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The transcriptome reveals the potential mechanism of 20E terminating diapause in cotton bollworm, *Helicoverpa armigera*

Zhaohong Ni[1], Yan Li[1], Shunchao Xia[1], Zhaolang Teng[1], Jianjun Guo[1], Jing Liao[1] and Haiyin Li[1*]

Abstract

Background Diapause is a crucial adaptive strategy employed across numerous insect species, endowing them to survive in unfavorable environments. *Helicoverpa armigera*, one of the most destructive pests globally, undergoes diapause in the pupa stage, which is essential for its survival during the overwintering period and ultimately determines the following year's population density. 20E is a primary hormone that regulates the process of pupae diapause. However, a comprehensive analysis of the mechanisms by which 20E regulates the initiation and termination of diapause in *H. armigera* remains lacking.

Results In the present study, exogenous 20E was initially administered to diapausing pupae, and the results demonstrated that 20E markedly enhanced the development and eclosion rate of diapausing pupae, indicating that 20E treatment effectively terminated the diapause of *H. armigera*. Subsequently, RNA-Seq was employed to construct a comprehensive transcriptome map of the 20E-induced termination of diapause. The results demonstrated that there were 2836 differentially expressed genes, including 1315 genes that were upregulated and 1521 genes that were downregulated, in the 20E injection group relative to the control group. KEGG and GO enrichment analysis showed that these genes were associated with various metabolic pathways. Moreover, additional analysis revealed that the majority of the pivotal genes associated with metabolism (including glycolysis/gluconeogenesis, glycerolipid, amino sugar and nucleotide sugar metabolism), cell signaling pathways (such as insulin, Wnt, MAPK signaling pathways), the cell cycle, and stress resistance exhibited altered expression following 20E injection. These findings suggest that 20E exerts its primary influence on metabolic processes, cell signaling pathways, cell cycle, and stress resistance during the termination of diapause.

Conclusions Our study presents a systematic and comprehensive analysis of the genes associated with 20E-induced diapause termination, thereby providing a foundation for elucidating the molecular mechanism of 20E regulating diapause. Furthermore, the findings lend support to the utilization of ecdysone analogs as pesticides in diapause-based pest management.

Keywords *Helicoverpa armigera*, Diapause, RNA sequencing, 20E, Pest control

Background

Diapause is a crucial adaptive strategy employed by numerous insects species, enabling them to survive in unfavorable environments and contributing to population reproduction [1]. The diapause process of insects is intricately associated with a variety of physiological changes, including arrest of development, reduced

*Correspondence:
Haiyin Li
hyli3@gzu.edu.cn
[1] Guizhou Provincial Key Laboratory for Agricultural Pest Management of the Mountainous Region, Institute of Entomology, Guizhou University, Guiyang 550025, PR China

metabolism, enhanced resistance, and lifespan extension [2]. Many agricultural pest species relay on diapause to synchronize their seasonal development with favorable seasons. For example, the cotton bollworm, *Helicoverpa armigera* (Hübner), a globally prevalent agricultural pest, enters diapause at the pupal stage when larvae sense shorter day lengths before the cold winter. If entering diapause, this period generally lasts for over three months, persisting until suitable environmental conditions arrive in spring. Then, the diapausing pupae resume development, thereby reproducing the next generation and initiating a new cycle of infestation [3, 4]. Diapause is vital for the survival of *H. armigera* during the overwintering period and ultimately determines the next year's population density. Therefore, studies focusing on diapause and exploiting tools to subvert the diapause process will offer potential strategies for pest management.

Hormones are crucial in regulating the growth, development and metamorphosis of insects, and the hormones and hormone analogs have been used as pesticides since the 1980 s [5]. Numerous studies have demonstrated that hormones are key regulators of insect diapause, including juvenile hormone (JH), diapause hormone (DH), and 20-Hydroxyecdysone (20E) [6–9]. Generally, JH is primarily associated with adult diapause (also known as reproductive diapause), and a reduction in JH titer triggers reproductive diapause in female, which has been well documented in many insects such as *Geleruca daurica, Harmonia axyridis*, and *Coccinella Septempunctata* [10–12]. DH has been proven to be a crucial hormone regulating embryonic diapause in the silkworm *Bombyx mori* [13]. 20E is another pivotal hormone that regulates insect diapause. As the active form of ecdysteroids, 20E is synthesized and secreted by the prothoracic glands (PGs) [14]. 20E exerts its function through a heterodimer composed of the ecdysone receptor (EcR) and ultraspiracle protein (USP), triggering downstream signal transduction via a strict cascade reaction [15, 16]. Many studies have demonstrated the role of 20E in pupal diapause. For example, lower levels of 20E have been observed in diapausing pupae of *Antheraea pernyi*, and exogenous 20E application initiates the termination of diapause [17, 18]. Similarly, our previous study in *Pieris rapae* revealed that 20E levels were reduced in diapause pupae, and 20E injection enhanced the expression levels of the target gene *HR3*, thereby breaking pupal diapause [19]. Indeed, pupal diapause in *H. armigera* is also regulated by 20E, with 20E injection effectively terminating the diapause [20]. However, a comprehensive analysis of the mechanisms by which 20E regulates the initiation and termination of diapause remains lacking.

At present, it has been uncovered that certain genes play a role in regulating diapause in *H. armigera*, such as those involved in the TGF-β signaling pathway, the insulin signaling pathway, and metabolic processes [21–23]. Additionally, some studies have shown that 20E can regulate Smad2, Smad4 and TGFβ receptor I (TGFβRI) in the TGF-β signaling pathway, as well as *Hexokinase* (*HK*) in metabolic processes [24, 25]. However, further investigation is required to determine whether additional genes can be regulated by 20E in the diapause regulation. RNA sequencing (RNA-Seq), a technology utilized for large-scale gene expression analysis, has gained widespread application in insect research [26]. In insect diapause, several studies have utilized RNA-Seq to compare gene expression profiles between diapause and nondiapause individuals, identifying the key genes and pathways involved in diapause [19, 27–30]. RNA-Seq will offers a comprehensive analytical tool for solving biological questions in insects.

In this study, we investigated the effect of 20E on pupal diapause of *H. armigera*. Subsequently, a comprehensive transcriptome map of 20E-induced diapause termination was constructed by utilizing RNA-Seq. Additionally, key downstream genes and signaling pathways implicated in 20E-induced diapause termination were identified and analyzed. Collectively, our study presents a systematic and comprehensive analysis of the gene association with 20E-induced diapause termination, which provides a foundation for elucidating the molecular mechanism of 20E regulating diapause. It also offers the support for the utilization of ecdysone analogs as pesticides in diapause-based pest management.

Materials and methods

Insect rearing and sampling

Insects were reared in the laboratory for several generations and the diapause rate approached 98%. The *H. armigera* larvae were reared in an artificial climate incubator with the temperature at 20℃, and the light/dark period is 10 h: 14 h to induce diapause pupae. The pupae samples were cleaned and dissected in pre-cooled 0.75% NaCl, and stored at − 80℃.

