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Comparative transcriptome analysis provides a glance into the regulation of the *Krüppel homolog 1* on the reproduction and diapause of the predatory ladybeetle, *Coccinella septempunctata*

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Abstract

As a typical natural predator of agricultural pests, the lady beetle, *Coccinella septempunctata*, has been commercially mass-reared and widely employed in in pest management. The lady beetles can enter a long-term reproductive diapause phase in response to short photoperiods and low temperatures, for maintaining population during the overwintering process. Insect diapause is a physiological adaptative strategy that is characterized by a cessation of ovarian development, lipid accumulation and extended lifespan. Diapause regulating improve the long-term storage of *C. septempunctata* and release of natural enemies at the right time. In our previous research, as a main output of the juvenile hormone pathway, *Krüppel homolog 1* (*Kr-h1*) was a key component of reproduction and diapause in *C. septempunctata*. However, the molecular mechanisms underlying the regulation of *C. septempunctata* reproduction and diapause by Kr-h1 transcription factor remains unknown. In this study, we utilized RNA-Sequencing to investigate the transcriptomic changes in *C. septempunctata* following RNA interference targeting the *Kr-h1* gene. DEGs analysis revealed significant transcriptional alterations between the *Kr-h1* knockdown group and the control group. Noteworthy findings include the downregulation of three genes related to reproduction (*follicle cell protein,vitelline membrane protein*, and *vitellogenin*) in the dsKr-h1 group, while genes involved in lipid metabolism, such as *lipase* and *fatty acid synthase*, were upregulated. These results suggested that *Kr-h1* plays a critical role in the regulation of both reproductive processes and lipid metabolism in *C. septempunctata*. Our findings provided valuable insights into the molecular mechanisms regulating reproduction and diapause in *C. septempunctata* and contributed to the expanding understanding of the role of *CsKr-h1* in insect physiology.

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Introduction

Reproductive diapause represents a sophisticated adaptation mechanism in insects, characterized by suspended ovarian development, enhanced lipid accumulation, and prolonged lifespan [1]. This physiological state enables insects to survive unfavorable environmental conditions, including temperature extremes, drought, and food scarcity [1]. Understanding the molecular underpinnings of diapause has significant implications for biological control programs [2, 3], particularly in optimizing the mass production and storage of beneficial insects such as *Harmonia axyridis* [4], *Chrysopa Formosa* [5], and *Trichogramma dendrolimi* [6].

The regulation of reproductive diapause is orchestrated by juvenile hormone (JH), with its absence or reduced titers serving as a primary trigger for diapause initiation [7]. This hormonal regulation has been well-documented in diverse insect species, including *Colaphellus bowringi*,*H. axyridis*, and *Culex pipiens*, where both suppressed JH biosynthesis and enhanced degradation contribute to diapause induction [8–12]. The molecular cascade through which JH governs the diapause-reproduction switch has been gradually elucidated, revealing a central role for the Methoprene-tolerant (Met) receptor and its downstream transcription factor, Krüppel-homolog 1 (Kr-h1) [13, 14]. Recent molecular studies have established that the JH-Met-Kr-h1 signaling axis serves as a master regulator of reproductive status. RNA interference targeting the *Met* gene in *Pyrrhocoris apterus* and *C. bowringi* halts reproduction and induces a diapause-like state [15, 16]. JH, together with its receptor Met, directly induces Kr-h1 expression [13, 17, 18]. The mechanistic details of this regulation have been particularly well-characterized in *C. bowringi*, where *Kr-h1* functions as a transcriptional switch, simultaneously activating reproduction-related genes while suppressing diapause-associated factors. Notably, downstream effectors of Kr-h1, including *triacylglycerol lipases* (*TGLs*) and *mini-chromosome maintenance 4* (*Mcm4*), have been identified as crucial mediators of ovarian development and lipid metabolism regulation [19]. The conservative nature of Kr-h1's role in reproductive regulation is evidenced across diverse insect taxa. For instance, silencing Kr-h1 in *Aedes aegypti* and *Nilaparvata lugens* impairs reproduction, highlighting its importance in JH-mediated reproductive processes [20–22]. Kr-h1 depletion leads to a reduction in *vitellogenin* (*Vg*) mRNA levels and blocks oocyte maturation in female migratory locusts [23]. These findings collectively establish Kr-h1 as a critical node in the JH-Met signaling network, coordinating various developmental and reproductive processes.

The seven-spotted ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), is a commercially valued biological control agent deployed against various agricultural pests, including aphids, whiteflies, mites, thrips, and lepidopteran eggs and larvae [24, 25]. This predator species is extensively mass-reared and utilized in integrated pest management programs across Europe, Asia, and North America [26, 27]. In Northern China, adult *C. septempunctata* undergoes winter diapause triggered by short photoperiods and low temperatures, characterized by arrested ovarian development, enhanced lipid accumulation, and extended longevity [28, 29]. Our previous investigations revealed that juvenile hormone (JH) deficiency, mediated through the upregulation of JH-degrading enzymes (*CsJeh* and *CsJheh*), plays a pivotal role in initiating and maintaining reproductive diapause in *C. septempunctata* [30]. The deficiency in JH leads to the downregulation of the *CsKr-h1* gene during reproductive diapause in *C. septempunctata* [30]. Furthermore, interfering with the *CsKr-h1* gene represses reproductive development by blocking vitellogenesis and ovarian growth [31]. These results suggest that *Kr-h1* is a critical regulator of the pathway that coordinates reproduction and diapause in *C. septempunctata*. However, the detailed molecular mechanisms underlying JH action in the regulation of *C. septempunctata* reproductive diapause by the Kr-h1 transcription factor remain unknown.

In this study, we aim to explore the role of the Kr-h1 transcription factor in regulating reproductive diapause in *C. septempunctata* by employing high-throughput RNA sequencing (RNA-seq). By uncovering the molecular function of Kr-h1 in diapause regulation, this research will provide a theoretical foundation for advancing our understanding of reproduction and diapause mechanisms and enhancing the mass production of biological control agents.

Materials and methods

Experimental insects and sampling

The *C. septempunctata* laboratory colony initially originated from field-collected adults from the wheat field at the east gate of the Chinese academy of agricultural sciences located in Haidian district, Beijing, China, in 2010. Offspring of those lady beetles were maintained at 24 ± 1 °C and 70% relative humidity (RH) under a long-day photoperiod condition (16 h:8 h, light/dark) and fed with fresh pea aphids (*Aphis glycines*) daily. The *C. septempunctata* laboratory colony reared at 24°C under a

long-day (16 L:8D) condition for ten days became reproductive. Our previous work indicates that the first 20 days after eclosion is the pre-diapause stage of *C. septempunctata* adult at the diapause induction condition of 18°Cunder a short-day (10 L:14D) [29].

