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DNA methyltransferase 3 participates in behavioral phase change in the migratory locust

Li Hou[a,b], Xuesong Wang[b], Pengcheng Yang[c], Beibei Li[a], Zhe Lin[a], Le Kang[a,b,c,**], Xianhui Wang[a,b,*]

[a] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China
[b] CAS Center for Excellence in Biotic Interactions, University of Chinese Academy of Sciences, Beijing, 100049, China
[c] Beijing Institutes of Life Science, Chinese Academy of Science, 1 Beichen West Road, Chaoyang District, Beijing, 100101, China

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ABSTRACT

DNA methylation plays important roles in the behavioral plasticity of animals. The migratory locust, *Locusta migratoria*, displays striking density-dependent phenotypic plasticity that can reversely transit between solitarious and gregarious phases. However, the role and the mechanism through which DNA methylation is involved in locust phase change remain unknown. Here, we investigated the expression levels of three DNA methyltransferase genes and their roles in the regulation of locust phase changes. All three *Dnmt* genes, namely, *Dnmt1*, *Dnmt2* and *Dnmt3* showed high expression levels in the brains of gregarious locusts. By contrast, only *Dnmt3* transcript rapidly responded to population density changes, decreasing during the isolation of gregarious locusts and steadily increasing upon the crowding of solitarious locusts. *Dnmt3* knockdown significantly reduced the phase-related locomotor activity, rather than the attraction index, in gregarious and crowded solitarious locusts. Transcriptome analysis showed that *Dnmt3* knockdown upregulated the genes related to metabolism and transporting activity and downregulated those associated with oxidative stress response. The expression level of the phase-core transcriptional factor, hormone receptor *HR3*, was significantly suppressed in the brain after *Dnmt3* knockdown. Moreover, there was significant overlap in the differentially expressed genes between *Dnmt3* RNAi and *HR3* RNAi data sets, suggesting HR3 may act as key transcriptional factor mediating Dnmt3-controlled gene expression profiles in locust brains. These findings suggest that *Dnmt3* transcription is involved in locust behavioral transition, implying the possible roles of DNA methylation in phase-related phenotypic plasticity in locusts.

1. Introduction

The migratory locust, *Locusta migratoria*, a worldwide agricultural pest, displays striking density-dependent phenotypic plasticity in response to population density changes (Uvarov, 1977). Locusts develop two phases, namely, solitarious and gregarious phases, in response to low- and high-density populations, differing significantly in their morphology, physiology, behaviors, and other life traits (Pener and Yerushalmi, 1998). Solitarious locusts are cryptic and avoid conspecifics, whereas gregarious locusts are active and seek out conspecifics (Pener and Simpson, 2009). Locusts can undergo reversible transition between two phases, termed as phase change, rapidly over only several hours (Simpson et al., 1999; Guo et al., 2011). Thousands of genes related to locust phase change have been identified at either short-term or long-term levels (Chen et al., 2010; Guo et al., 2011). Moreover, maternal and paternal phase states and gene expression patterns can be inherited by the next generations (Maeno and Tanaka, 2008; Chen et al., 2015). Recently, several studies have proposed the involvement of epigenetic mechanisms in the regulation of phase transition and transgenerational events in locusts (Wang and Kang, 2014; Ernst et al., 2015).

DNA methylation is an important epigenetic modification, especially for the methylation of the fifth position on the pyrimidine group (5-mC) (Zemach et al., 2010). In vivo DNA pyrimidine methylation is catalyzed by different kinds of methyltransferases, including Dnmt1, Dnmt2, and Dnmt3 (Kay et al., 2018; Li et al., 2018, 2019). Dnmt1

* Corresponding author. Institute of Zoology, Chinese Academy of Sciences, China.
** Corresponding author. State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China.
E-mail addresses: lkang@ioz.ac.cn (L. Kang), wangxh@ioz.ac.cn (X. Wang).