20E treatment

20E powder was purchased from Sangon Biotech (Shanghai, China), and dissolved in dimethyl sulfoxide (DMSO) to achieve a final concentration of 0.5 μg/μL. The 30-day-old diapause pupa was injected with 2 μL DMSO or 20E. After injection, the injected pupae were fed in long-day conditions (14 h light: 10 h dark) at a temperature of

26℃. The disappearance of diapause pupae's stemmata (indicating the termination of diapause) was assessed on the 4 th day, and the eclosion rate was calculated on the 10 th day.

RNA isolation, and RNA sequencing

The pupae (a total of 30 individuals) were collected at 24 h after injection, and three biological replications were prepared for each treatment. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and purity of the extracted RNA were checked using NanoDrop 2000 (Thermo, USA), and RNA integrity was checked by agarose gel electrophoresis. The and RIN values were determined using 2100 Bioanalyzer (Agilent Technologies, USA). Sequencing libraries were constructed according to standard procedures using the Illumina TruseqTM RNA Sample Preparation Kit and the cDNA libraries were sequenced and analyzed on the Illumina Novaseq 6000 platform at Majorbio. The raw sequencing data from this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number PRJNA1164935.

Differentially Expressed Genes (DEGs) analysis

These clean reads were filtered from the original reads were used to locate the *H. armigera* reference genome (GCF_023701775.1). Each sample mapping to read by StringTie (https://ccb.jhu.edu/software/stringtie/) for assembly. The DEGs analysis between groups was performed using the software DESeq2 (Version 1.24.0) and the expression quantification software RSEM (Version 1.3.3) was used to quantify the expression levels of genes in terms of transcripts per million reads (TPM). The heatmaps of DEGs were calculated at log(TPM + 1) and imaged by Chiplot (https://www.chiplot.online/). Based on the expression quantification results, differential gene analysis between groups was performed using the software DESeq2 (Version 1.24.0), with screening thresholds of |log2 FC| > = 2 and Padjust < 0.05.

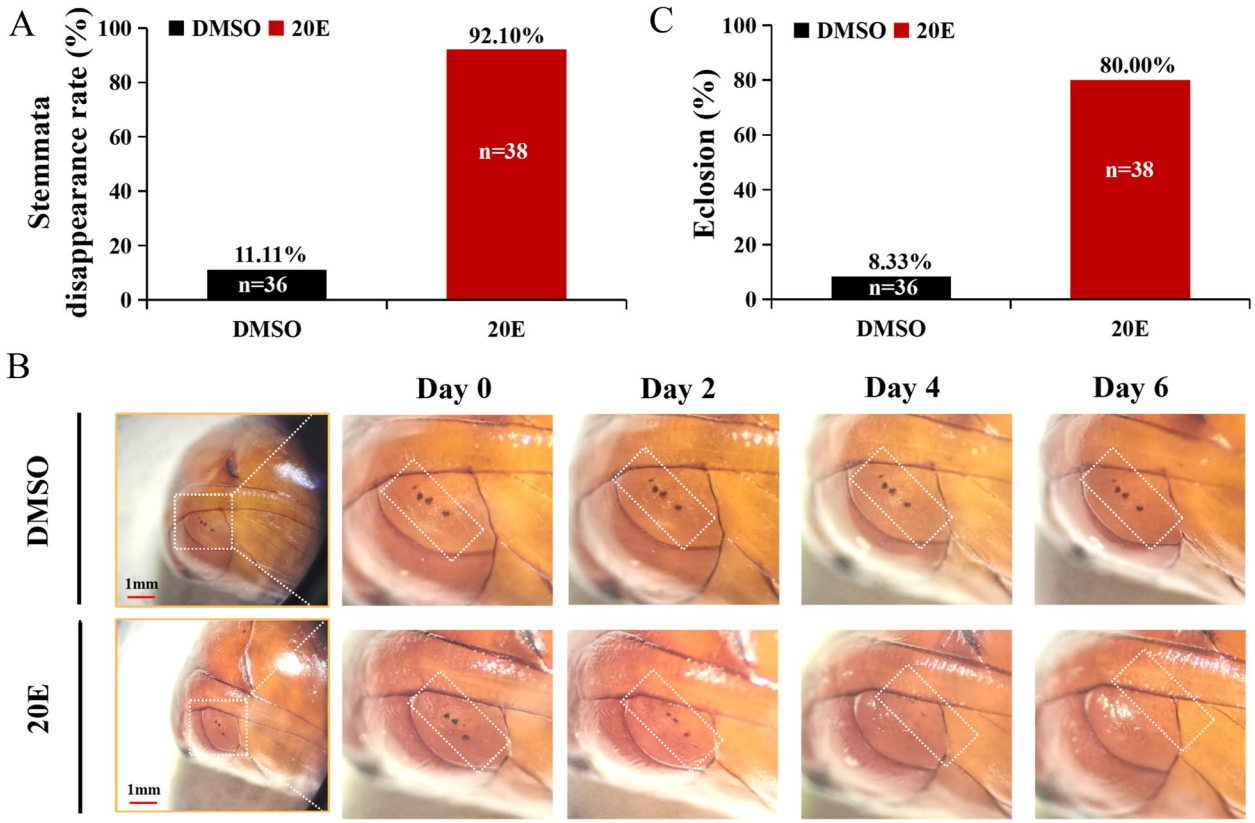

Fig. 1 20E injection breaks the pupal diapause. **A** The rate of stemmata disappearance in diapause pupae after 20E injection. **B** Photographs of diapause pupae stemmata changes after 20E injection. **C** The eclosion rate of diapause pupae after 20E injection. The 30-day-old diapause pupae were injected with DMSO or 1 μg 20E, and the disappearance of diapause pupae's stemmata was assessed on the 4 th day, and the eclosion rate was calculated on the 10 th day after injection. DMSO, *n* = 36; 20E, *n* = 38