To evaluate the relative mRNAs expression of the *CsKr-h1* gene at different stages, we sampled the one-day-old female adults, three-day-old female adults, five-day-old female adults and ten-day-old female adults of *C. septempunctata* under non-diapausing conditions and under diapause-inducing conditions. All samples were collected, cleaned, and frozen using liquid nitrogen. The samples were then stored in a refrigerator (-80 °C) until analyses were done. Each treatment was performed with three biological replicates, and each replicate was made up of four female adults.

RNA interference (RNAi)

To further elucidate the molecular mechanisms of JH-mediated diapause regulation through Kr-h1, we conducted RNAi experiments targeting specific genes. The synthesis of dsRNA and the injection of double stranded RNA are based on the previous methods [31]. Specifically, as follows: Double-stranded RNA (dsRNA) against green fluorescent protein (GFP) was used as the control. The 344 bps regions of *CsKr-h1* were amplified from cDNA using the PCR technique with the specific primers dsCsKr-h1 (Table S1). the MEGAscript T7 High Yield Transcription Kit (Invitrogen, Carlsbad, CA, USA) were used to synthesize dsRNAs. Female beetles of uniform size were injected with 1 μL of dsRNA solution (2 μg/μL) at the internode membranes between the second and third abdominal segments, with dsGFP-injected specimens serving as controls. Whole-body samples were collected 48 h post-injection for transcriptome sequencing and RT-qPCR analyses to investigate the downstream molecular cascades regulated by *CsKr-h1* and RNA interference efficiency. A total of 3 independent biological samples were included for each group, and each biological replicate contains 4 females.

Construction of cDNA libraries and sequencing

Total RNAs were extracted from RNAi-individuals using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The samples were divided into two parts: one for the construction of cDNA library and high-throughput sequencing, and the other for subsequent qRT-PCR. RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). The samples with a 28 S/18S ratio > 1.5 and an RNA integrity number (RIN) > 7 were selected for the subsequent analysis. The next step involved the enrichment of mRNA using magnetic beads with Oligo (dT). The mRNA was fragmented using a fragmentation buffer, after which the resulting fragments underwent conversion into first-strand cDNA using N6 random primers. Subsequently, a second-strand cDNA synthesis was undertaken to continue the process. These cDNA fragments were then ligated with adapters. PCR amplification was performed, and the resulting products were used for the cDNA library construction, following the protocols provided by the manufacturer. Finally, the generated cDNA libraries were subjected to sequencing through the BGISEQ-500 platform, utilizing a 50 bp single-end strategy (BGI, Shenzhen, China).

Transcriptome sequencing data analysis

RNA-Seq was used to compare gene expression profiles between Kr-h1-depleted and control females. Three biological replicates of each sample were used to construct cDNA libraries, which were then subjected to RNA-seq; the data were pooled for analysis. Gene expression levels were calculated using the FPKM (fragments per kilobase of exon per million mapped fragments) method. After obtaining the expression of all RNAs, principal component analysis and correlation analysis will be carried out. Differentially expressed genes (DEGs) were identified using the DESeq package. To identify differentially expressed transcripts, the criteria were $\log2^{|(\text{fold change})|} \geq 2$ and adjusted $p < 0.05$. Candidate unigenes were selected according to the NCBI non-redundant (NR) protein sequences annotation. The raw data from sequencing are available at the NCBI under BioProject PRJNA1158213.

Gene ontology and Kyoto encyclopedia of genes and genomes analyses

The DEGs were analyzed for Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, with a p-value threshold of < 0.05 for further analysis. The GO classification consists of three categories: cellular component, molecular function, and biological process. Heat map analysis was conducted using OmicShare tools (https://www.omicshare.com/tools) with the FPKM values of genes that were associated with lipolysis, lipogenesis, insect reproduction, and diapause.

Gene quantitation by qRT-PCR

Transcript abundance was measured using real-time PCR (qRT-PCR). Total RNA was extracted from the insect samples using the TRIzol Reagent, and 1 μg of total RNA was reverse transcribed to cDNA using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) under the following conditions: 42 ℃ for 30 min, and 85 ℃ for 5s. Subsequently, real-time PCR was done using TORO-Green® 5G qPCRPremix (Toroid Technology Limited) and a LightCycler® 96 Instrument (Roche, Switzerland). All reactions were run in triplicates, with a total volume

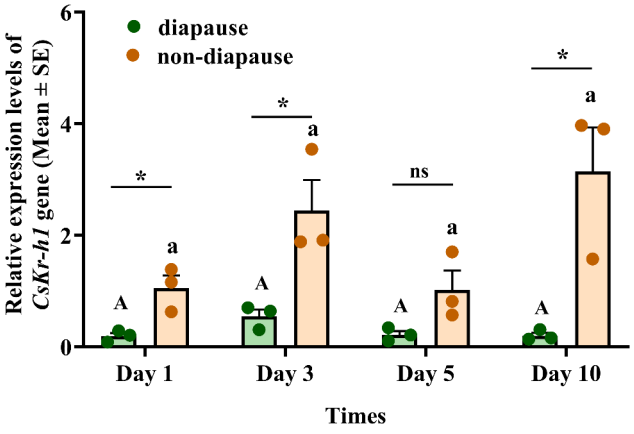

Fig. 1 Time expressions of *CsKr-h1* under diapause/non-diapause condition. The mRNA expression levels of *CsKr-h1* were measured at various time points, and data are presented as Mean ± SE of triplicate biological replicates. The data were statistically analyzed by *t*-test between expressions of *CsKr-h1* under diapause and expressions of *CsKr-h1* under non-diapause condition (*$p < 0.05$, "ns" ≥ 0.05); The time expressions of *CsKr-h1d*were statistically analyzed by one-way ANOVA followed by Tukey's HSD (The letters "a" indicate significant difference at the 0.05 level under non-diapause condition; The letters "A" indicate significant difference at the 0.05 level under diapause condition)

of 20 μL, and each reaction consisted of 10 μL TORO-Green Premix, 0.8 μL of each specific primer (Table S1), 1 μL sample cDNA, and 7.4 μL nuclease-free water. PCR amplification of the genes was conducted under the following conditions: 95 ℃ for 5 min, followed by 40cycles at 98 ℃ for 10 s and 55–59 ℃ for 20 s. A dissociation step cycle (95 ℃ for 10 s, 65 ℃ for 60 s, and from 65 °C to 97 °C in increments of 0.2 °C / s; 5 readings / °C) was added for the melting curve analysis. When the reactions were complete, CT values were determined using fixed threshold settings. The relative expression levels of genes were analyzed using the comparative $2^{-\Delta\Delta Ct}$ quantitation method [32], and abundance was normalized to the *CsActin* transcript level [30, 33]. A total of 3 independent biological samples were included for each group, and three technical replicates of each biological sample were processed for all reactions.

