, it did not fully overlap (fig. S9).

Signaling pathway related to diapause

Diapause preparation is physiologically stimulated by external environmental signal and regulated by endogenous hormones.

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Although it seems to be a cessation state, active processes or syndrome by minimal metabolism level enhances resistance to adverse environments (Koštál, 2006). In most winter diapause, universal checkpoint mechanisms between diapause and non-diapause status include metabolic and signaling pathways such as prothoracicotropic hormone receptor signaling transduction (Dong *et al*., 2014; Kučerová *et al*., 2016), cross-talk between the brain and fat body in tricarboxylic acid cycle regulate hormone biosynthesis (Xu *et al*., 2012), MAPK signaling pathway (Dong *et al*., 2014; Tu *et al*., 2015; Yocum *et al*., 2015; Hao *et al*., 2016; Kučerová *et al*., 2016), insulin signaling pathway (Sim and Denlinger, 2008; Zhai *et al*., 2019), regulation of forkhead transcription factor (FOXO) (Sim and Denlinger, 2008; Sim *et al*., 2015), target of rapamycin (mTOR) (Kučerová *et al*., 2016), Wnt (Ragland *et al*., 2011; Koštál *et al*., 2017), mitogen-activated protein kinase signaling pathway (Ragland *et al*., 2011), and Ca^{2+} signaling (Dong *et al*., 2014; Zhao *et al*., 2017). GO and KEGG analyses showed no significant differential enrichment in the signaling-related categories and pathways. Among the primary diapause-regulated pathways, 11 were regulated both in summer and winter diapauses, including metabolic pathway, pyruvate metabolism, purine metabolism pathway, biosynthesis of secondary metabolites, etc. Thus, the overlap in GO and KEGG pathways between summer and winter diapauses suggests involvement in the universal regulation mechanisms. In WD preparation, differential enrichment occurred in amino sugar and nucleotide sugar metabolism, pyrimidine metabolism, galactose metabolism, pentose and glucuronate interconversions, and ribosome biogenesis. This observation suggests that these candidate pathways involved in energy storage and utilization, signal sensors, or transduction were important for WD in *P. melete*.

Gene expression differences between summer and winter prediapauses

Diapause is an alternative developmental program that is initiated in response to and occurs well in advance of physiologically limiting environmental factors as a token stimulus, which is often photoperiod. Although several components of the diapause preparation have been elucidated, key functional genes in regulatory networks related to these complex changes are yet to be fully identified. Candidate key genes that play universal regulatory roles in diapause are mainly involved in depression of metabolic (Koštál *et al*., 2017; Leal *et al*., 2018), changes in photo-transduction, circadian or clock rhythm, and cell-cycle arrest (Poelchau *et al*., 2013*b*; Yocum *et al*., 2015), changes in signaling pathway related to insulin (Poelchau *et al*., 2013*b*; Yocum *et al*., 2015), TGF-β and Wnt pathway (Hickner *et al*., 2015), up-regulation of stress response genes (King and MacRae, 2015), and circadian clocks in time measurement that decides whether to enter diapause or not (Hand *et al*., 2016; Santos *et al*., 2018). Our analysis screened out 35 DEGs that were linked to SD or WD preparation induced by different light treatments (fig. 2). We suggest that the current study provides valuable data for subsequent functional analysis of this set of differential expression genes, which could provide further insight into the molecular mechanisms of seasonal adaptation in *P. melete* and other related pests.

In adaptation to major diverse environmental factors, many of the phenotypic responses during summer and winter diapauses are the consequence of biological, physiological, or molecular pathways. In this study, we performed comparative transcriptome profiling among preparation of non-diapause, summer and winter diapause and identified partial overlapping in universal and differential transcription regulatory pathways involved in the preparation of summer and winter diapauses.

Further studies on adaptation strategies to over-summering and over-wintering in non-model species are needed to fully understand the molecular mechanisms of these universal and differing responses to environmental changes. In the cabbage butterfly *P. melete* specifically, future studies on differential gene expression during the dynamic progression, maintenance, and termination of diapause and comparisons between summer and winter diapauses would provide additional insights into the entire process, in particularly regulatory mechanism transcriptome-wide.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485321000018.

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Author contributions.
H. J. X. conceived the study. H. J. X. designed the experiments. H. J. X. and T. J. conducted the preliminary work. H. J. X. and T. J. collected data. H. J. X., T. J. and Y. Z. analyzed the data. H. J. X. and T. J. wrote the first draft, other authors provided editorial advice. All authors read and approved the final manuscript.

Conflict of interest. The authors declare no conflicts of interest or any involvement, financial or otherwise, that might potentially bias the work.

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