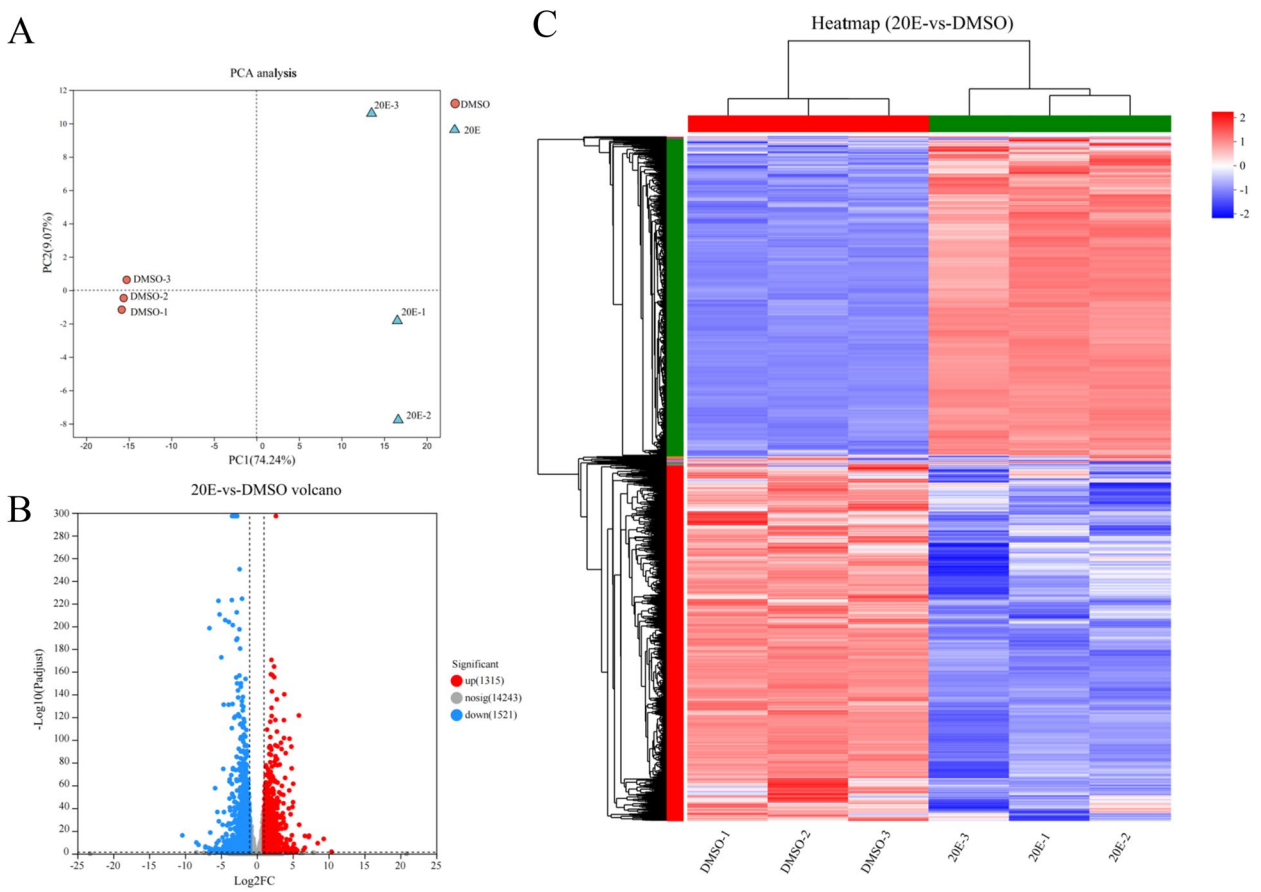

Fig. 2 RNA-Seq analysis and identification of differentially expressed genes (DEGs). **A** Principal component analysis (PCA) of samples from different treatments of diapause pupae. **B** Volcano plots showing the DEGs after 20E injection. **C** Heatmap of DEGs post- 20E treatment. Each column represents a replicate, and color bar indicates expression level from high (red) to low (blue)

GO and KEGG functional enrichment for DEGs

To assess the biological function of the differentially expressed genes, enrichment analysis of DEGs was performed using the ClusterProfile package. The Goatools software (Version 0.6.5) and KOBAS (Version 2.1.1) were utilized for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, respectively. The DEGs were categorized into the molecular function (MF), cellular component (CC), and biological process (BP) categories. Adjusted p-values <0.05 and Q-values <0.05 were considered significantly enriched.

Quantitative Real-Time PCR (qRT-PCR)

The qRT-PCR was performed using a Bio-Rad CFX96 PCR system (Bio-Rad, USA). The reference gene *Ribosomal protein L32* (*RPL32*) was employed to calibrate the sample-to-sample variation and normalize the target gene expression [31]. PCR reaction was performed in a 10 μL reaction mixture containing 5 μL SYBR 2× Taq II (Genstar, China), 0.4 μL of each primer, 0.5 μL cDNA template, and 4.1 μL ddH_2O. The amplification procedure was as follows: 95℃ for 2 min, followed by 40 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 10 s. The $2^{-\Delta CT}$ method was used to calculate the genes'relative expression and the two-tailed student T-test was used to statistics analysis. The primers for qRT-PCR were designed by Primer Premier 5 and the primer sequences are shown in Table S1.

Results

20E injection breaks the pupal diapause

When the larvae of *H. armigera* receive a short day lengths signal, the pupae will enter diapause within 8–10 days, and the diapausing individual maintain this state for over three months [32]. To investigate the effect of 20E on diapause, 1 μg of 20E was injected into 30-day-old diapause pupae. The results showed that 92.10% of the diapause pupae's stemmata had disappeared (indicating the termination of diapause) on the 4th day in the 20E injection group. In contrast, the DMSO injection group exhibited an 11.11% disappearance rate (Fig. 1A, B). In addition, the eclosion rate of diapause pupae injected

with 20E was 80.00%, significantly higher than that of DMSO (8.33%) (Fig. 1C).

RNA-Seq analysis and identification of DEGs

To comprehensively explore the genes respond to 20E that terminates pupal diapause, RNA-Seq was performed at 24 h after 20E injection on *H. armigera* pupae. The percentage of bases with Phred scores at the Q30 level ranged from 88.71% to 89.16%, and 88.20% to 95.57% of clean reads were mapped to the reference genome (Table S2). High repeatability was found in the samples of the same treatment, but there was not in the samples with different treatment by principal component analysis (PCA) (Fig. 2A). Based on the screening thresholds of |log2 FC|> =2 and Padjust <0.05, identifying a total of 2836 differentially expressed genes (DEGs) between the DMSO injection group and the 20E injection group. Specifically, compared to the DMSO group, 1315 genes were upregulated and 1521 genes were downregulated following 20E injection (Fig. 2B). Figure 2C visualizes the differences in gene expression between the treatments, based on the TPM values of 2836 genes.

Analysis and identification of DEGs in GO and KEGG

To further gain insights into possible roles of these DEGs, a KEGG enrichment analysis was performed, and highlighting the top 20 enriched terms. The results indicated that the upregulated genes were significantly enriched