The assessement of potential off-targets of dsCsKr-h1

For reasonably identify the downstream genes of *CsKr-h1*, the potential off-targets of dsCsKr-h1 in *C. septempunctata* were evaluated using "dsRIP" (https://dsrip.uni-goettingen.de/efficiency) with 0 mismatches per siRNA for off-target prediction [34–36].

Data analysis

Statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The differences in gene expression between the dsRNA-injected sample and the control sample was evaluated using Student's *t*-test ($P < 0.05$).

Table 1 Statistics of the RNA-seq data in each of the three replicates

Sample	Clean Bases (Gb)	Q20 (%)	Q30 (%)	Mapped Reads (%)	Unigene Number
dsKr-h1_1	44.43	98.22	93.56	91.96	44,425,550
dsKr-h1_2	44.21	98.07	93.12	91.93	44,207,266
dsKr-h1_3	44.36	98.05	93.08	92.39	44,355,750
dsGFP_1	44.06	98.27	93.82	90.88	44,060,812
dsGFP_2	45.28	98.36	94.11	90.66	45,280,276
dsGFP_3	44.76	98.28	93.84	91.78	44,755,588

Results

Stage-specific transcript abundance of the *CsKr-h1* gene

We investigated the transcriptional profiles of the *CsKr-h1* at the diapause induction phase (D1, D3, D5, and D10) and during the corresponding developmental state (N1, N3, N5, and N10) of *C. septempunctata* females, respectively (Fig. 1). In the diapause induction-phase and non-diapause induction-phase, there were no significant difference in the mRNA abundances of the *CsKr-h1* gene at different times. However, compared to non-diapause females, *CsKr-h1* consistently showed lower expression levels throughout the diapause induction stages (Fig. 1).

Overview of the RNA-Seq of *C. septempunctata* samples

Firstly, compared to the dsGFP control, *CsKr-h1* mRNA abundance was reduced by 92.99% on the 2th day post dsRNA injection (Figure S1A). Secondly, overview of the RNA-Seq of *C. septempunctata* samples were collected from both the control and the RNAi groups on day 2 after the injection of double-stranded RNA to form RNA-seq samples, namely dsKr-h1 for the knockdown group; dsGFP for the control group. After quality control screening, about 264 Gb of the total raw reads (about 44 Gb for each sample) were generated. As the data show, the percentage of Q30 bases of all products exceeded 93%, indicating high quality sequences. Through alignment with the reference genome, the mapping rate exceeded 91% and the unigenes obtained exceeded 44,060,812 for each RNA-seq sample (Table 1). All sequence reads were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) (accession SRR30589023-SRR30589028) under Bioproject PRJNA1158213.

Identification of DEGs

To identify potential Kr-h1-regulated regulatory genes, DEGs between the knockdown group and the control group were analyzed. Fragments per kilobase per million reads (FPKM) is a common measure for estimating

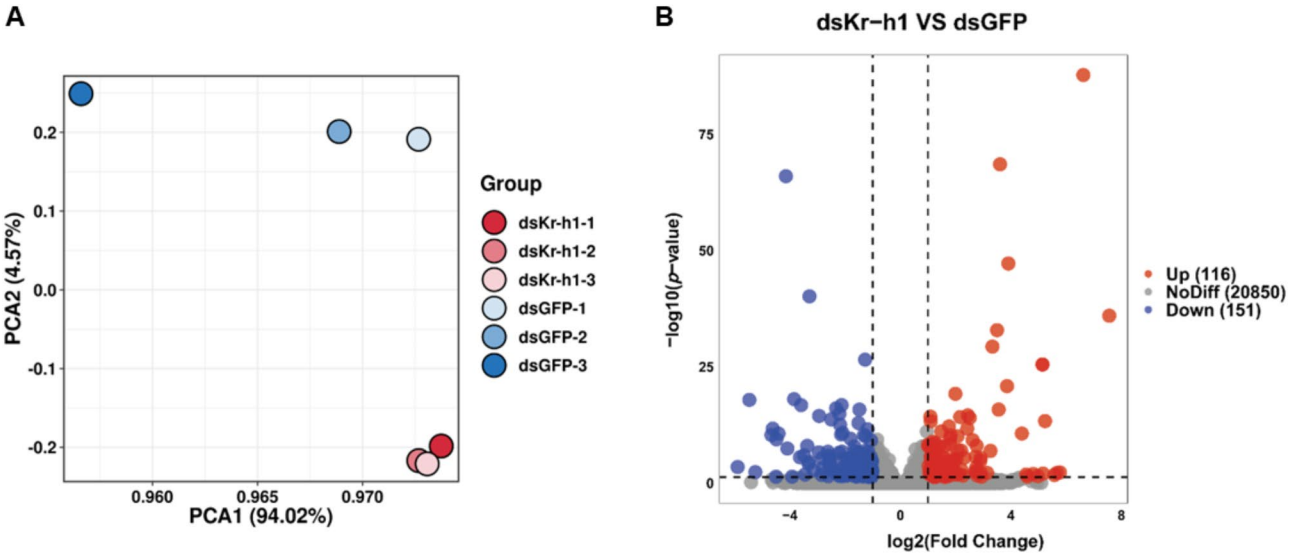

Fig. 2 Identification of DEGs between dsKr-h1 and dsGFP. (**A**) Principal component analysis (PCA) plot of the gene expression profile dsKr-h1 and dsGFP (**B**). The Volcano plot depicting the expression profiles of differentially expressed genes in the transcriptomes of dsKr-h1 and dsGFP. Compared with the controls, the red and blue dots, respectively, indicate upregulated and downregulated genes expressions in dsKr-h1, while the gray dots represented equally expressed genes (log2|(fold change)| < 2 and adjusted *p*-value ≥ 0.05), while the bule and red points represented equally expressed genes (log2|(fold change)| ≥ 2 and adjusted *p*-value < 0.05)

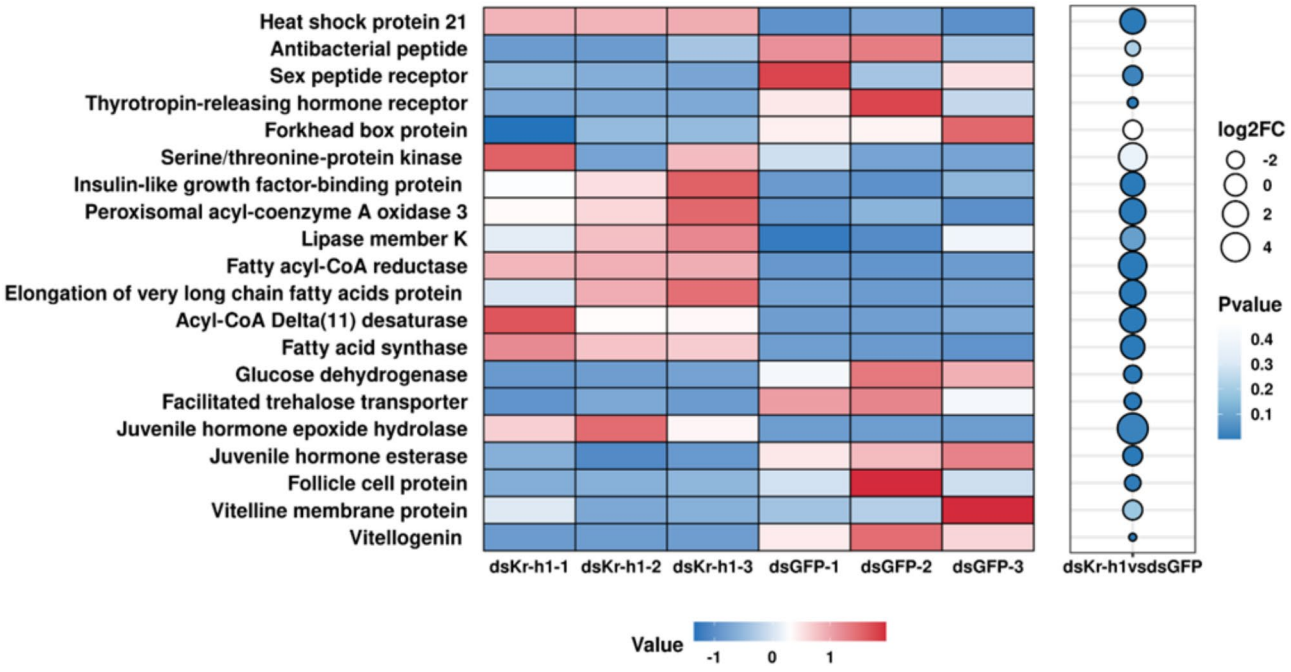

Fig. 3 Hierarchical clustering showed the DEGs and their expression levels from diapause-related between dsKr-h1 and dsGFP

gene expression levels in transcriptome sequencing data analysis. The overall trends in gene expression were similar between the samples (Figure S1B). A distinct separation in the PCA plot revealed large variations in the gene expression profiles between dsKr-h1 vs. dsGFP (Fig. 2A). The DEG analysis results revealed a total of 267 DEGs in the "dsKr-h1 vs. dsGFP" comparison. Of these, 116 genes were upregulated and 151 were downregulated (Fig. 2B). In these DEGs, three reproductive related genes, *follicle cell protein,vitelline memberane protein* and *vitellogenin were* downregulated in dsKr-h1, compared to dsGFP (Fig. 3). Compared to dsGFP, glucose dehydrogenase gene and facilitated trehalsose transporter gene also were downregulated in dsKr-h1(Fig. 3). But five genes in lipid metabolism pathway, lipase member K, fatty acyl-CoA reductase, elongation of very long chain fatty

acids protein, acyl-CoA Delta (11) destaurase and fatty acid synthase were upregulated in dsKr-h1, compared to dsGFP (Fig. 3).

Pathway enrichment analysis of DEGs

To investigate the functional roles of the DEGs between the dsKr-h1 and dsGFP, GO annotation and KEGG pathway enrichment analyses were carried out. Only significantly enriched GO terms and KEGG pathways are presented in this study. The GO analysis of DEGs highlighted their primary associations with cellular components (Fig. 4A), molecular functions (Fig. 4B) and biological processes (Fig. 4C). KEGG pathway analysis revealed significant enrichment in 25 pathways, with notable enrichments in lipid metabolism pathways, including glycerolipid metabolism (ko00561), biosynthesis of unsaturated fatty acids (ko01040), fatty acid elongation (ko00062), and fatty acid degradation (ko00071). In addition, several amino acid metabolism pathways, such as valine, leucine and isoleucine degradation (ko00280), tryptophan metabolism (ko00380), glycine, serine and threonine metabolism (ko00260), and cysteine and methionine metabolism (ko00270), were also significantly enriched in the dsKr-h1 versus dsGFP comparison (Fig. 5).

Validation of gene expression by qRT-PCR

To verify the accuracy of the transcriptome data, 9 DEGs from each comparison that showed significantly different expression levels in the qRT-PCR analysis were randomly selected or validation by qRT-PCR. Positive values indicate upregulation, while negative values represent downregulation. As shown in Fig. 6, the expression patterns of the DEGs identified by qRT-PCR were generally consistent with those obtained from RNA-Seqencing. Although there were minor discrepancies in the relative expression levels, the overall trends confirmed the reliability of the transcriptome sequencing results.