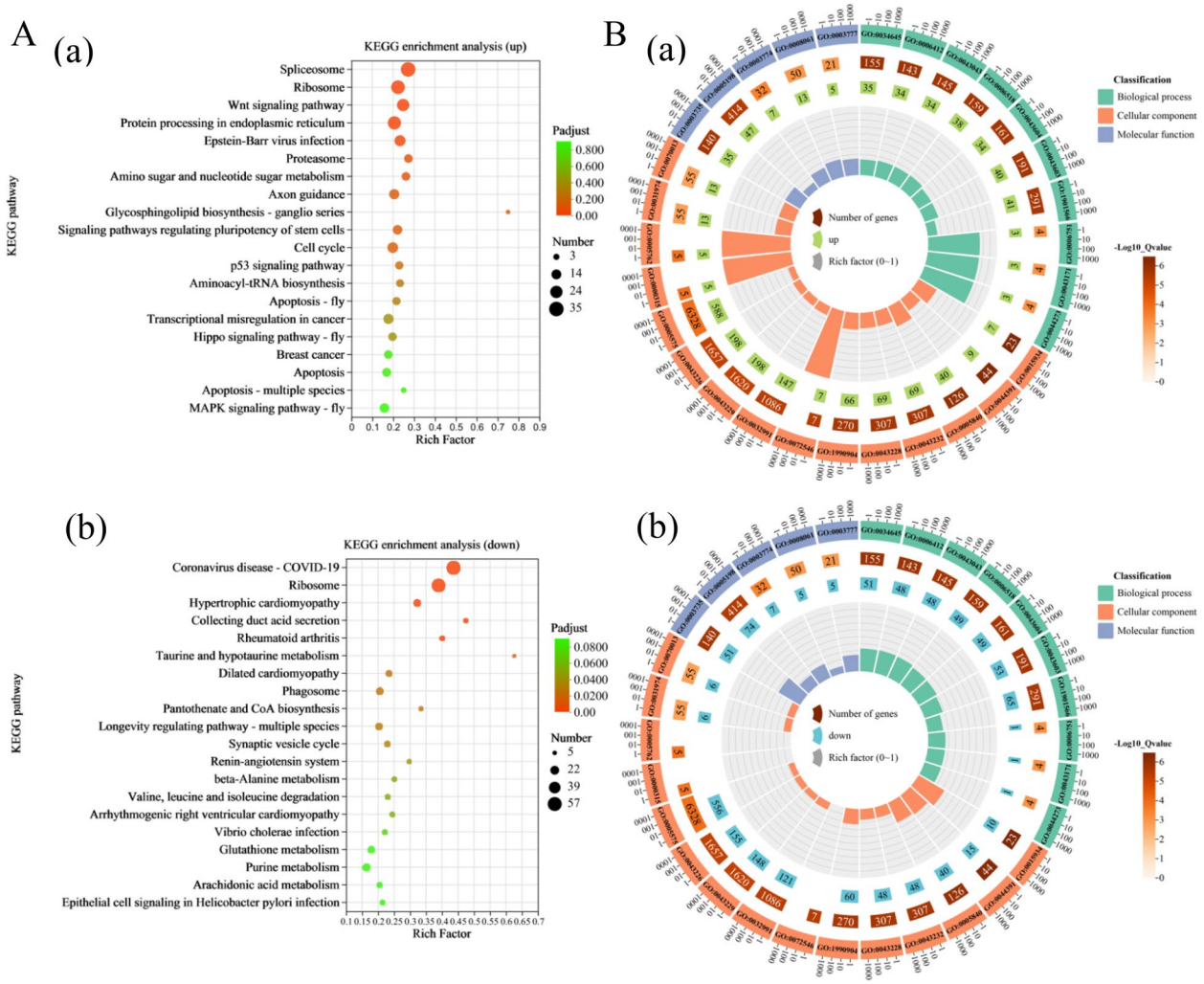

Fig. 3 GO and KEGG analysis of DEGs. **A** KEGG enrichment analysis of upregulated (a) and downregulated (b) genes after injected 20E. **B** The top 30 significant GO terms analysis of the upregulated (a) and downregulated (b) DEGs. Three colors represent three categories: Biological processes (BP), Cellular components (CC), and Molecular functions (MF). Outside the circle is a sitting scale for the number of genes. Different colors represent different classifications. The second circle is the total number of DEGs. The third circle is the number of upregulated or downregulated genes, green genes are upregulated, blue genes are downregulated. The fourth circle is the rich factor value for each classification (the number of foreground genes in that classification divided by the number of background genes), each cell of the background auxiliary line represents 0.1

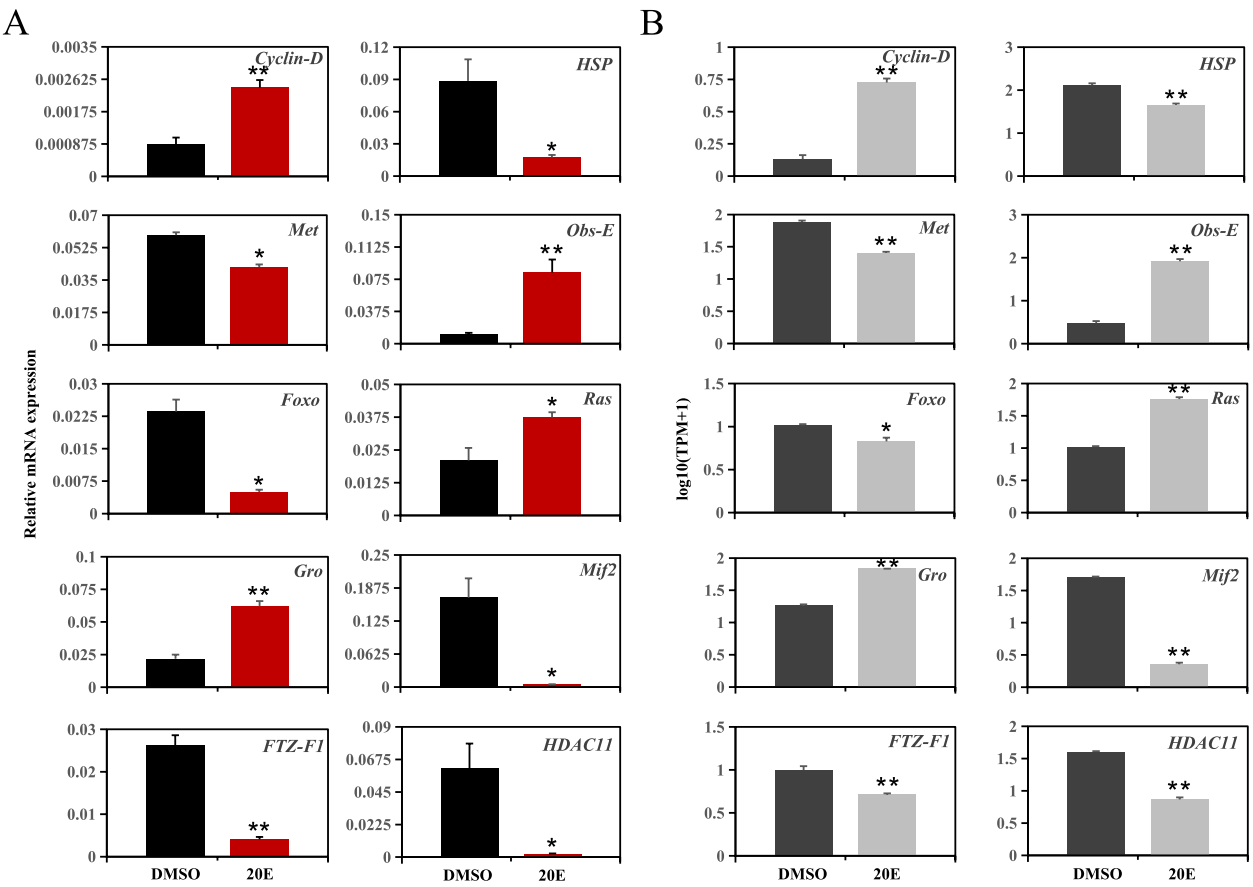

Fig. 4 qRT-PCR validation of RNA sequencing data. **A** The genes relative expression levels determined by qRT-PCR. **B** The normalized expression level (TPM) of genes in RNA-Seq. Each point represents the mean ± SEM of three independent replicates. *, $P < 0.05$; **, $P < 0.01$ (determined by the two-tailed student T-test)

in Wnt signaling pathway, MAPK signaling pathway and cell cycle, while the downregulated genes were predominantly enriched in the metabolic pathway, longevity regulating pathway, taurine and hypotaurine metabolism and arachidonic acid metabolism (Fig. 3A). In order to further identify the key terms that DEGs enriched, a Gene Ontology (GO) enrichment analysis was conducted and the DEGs were significant enrichment in BP (Biological process), CC (Cellular component) and MF (Molecular function). The top 30 significant GO terms were analysed in Fig. 3B. Thereinto, the upregulated genes were mainly enriched in amino acid process (GO:0005762), protein process (GO:0000315) and ER membrane protein complex (GO:0072546) (Fig. 3B(a)). Meanwhile, the downregulated genes were mainly enriched in structural molecule activity (GO:0005198), structural constituent of ribosome (GO:0003735) and organonitrogen compound biosynthetic process (GO:1,901,566) (Fig. 3B(b)). Ten genes were chosen for qRT-PCR validation, and the expression levels of these chosen genes were consistent with the trends observed in the RNA sequencing data (Fig. 4).