Potential off-targets of dsCsKr-h1

The analysis revealed that dsCsKr-h1 was designed to only specifically target *CsKr-h1* gene (XM_044894324.1: *C. septempunctata Krueppel homolog 1-like* (LOC123310706), transcript variant X1 and

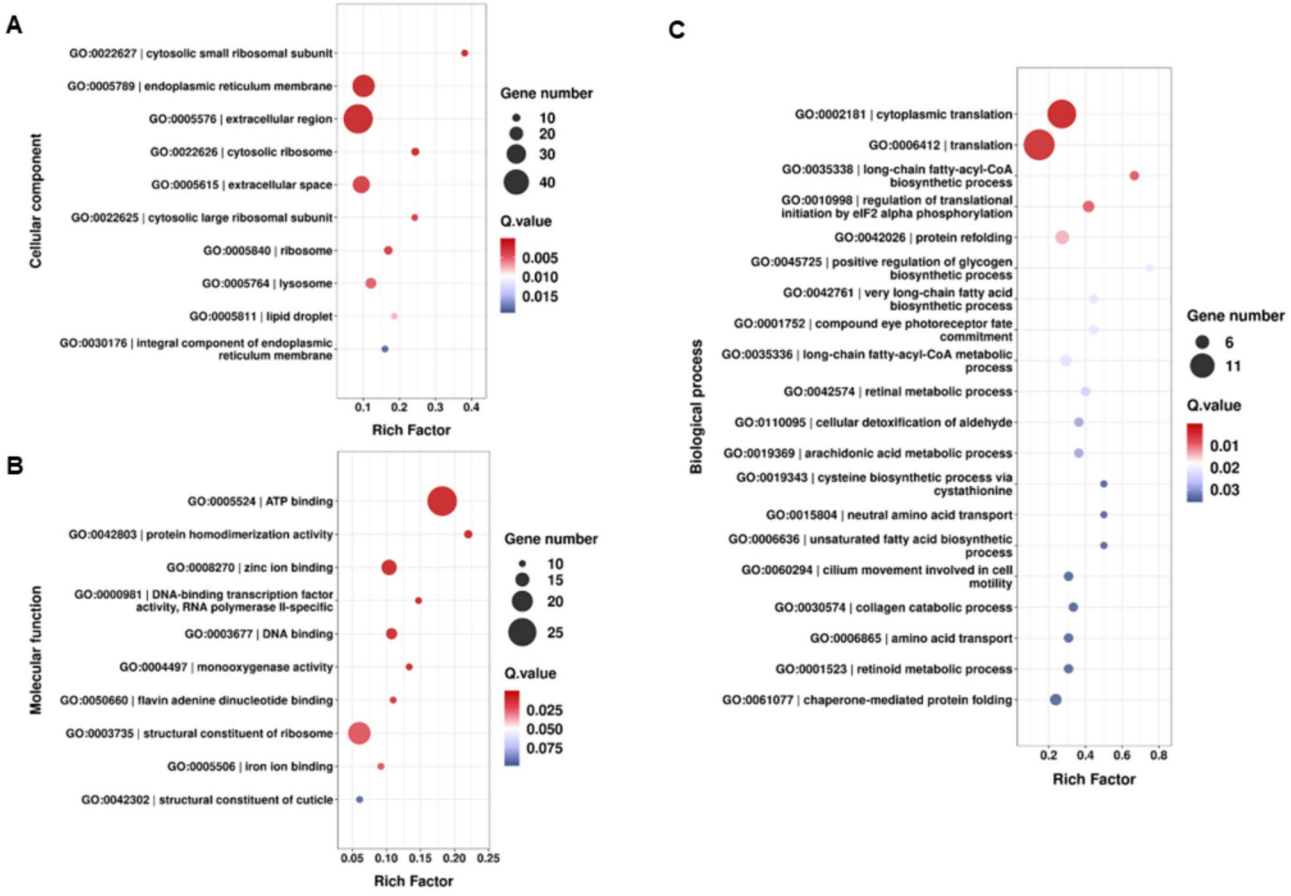

Fig. 4 Gene ontology (GO) classification analysis of DEGs between dsKr-h1 and dsGFP. (**A**) Cellular component, (**B**) Molecular function, (**C**) Biological process. The X-axis showed the rich factor indicating the percentage of DEGs in the pathway. The Y-axis showed the GO categories. The gene numbers of DEGs and Q-value are shown on the right

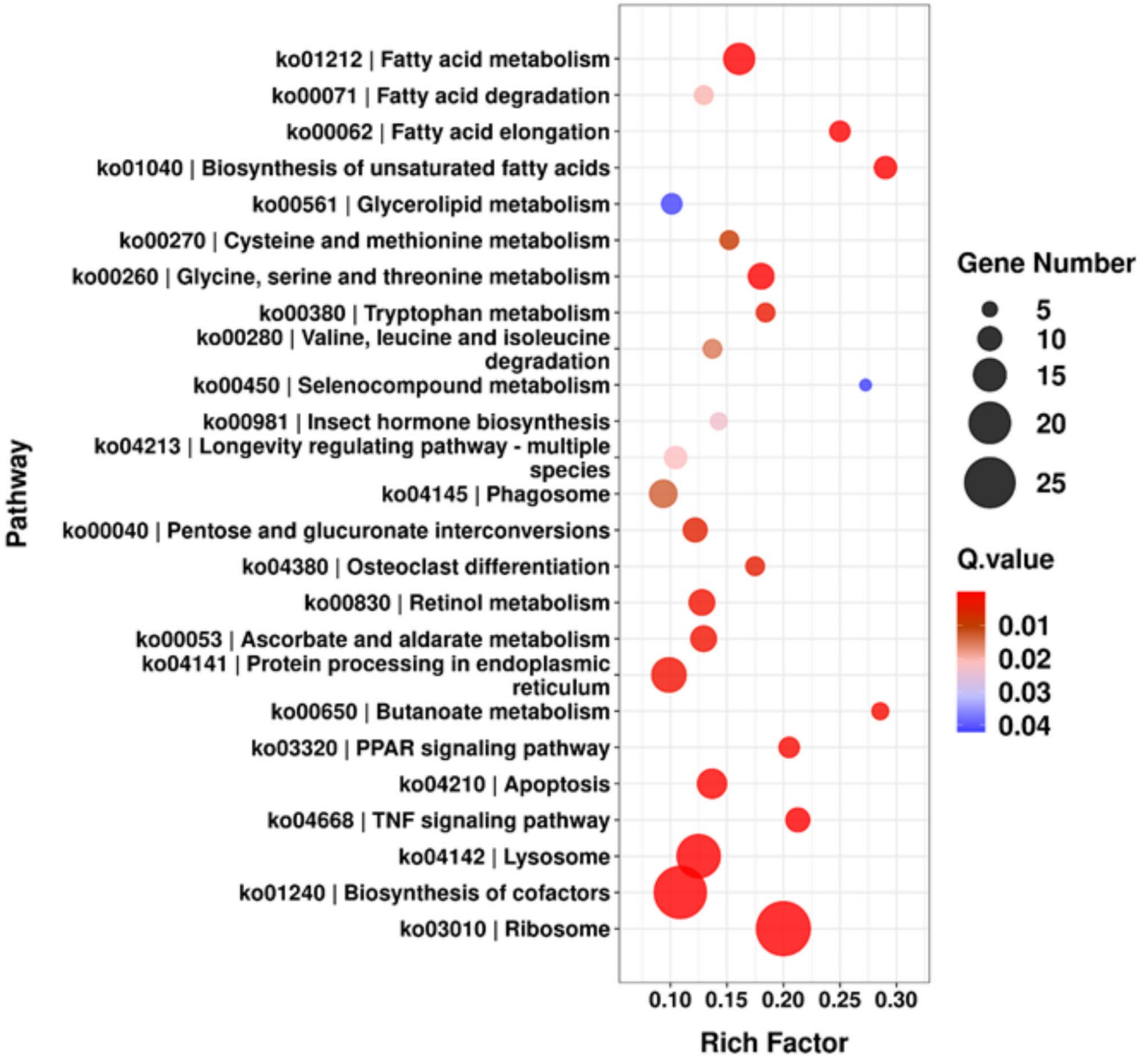

Fig. 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) classification analysis of DEGs between dsKr-h1 and dsGFP. The X-axis showed the rich factor indicating the percentage of DEGs in the pathway. The Y-axis showed the kegg categories. The numbers of DEGs and Q-value are shown on the right

XM_044894325.1: *C. septempunctata Krueppel homolog 1-like* (LOC123310706), transcript variant X2), and no potential off-target effects were detected, theoretically (Figure S1C; Table S2).

Discussion

Reproductive diapause in insects is primarily associated with a decline in JH levels [7]. Several studies have demonstrated that Kr-h1, a key transcription factor downstream of JH, plays a pivotal role in regulating reproductive processes in insects [14, 19, 21, 23]. Guo et al. (2021) identified the DNA replication gene *mini-chromosome maintenance 4* (*Mcm4*) and two triacylglycerol lipase genes as important downstream targets of Kr-h1, which contributes to reproductive plasticity in *C. bowringi* [19]. In our previous research, we showed that the downregulation of the *CsKr-h1* gene, induced by JH deficiency, is a critical factor in the initiation of reproductive diapause in *C. septempunctata* [30, 31]. However, the specific downstream targets regulated by Kr-h1in *C. septempunctata* during diapause remain unclear. In this study, we aimed to identify genes and pathways associated with reproductive diapause that are regulated by Kr-h1 in *C. septempunctata*, using RNAi to knock down *CsKr-h1* expression and conducting transcriptomic analysis. According to RT-qPCR results, compared with

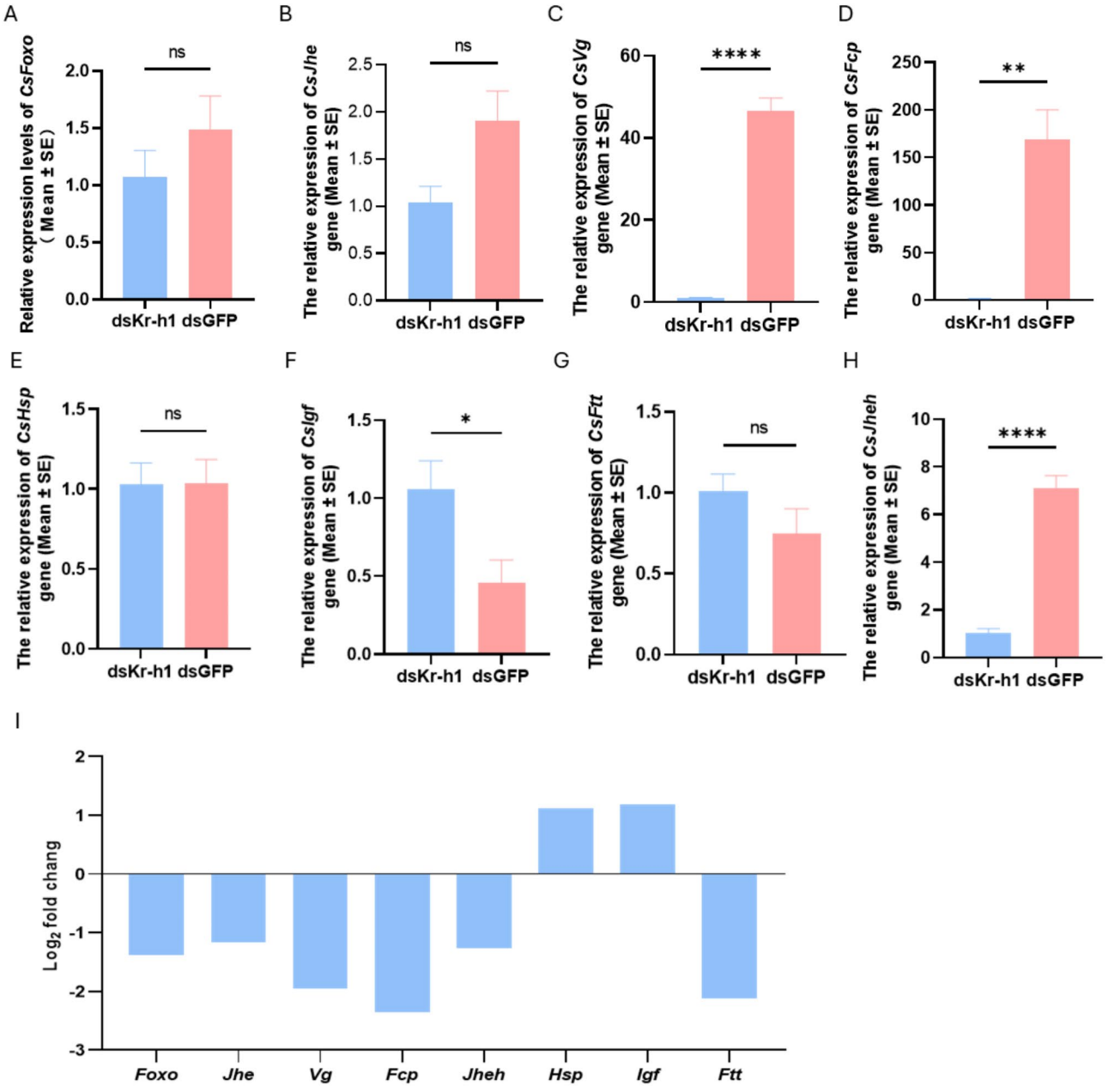

Fig. 6 Validation of gene expression by qRT-PCR. The relative expression of *CsFoxO* (**A**), *CsJhe* (**B**), *CsVg* (**C**), *CsFcp* (**D**), *CsHsp* (**E**), *CsIgf* (**F**), *CsFtt* (**G**), and *CsJheh* (**H**) in the dsKr-h1 and dsGFP group. Relative expression trends of the nine genes from the transcriptome (**I**). *CsActin* expression was used as a reference gene. Error bars represent the standard error (SE). The experiments were conducted with three independent replicates, *, $p < 0.05$, **, $p < 0.01$, and ***/**** $p < 0.001$

non-diapause females, the transcription level of *CsKr-h1* was significantly reduced in diapause females (Fig. 1). On the other hand, in our previous studies, RNA interference with the *CsKr-h1* gene significantly inhibited ovarian development of non-diapause *C. septempunctata* [31]. Therefore, we used non-diapause beetles for RNAi. There is a limitation in our study because a more proper experimental design would be to include diapausing beetles in RNA-seq. But, the high efficiency of RNAi-*Kr-h1* (Figure S1A) and no potential off-target effects of dsCsKr-h1 (Figure S1C; Table S2) make our research results convincing.