Analyze the DEGs within metabolic pathways

Diapause is characterized by a notably low metabolic rate. Based on RNA-Seq data, it was observed that the expression of several metabolism-related genes were altered following 20E injection. Specifically, within the glycolysis/gluconeogenesis metabolic pathway, 8 genes (including *GPI*, *ALDO*, *GAPDH*, *MINPP1*, *PCK*, *ALDH*, *ALDH1 A1*, and *ALDH7 A1*) were significantly downregulated, while 2 genes (*aceE* and *LDH*) were upregulated post- 20E injection (Fig. 5A). Besides, in the glycerolipid metabolism pathway, 10 genes (including *ALDH*, *ALDH1 A1*, *ALDH7 A1*, *AGPAT1,2*, *AGPAT8*, *DGK*, *CEL*, *PL*, *PLRP1*, and *PLRP2*) were downregulated and 2 genes (including *PLPP* and *MOGAT*) were upregulated in the 20E injection group (Fig. 5B). Additionally, in the amino sugar and

nucleotide sugar metabolism pathway, 9 genes demonstrated differential expression, with 7 genes (including *E3.2.1.14*, *HEXs*, *SAS*, *GALK2*, *GALE*, *GFPT,* and *UGDH*) being upregulated and 2 genes (*CHS1* and *GALT*) downregulated (Fig. 5C).

DEGs within key signaling pathway associated with diapause

In comparsion, diapause individuals tend to exhibit increased longevity relative to their nondiapause individuals. Several studies have demonstrated that longevity-related pathways such as insulin and TOR signaling are integral to the regulation of diapause. In the present study, the administration of 20E was observed to induce alterations in the expression of genes associated with longevity pathways. For example, within the classical longevity pathway insulin signaling pathway, the *PI3K*, *GRB2*, *Ras*, *S6K*, *PDK1,2* and *Foxa2* genes were significantly upregulated, whereas the *Foxo*, *IRS*, *INS*, *AMPK*, *PEPCK*, *eIF4E*, *PP1* and *PHK* genes were significantly dowonregulated (Fig. 6A). Furthermore, our analysis revealed significant alterations in genes associated with other critical signaling pathways following 20E injection, including those within the Wnt pathway (Fig. 6B) and the MAPK pathway (Fig. 6C).

Analyze the DEGs associated with cell cycle progression

Cell cycle progression is fundamental for the growth and proliferation of new tissues. Diapause, or developmental arrest, is accompanied by certain arrest of some cell cycles. In this study, we analyzed the changes of genes that regulating cell cycle in diapause individuals after 20E injection. The finding revealed that most of the genes related to cell cycle progression were significantly upregulated, including the cyclin family genes (*Cyclin-A*, *Cyclin-B*, *Cyclin-D*, *Cyclin-E*), the protein kinases *CDK4,6*, as well as other relevant genes such as *Cdc25*, *PLK1*, *PCNA*, *HDAC1*, *Skp2*, *Msp1*, *Cdc20*, *APC1*, *ORC1*, *and MCM3.* Conversely, *E2F4.5* and *ORC4*, which links to the cell cycle, were significantly downregulated in the 20E treatment group (Fig. 7).

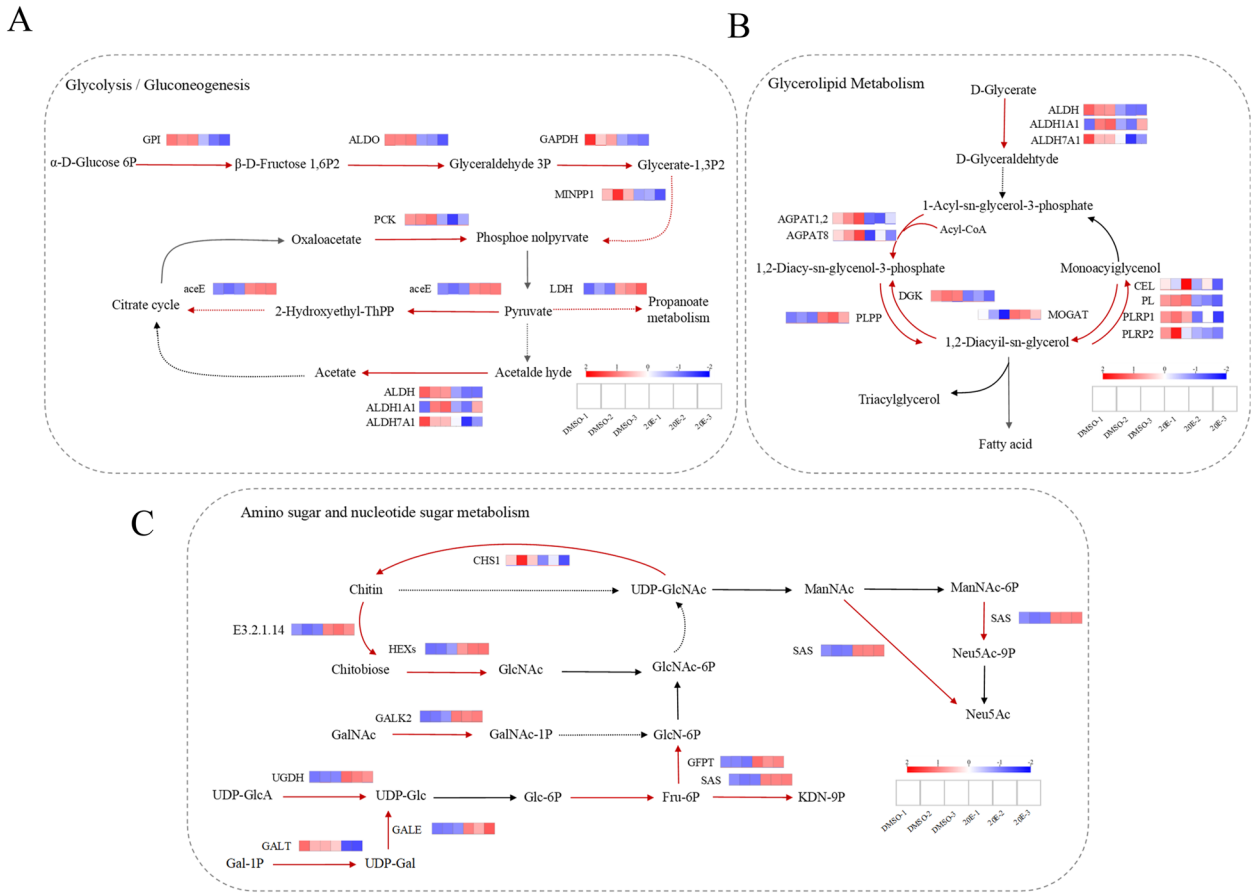

Fig. 5 Analyze the DEGs within metabolic pathways. Panoramagram of DEGs involved in (**A**) Glycolysis/Gluconeogenesis, (**B**) Glycerolipid Metabolism, (**C**) and Amino sugar and nucleotide sugar metabolism. Color bar indicates expression level from high (red) to low (blue)