Our results showed a significant reduction in *CsKr-h1* expression as early as day 2 post-dsRNA treatment, confirming the effectiveness of the RNAi approach. qRT-PCR analysis of selected differentially expressed genes further validated the reliability of the transcriptomic data. Following *CsKr-h1* knockdown, females *C. septempunctata* exhibited a complete absence of vitellogenesis and oogenesis, which is consistent with our

previous findings [31]. This suggests that *Kr-h1* plays a central role in regulating ovarian development by suppressing oogenesis in *C. septempunctata*. To investigate this further, we focused on *vitellogenin* (*Vg*) and related genes, which are integral to the process of vitellogenesis. As is well-established, vitellogenesis is the process by which vitellus accumulates, involving the massive heterosynthetic synthesis of *vitellogenin* (*Vg*), which is then deposited in the oocyte. The expression levels of *Vg* are considered key indicators of oogenesis efficiency [33]. In fact, multiple studies have reported the core role of Kr-h1 as a transcription factor in the insect reproductive regulatory network by regulating the expression of *vitellogenin* (*Vg*) [19, 23, 37]. In addition to downregulation of the *Vg* gene, we also observed a significant reduction in the expression of two other reproductive-related genes, *vitelline membrane protein* (*Vmp*) and *follicle cell protein* (*Fcp*), in *CsKr-h1*-silenced females. The vitelline membrane, which is essential for egg formation and embryo development, is the first layer secreted by follicular cells and interacts with the oocyte membrane [38–40]. Vmp is a major component of the vitelline membrane and is synthesized and secreted by follicular epithelium cells in the insect ovary [41, 42]. In *Plutella xylostella*, the expression of *PxVmp26* is specifically upregulated in females, with higher expression in ovarian tubes containing incomplete yolk compared to those with complete yolk. In CRISPR/Cas9-induced *PxVmp26* mutants, alterations in egg size, damage to the vitelline membrane structure, and egg collapse were observed [43]. In melon fly, *Zeugodacus cucurbitae* (Coquillett), suppression of the *ZcVmp26Ab* transcription by RNA interference significantly caused the increased desiccation and low hatchability of newly laid eggs [44]. The downregulation of *Vg*,*VmP* and *Fcp* in RNAi-*CsKr-h1* females suggests a significant disruption in ovarian development, further supporting the conclusion that *CsKr-h1* plays a crucial role in regulating key genes involved in vitellogenesis and oogenesis. These findings underline the importance of *CsKr-h1* in the reproductive processes of *C. septempunctata* and provide insight into potential pathways for future research on insect reproduction. However, the mutual regulation relationship between these three reproductive related genes and this gene needs further confirmed by dual-luciferase assay to confirm the relationship between the gene and the downstream gene. Although there is currently no strong evidence to suggest a direct interaction between *Kr-h1* and *Vmp*, the direct regulation of insect *Vmp* by transcription factor E93 has been reported [45]. Remarkablely, studies on the interaction between two transcription factors, Kr-h1 and E93, in regulating insect ovarian development have been reported [46, 47], suggesting that Kr-h1 potentially mediates the expression of *Vmp* gene via E93-dependent regulation.

In this study, we also observed a significant downregulation of the *forkhead box O transcription factor* (*FoxO*) gene in RNAi-mediated *CsKr-h1* knockdown females. This may reflect a co-regulatory relationship between FoxO and Kr-h1. Transcriptional co-regulation, where multiple transcription factors interact at their genomic binding sites, is critical for integrating signals from various pathways to regulate developmental and metabolic networks [48]. In both vivo and in vitro studies in *Drosophila*, Kr-h1 was found to physically and genetically interact with dFoxO to modulate the transcriptional activation of the *insulin receptor* (*InR*) and the adipose lipase *brummer* (*bmm*), which are crucial for maintaining metabolic homeostasis and coordinating organismal growth [49]. This transcriptional co-regulation by Kr-h1 and dFoxO may represent a conserved mechanism across species. In *Geleruca daurica*, the *Kr-h1* gene was significantly upregulated in female adults following topical application of the JH analog methoprene, accompanied by a down-regulation of the *FoxO* gene [18]. Previously, we demonstrated that both FoxO and Kr-h1 play key roles in regulating diapause in *C. septempunctata* by modulating the expression of the *Vg* gene [31]. Specifically, FoxO inhibits *Vg* expression, while Kr-h1 promotes it. Thus, the coordinated regulation of FoxO and Kr-h1 is essential for controlling *Vg* expression during diapause in *C. septempunctata*.

Insect can alter their body compositions in anticipation of diapause to endure seasons with limited food availability. Accumulation of lipid reserves is observed during the preparation for diapause in diapause insects. Ovarian development during diapause in *C. septempunctata* is typically associated with substantial lipid accumulation. In this study, we also identified significant upregulation of six genes related to lipid metabolism, including *fatty acid synthase* (*Fas*), *acyl-CoA delta (11) desaturase* (*FADΔ11*), *elongation of very long chain fatty acids* (*ELO*), *fatty acyl-CoA reductase* (*FAR*), *lipase member K* (*LIPM*), and *peroxisomal acyl-coenzyme A oxidase* (*ACOX*) following silencing of *CsKr-h1*. In addition to these genes, previous studies have identified several key genes involved in lipid biosynthesis during *C. septempunctata* diapause, including *FasFADΔ11,acetyl-CoA carboxylase* (*ACC*), *long-chain fatty acid-CoA ligase* (*ACSL*), *elongase of very-long-chain fatty acids* (*ELO*), and *very-long-chain 3-oxoacyl-CoA reductase* (*KAR*) [50]. In other insects, the knockdown of various genes related to de novo lipogenesis enzymes, including *ACC,Fas1* [51] and *Fas2* [52] and fatty acid transportation genes, including *fatty acid transport protein* (*FATP*) and *fatty acid binding protein* (*FABP*) [51] prevented lipid accumulation during the preparatory phase. These genes are essential for fatty acid synthesis during diapause. Based on these findings, we hypothesize that silencing *CsKr-h1* disrupts ovarian development

while enhancing lipid synthesis and metabolic activity, potentially contributing to increased lipid accumulation during diapause.

Additionally, we observed downregulation of the *glucose dehydrogenase* (*GPDH*) gene in female RNAi-*CsKr-h1 C. septempunctata*. GPDH (or G6PDH), a typical NADP+-dependent oxidoreductase, plays a crucial role in insect metabolism, biosynthesis, and cellular redox balance [53, 54]. Previous research has demonstrated that silencing of *CsKr-h1* induces a diapause-like state in female *C. septempunctata*, characterized by a significant reduction in oxidative metabolism [55]. The observed downregulation of GPDH expression may thus be linked to the reduced oxidative metabolism following RNA interference of *CsKr-h1*. Additionally, GPDH activity is critical during embryonic development, with increased GPDH activity observed during embryonic growth in *Caenorhabditis elegans* [56]. Therefore, the inhibition of ovarian development could also contribute to the decreased *GPDH* expression observed in female adults after RNA interference of the *CsKr-h1* gene.

Insects accumulate substantial nutrient reserves, including sugars and lipids, during the diapause preparation phase [57, 58]. Among these nutrients, trehalose serves as the primary sugar in insect hemolymph. Trehalose not only provides an energy source but also protects proteins and cellular membranes against dehydration, desiccation, heat, cold, and oxidative stress [59]. It is transported from the fat body to the hemolymph and distributed to other tissues through specialized trehalose transporters (TRET) [60]. Trehalose transporter 1 (TRET1) is a high-capacity, facilitated transporter that mediates the uptake of exogenous trehalose into cells. In this study, TRET1 expression was downregulated in diapause females following RNAi-mediated silencing of *CsKr-h1*, suggesting a potential role for TRET1 in the diapause process of *C. septempunctata*. However, further investigation is needed to confirm the precise involvement of TRET1 in diapause regulation. Notably, multiple TRET isoforms have been predicted across various insect species, yet their functionality and regulatory mechanisms remain largely unexplored. For example, two TRET isoforms, TRET1-1 and TRET1-2, have been identified in *Drosophila melanogaster* [61], and two TRET1 isoforms, TRET1a and TRET1b, were characterized in *C. bowringi*, with TRET1a being highly expressed in the fat body of diapause females, while TRET1b was predominantly expressed in the ovaries of non-diapause females [62]. Additionally, numerous TRET isoforms have been identified in *Bombyx mori,P. xylostella*, and *Helicoverpa armigera*, suggesting significant diversity in TRET isoforms among Lepidopteran species [59]. These findings highlight the complexity and diversity of TRET transporters across insect species, suggesting that the regulation of trehalose transport plays an integral role in diapause and other physiological processes. However, much remains to be understood regarding the specific roles of TRETs in diapause and their potential as targets for manipulating diapause in insects.

Conclusions

In this study, we utilized RNA-Seq to investigate the transcriptomic changes in *C. septempunctata* following RNA interference targeting the *Kr-h1* gene. DEGs analysis revealed significant transcriptional alterations between the *Kr-h1* knockdown group (dsKr-h1) and the control group (dsGFP). Noteworthy findings include the downregulation of three genes related to reproduction (*follicle cell protein,vitelline membrane protein*, and *vitellogenin*) in the dsKr-h1 group, while genes involved in lipid metabolism, such as lipase and fatty acid synthase, were upregulated. These results suggested that *Kr-h1* plays a critical role in the regulation of both reproductive processes and lipid metabolism in *C. septempunctata*. Our findings provided valuable insights into the molecular mechanisms regulating reproductive and diapause in *C. septempunctata* and contributed to the expanding understanding of the role of *CsKr-h1* in insect physiology. However, further research is needed to explore the functional roles of the identified DEGs in greater detail, particularly those related to lipid metabolism and reproduction. Moreover, investigating the broader impacts of *Kr-h1* knockdown on insect development and its potential for manipulating diapause would offer valuable practical applications. We can develop an efficient Kr-h1 inhibitor to promote female diapause in the future, given that interference with Kr-h1 inhibits female ovarian development. Further studies should also focus on elucidating the regulatory networks that connect *Kr-h1* to other critical biological pathways in *C. septempunctata*.

Abbreviations

JH	Juvenile hormone
Met	Methoprene-tolerant
Kr-h1	Krüppel-homolog 1
TGLs	Triacylglycerol lipases
Mcm4	Mini-chromosome maintenance 4
Vg	Vitellogenin
RNA-seq	RNA sequencing
dsRNA	Double-stranded RNA
DEGs	Differentially expressed genes
FPKM	fragments per kilobase of exon per million mapped fragments
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
Vmp	Vitelline membrane protein
Fcp	Follicle cell protein
FoxO	Forkhead box O transcription factor
InR	Insulin receptor
Bmm	Brummer
Fas	Fatty acid synthase
FADΔ11	Acyl-CoA delta (11) desaturase
ELO	Elongation of very long chain fatty acids
FAR	Fatty acyl-CoA reductase
LIPM	Lipase member K

ACOX	Peroxisomal acyl-coenzyme A oxidase
ACC	Acetyl-CoA carboxylase
ACSL	Long-chain fatty acid-CoA ligase
ELO	Elongase of very-long-chain fatty acids
KAR	Very-long-chain 3-oxoacyl-CoA reductase
GPDH	Glucose dehydrogenase
TRET	Trehalose transporters
FATP	Fatty acid transport protein
FABP	Fatty acid binding protein

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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J.J C.: Writing–original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. X.X. L.: Writing–original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. S.D. H.: Validation, Investigation. Z.H. L.: Investigation. M.S. Z.: Formal analysis. Y.Y L.: Writing–review & editing. L.S. Z.: Writing–review & editing, Resources, Project administration. All authors reviewed the manuscript.

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Data availability
The datasets generated during the current study are available in the Sequence Read Archive of the National Center for Biotechnology Information repository, (accession SRR30589023-SRR30589028) under Bioproject PRJNA1158213.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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