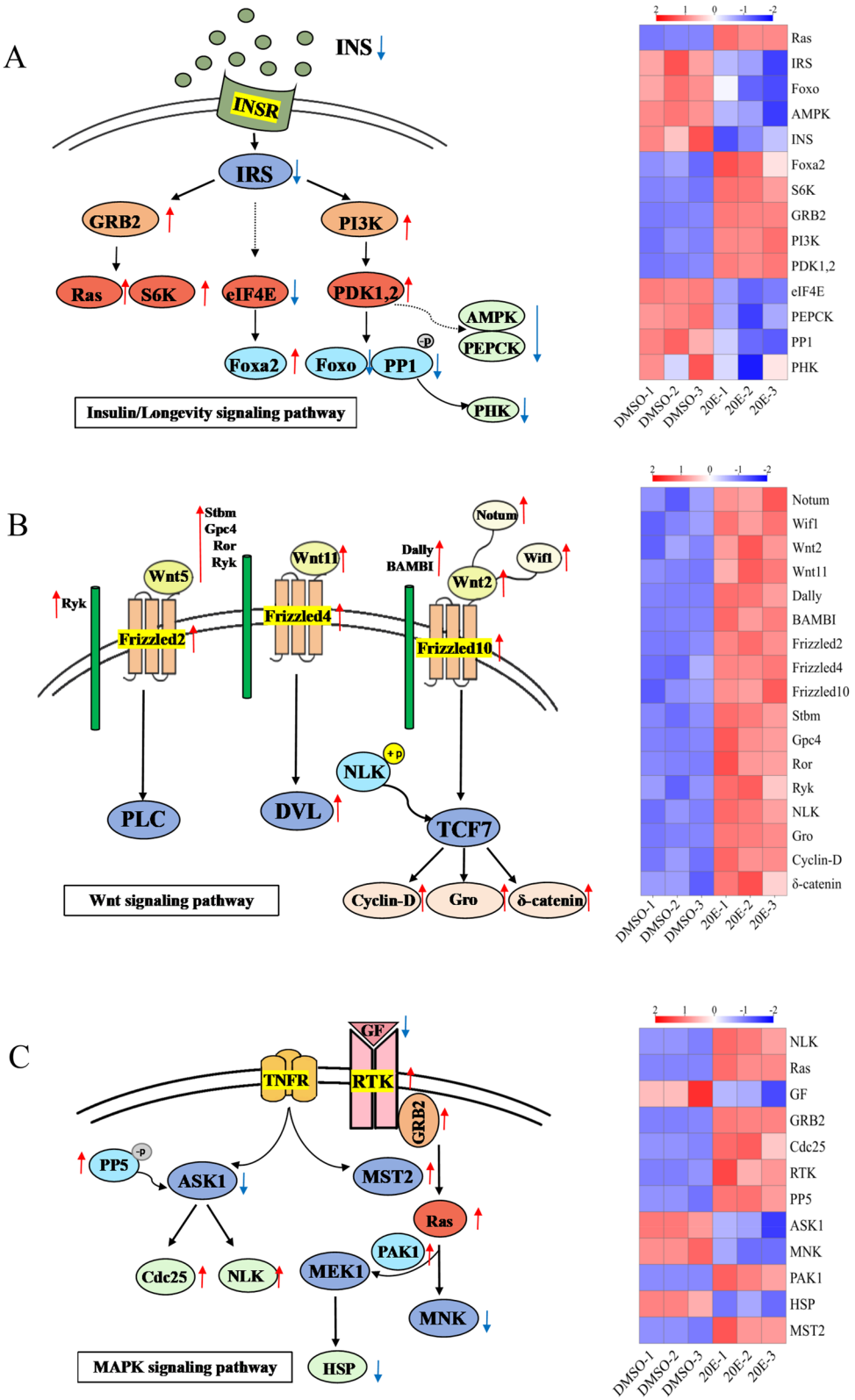

Fig. 6 DEGs within key signaling pathways associated with diapause. **A** Genes involved in insulin/longevity signaling pathway. **B** Genes involved in Wnt signaling pathway. **C** Genes involved in MAPK signaling pathway

Analyze the DEGs associated with stress resistance

The high stress resistance observed in diapause insects is a key factor for their successful survival during diapause, and the process of terminating diapause is frequently accompanied by changes in stress resistance. In our study, we identified the top 50 genes based on log2 FC value, of which 25 genes were closely related to stress resistance. Specifically, there were 9 genes (e.g., *ECHs*, *CP1*, *Obs*-E, *PCP52*, *PCPC1*, *Prisilkin-39*, *Hornerin*, *xP2,* and *EGF-like*) involved in the synthesis and decomposition of cuticle, 7 genes (e.g., *TEFS2-like*, *ACN2*, *CBP*, *lysozyme*, *SCL22*, *NOTCH1,* and *Mif2*) related to immune and antioxidant, 6 genes (e.g., *FabG*, *MCT9*, *MCT14*, *UGT2*, *UGT5,* and *P450*) involved in detoxifying enzymes, and 3 genes (e.g., *Tret1*, *PNLIP* and *RgN*) linked to cold resistance (Fig. 8).

Discussion

Diapause is a phenomenon observed in numerous agricultural pests, enabling their survival under harsh environmental conditions and promoting synchronized population development, which ultimately enhances reproductive success. Consequently, an investigation into methods of disrupting the diapause process, thereby reducing the pests'resistance to adverse environments, could facilitate the attainment of effective pest control. It has been demonstrated that ecdysone is the primary hormone regulating pupal diapause, and ecdysone treatment effectively terminates pupal diapause [33, 34]. Moreover, the findings of present studies have demonstrated that ecdysone is an effective agent for disrupting pupal diapause in the *H. armigera*. In light of the aforementioned findings, utilizing ecdysone analog pesticides to interfere with the diapause process presents a significant application potential for the pest management in agricultural production. Furthermore, this study performed a comprehensive analysis of downstream response genes subsequent to ecdysone-induced diapause termination, identifying several key genes and pathways. The resulting data will provide support for the utilization of ecdysone-based pesticides.

The decline in metabolism is a defining feature of insects during diapause [35, 36]. Previous studies have indicated that when insects enter diapause, there are alterations in glycolysis and gluconeogenesis, accompanied by a reduction in aerobic metabolism, which collectively facilitate metabolic depression [37, 38]. Furthermore, it has been demonstrated that the activity of the citric acid (TCA) cycle in diapause pupae of *H. armigera* is diminished due to a deficiency of 20E [39]. In this study, we observed that the expression of aceE, a key enzyme of the pyruvate dehydrogenase complex that enhances TCA cycle activity, was upregulated following 20E injection. These findings suggest that 20E may

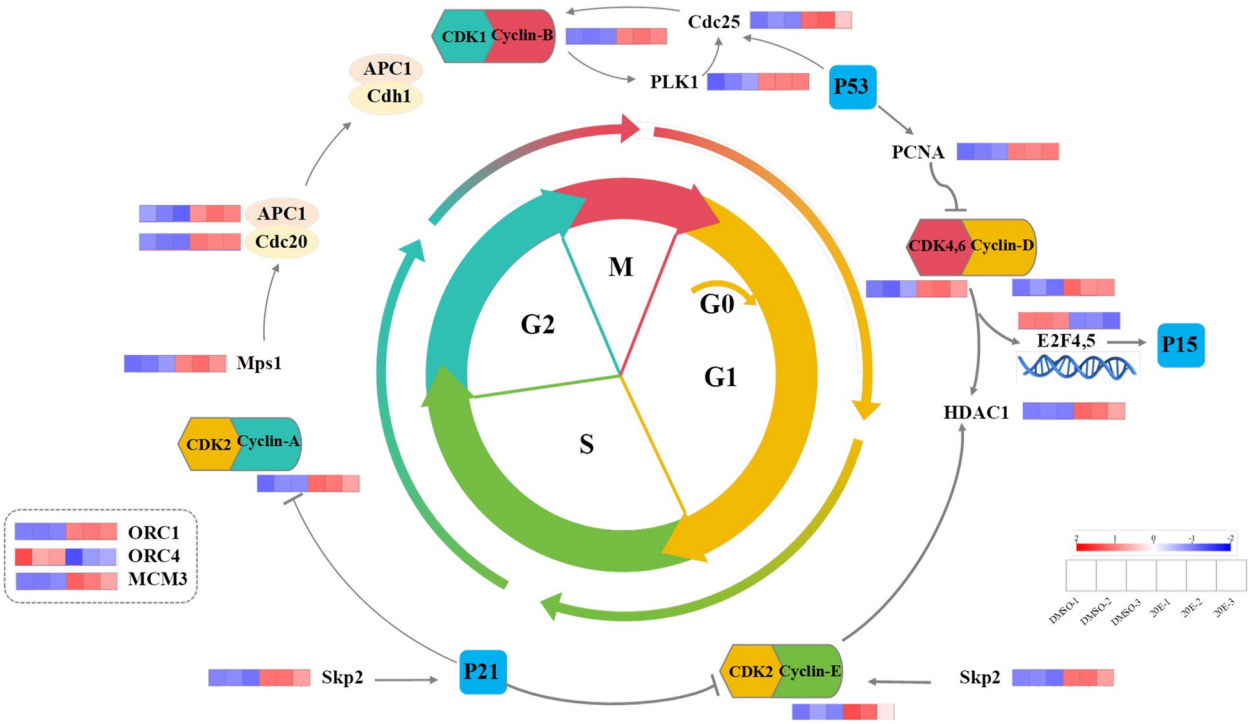

Fig. 7 Analyze the DEGs associated with cell cycle progression. Different colors represent different phases. M, mitotic phase; G0, quiescent phase; G1, DNA pre-synthetic phase; S, DNA synthesis phase; G2, DNA post-synthetic phase

regulate the expression of the aceE gene to enhance TCA cycle activity, thereby regulating diapause. It is noteworthy that our findings also revealed that certain genes, including *GPI*, *ALDO*, *GAPDH*, and *MINPP1*, involved in the glycolytic/gluconeogenesis pathway, exhibited a reduction in expression following 20E injection. These genes encode enzymes that are capable of catalyzing bidirectional reactions, thereby participating in both glycolysis and gluconeogenesis [40–43]. It is therefore necessary to investigate further the mechanisms by which these genes, which are downregulated by 20E treatment, maintain their functional balance in glycolysis and gluconeogenesis. Similarly, lipids serve as crucial energy storage substances for diapausing insects [44, 45], and our results showed that the expression of several genes regulating glycerolipid metabolism was altered following 20E injection. These findings imply that 20E influences diapause not only by regulating glycolysis and gluconeogenesis but also by modulating lipid metabolism. In this study, a notable decrease in the chitin synthetase gene *CHS1* and an increase in the hydrolytic enzyme chitinase gene (*E3.2.1.14*) were observed following 20E injection. Similarly, Wang reported that the injection of exogenous 20E regulates chitin metabolism in *Bactrocera minax* [46]. Furthermore, a previous study demonstrated that *CHSs* genes are upregulated to harden the cuticle of diapausing in insects [47]. These results collectively support the hypothesis that a decrease in chitin may be a sign for the transition from diapause to development in insects.

The lifespan of insects in a state of diapause was notably extended. Numerous studies have elucidated that longevity regulatory pathways, such as the insulin, Wnt, and MAPK signaling pathways, are integral to the regulation of diapause [48–50]. The present study observed that the administration of 20E resulted in substantial alterations in the expression of several genes, including *Foxo*, *S6 K*, and *AMPK*, within longevity signaling pathways. These observations suggest that 20E, as a principal regulator, regulates the initiation or termination of diapause by modulating most longevity signaling pathways. Additionally, several studies have indicated that insulin signaling stimulates ecdysteroidogenesis [51–53]. These findings imply that 20E, in conjunction with multiple signaling pathways, may participate in the regulation of diapause through a network interaction pattern.

During diapause, the change of cell cycle is an important factor that can not be ignored. The cell cycle comprises G1, S, G2, M, and G0 phase, with different insects species exhibiting phase-specific inhibition during diapause. For example, in the *Bombyx mori*, embryonic diapause results in cell cycle arrest at the G2 phase, whereas in *Sarcophaga crassipalpis*, brain cells in diapausing pupae are arrested in the G0/G1 phase [54, 55]. Nevertheless, the precise stage at which the cell cycle is arrested during diapause in *H. armigera* remains uncertain. Our study revealed that the majority of genes associated with the cell cycle, particularly those involved in the G1 and G2 phases, exhibited a significant increase in expression upon the termination of diapause. For example, the *PCNA* gene, which is involved in the G1/S phase transition, has been shown to be highly expressed after the termination of diapause in *S. crassipalpis* [56]. Our findings showed that *PCNA* was significantly upregulated after 20E injection, which is consistent with the previous study in *S. crassipalpis*. Additionally, our comprehensive transcriptomic analysis revealed that a multitude of cell cycle regulators, including *Cyclin-A*, *Cyclin-B*, *Cyclin-D*, *Cyclin-E*, and *CDK4,6*, exhibited elevated expression levels following the termination of diapause by 20E. These results suggest that the cell cycle is implicated in the termination of *H. armigera* diapause in response to 20E. Moreover, it can be postulated that the cell cycle may be arrested in the G1/G2 phase during the diapause period, a pattern that has been observed in *S. crassipalpis*.

The elevated resistance is of paramount importance for the survival of insects that undergo diapause in unfavorable environments [57]. In our study, most of the top 50 log2 FC genes were found to be associated with stress resistance. Specifically, *ACN2*, *CBP*, and *lysozyme* were identified as regulators of the immune and antioxidant response [58–60]. It can thus be inferred that the significant upregulation of these genes following the injection of 20E may serve to mitigate bacterial infections or oxidative damage in diapause pupae due to prolonged diapause. Previous studies have shown that the synthesis and metabolism of the insect cuticle are one of the main factors affecting stress resistance [61]. In our data, the key genes involved in cuticle synthesis, including *Obs-E*, *PCP52*, and *prisilkin- 39*, were found to be significantly upregulated. These results serve to further substantiate the important role of the cuticle in insect stress resistance. In addition, our findings indicate that the cold resistance genes *Tret1*, *PNLIP*, and *RgN* were significantly downregulated following the termination of diapause induced by 20E. It is postulated that the reason for this may be as follows: to adapt to low temperatures, diapausing *H. armigera* must accumulate substantial quantities of antifreeze substances, and the termination of diapause occurs only when temperatures warm up. Consequently, when the diapause terminates under suitable thermal conditions, insects no longer require antifreeze properties and can instead utilise some of these antifreeze substances as energy sources necessary for development.

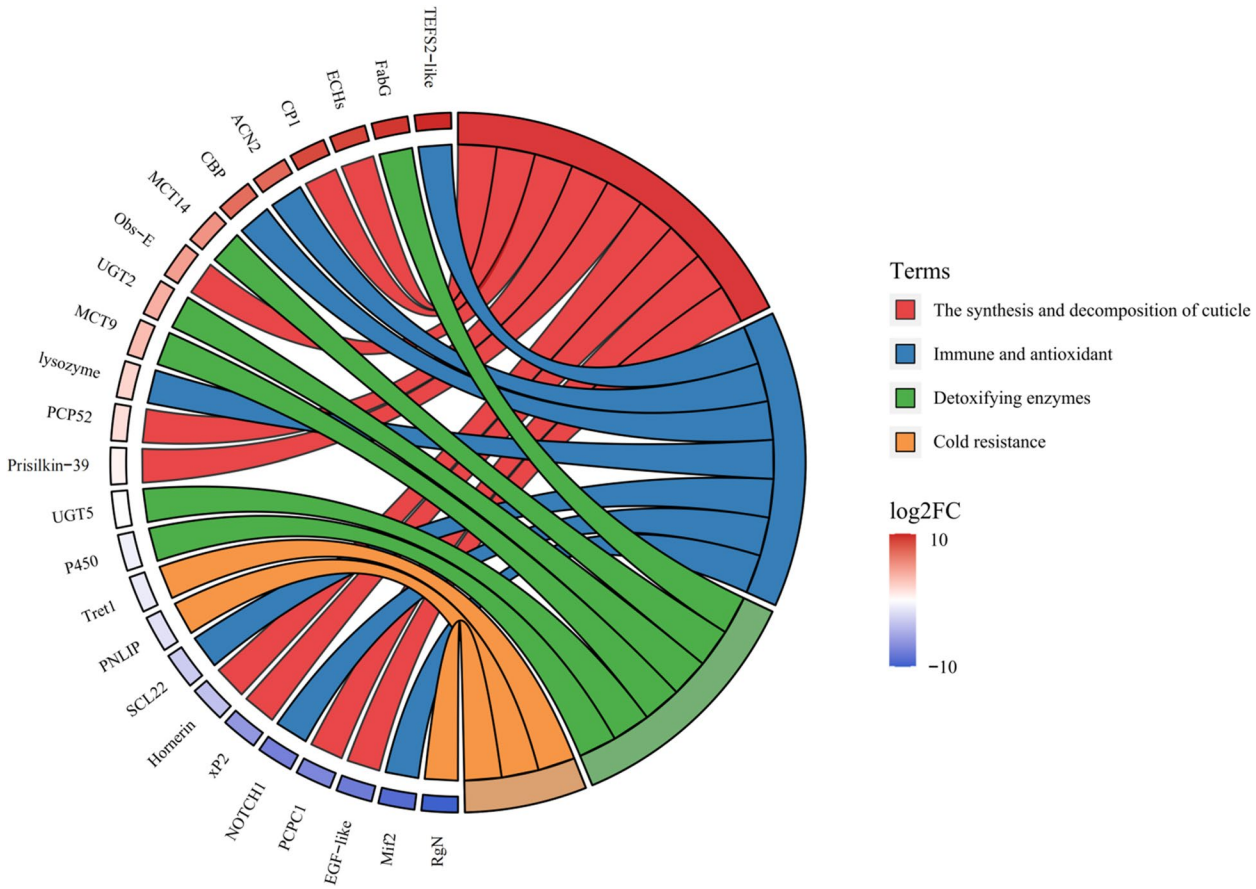

Fig. 8 Analyze the DEGs associated with stress resistance. The functional classification of the top 50 log2 FC (log2 fold change) genes involves the synthesis and decomposition of cuticle, immune and antioxidant, detoxifying enzymes, and cold resistance

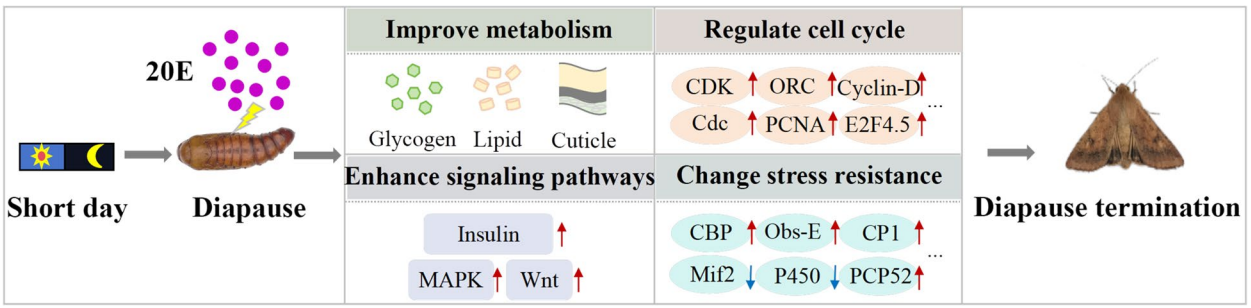

Fig. 9 A schematic drawing of 20E terminating diapause through regulating metabolism, signaling pathways, cell cycle, and stress resistance

Conclusion

In conclusion, our study confirms the role of 20E in effectively terminating diapause in *H. armigera*. A comprehensive analysis of the genes responding to 20E, which terminates diapause, was conducted using RNA-Seq. Further analysis indicates that 20E primarily improves metabolism, enhances cell signaling pathways, regulates the cell cycle, and alters the expression of genes

associated with stress resistance during diapause termination (Fig. 9). This study not only provides a deeper understanding of the molecular mechanisms by which 20E regulates diapause in *H. armigera*, but also supports the potential use of ecdysone analogs as pesticides in diapause-based pest management strategies.

Abbreviations

20E	20-Hydroxyecdysone
JH	Juvenile hormone
DH	Diapause hormone
EcR	Ecdysone receptor
USP	Ultraspiracle protein
RNA-Seq	RNA sequencing
DMSO	Dimethyl sulfoxide
DEGs	Differentially expressed genes
TPM	Transcripts per million reads
GO	Gene ontology
KEGG	Kyoto encyclopedia of genes and genomes
PCA	Principal component analysis
TCA	The citric acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11572-3.

Supplementary Material 1: Table S1. The primers used for qRT-PCR. Table S2. Sequencing data quality.

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Not applicable.

Authors' contributions
HL: Conceptualization, Supervision, Writing—review and editing, Funding acquisition. ZN: Methodology, Investigation, Writing—original draft. YL: Validation, Data curation, Writing—review and editing. JL, SX, JG, ZT: Software, Data curation.

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Data availability
Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive database with the primary accession code PRJNA1164935.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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