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enzyme has commonly been termed as “maintenance methyltransferase” on the basis of its strong preference for hemimethylated substrates (Gruenbaum et al., 1982; Bestor and Ingram, 1983). Dnmt3 enzyme can methylate unmethylated DNA and is designated as de novo methyltransferase (Jones, 2012). Dnmt2 enzyme has been implicated in tRNA methylation (Goll et al., 2006). A series of studies have suggested that DNA methylation plays essential roles in manifesting gene expressions, alternative splicing, gene imprinting, and other biological processes (Li et al., 1993; Mohn and Schubeler, 2009). Recently, DNA methylation states are involved in phenotypical plasticity in several social insects, such as honey bees (Foret et al., 2012; De Loof et al., 2013), ants (Bonasio et al., 2012), and bumblebees (Morandin et al., 2019). However, the distinct mechanisms of DNA methylations in regulating insect phenotypical plasticity remain unclear.

The locust genome displays a relatively high DNA cytosine methylation level (1.6%) compared with that of other insect species investigated (0.1%–0.3%) (Bonasio et al., 2012; Wang et al., 2014; Libbrecht et al., 2016). Over 90 genes associated with synaptic plasticity have been proposed to have differential methylation between gregarious and solitarious locusts (Wang et al., 2014). Another study has shown that those genes involved in DNA methylation show differential expression levels between two phases of locusts, especially at the embryonic stage (Robinson et al., 2016). Evidence from the desert locust also supports the notion that DNA methylation fingerprint differs significantly between the long-term crowd-reared and solitarily reared locusts; they obviously changed after either acute or chronic crowding treatments (Mallon et al., 2016). Some DNA methylation-associated genes, such as *Dnmt1*, *Dnmt2*, and Methyl-CpG-binding domain gene, have been reported in the migratory locusts (Robinson et al., 2011). However, the methyltransferase system responsible for DNA methylation has not been fully characterized in locusts thus far; as such, their roles in phase change regulation need to be further explored.

In this study, we identified three DNA methyltransferase genes, especially for confirming the existence of Dnmt3 and their expression patterns associated with locust phase change were analyzed. We further investigated function of *Dnmt3* in locust phase-related behaviors by using RNA interference and transcriptome analyses. This work provides insights into the epigenetic mechanism underlying phenotypical plasticity of the migratory locust.

2. Materials and methods

2.1. Insects

Gregarious and solitarious locusts were obtained from the same colony and raised in the Institute of Zoology, Chinese Academy of Sciences, Beijing. Gregarious nymphs were maintained in large cages (40 cm × 40 cm × 40 cm) at a density of 400–500 insects per cage. Solitarious nymphs were reared separately in metal boxes (10 cm × 10 cm × 25 cm) supplied with charcoal-filtered compressed air. Both colonies were maintained at 30 ± 2 °C under a 14:10 light/dark photocycle regime and a diet of fresh wheat seedling and bran (Guo et al., 2011).

2.2. Identification of DNMT gene sequences

The amino acid sequences of DNMT1, DNMT2, and DNMT3 from *Apis mellifera* and other insect species were used to search for DNA methyltransferase homologs in the locust genome and transcriptome database by utilizing the TBLASTN algorithm. The putative open reading frames and amino acid sequences of locust DNA methyltransferases were analyzed through the ExPASy server (https://web.expasy.org/translate/), and protein domains were predicted with online SMART software (http://smart.embl-heidelberg.de/). The cDNA sequences of three DNA methyltransferase were validated through RT-PCR by using gene-specific primers together with subsequent Sanger sequencing. cDNA sequences of three DNA methyltransferases have been submitted to GenBank database of the National Center for Biotechnology Information (NCBI) (accession no: MN815770, MN815771, MN815772 for *Dnmt1*, *Dnmt2*, and *Dnmt3*). The phylogenetic relationship of DNA methyltransferases in the migratory locust and other representative insect species was analyzed by using the neighbor-joining method with a Poisson model and 1000 bootstrap replications of MEGA5.0 software.

2.3. Sample preparation

The brains of fourth-instar nymphs of gregarious and solitarious locusts were collected to determine the phase-related mRNA levels of three *Dnmt* genes. For the crowding treatment, 10 fourth-instar solitarious nymphs were reared together with 20 gregarious locusts at the same developmental stage in a small cage (10 cm × 10 cm × 10 cm). After 0, 4, 16, and 64 h crowding, the brains of solitarious nymphs were dissected and frozen in liquid nitrogen. Isolation treatment was performed by separately raising the fourth-instar gregarious nymphs. After 0, 4, 16, and 64 h isolation, the brains of gregarious nymphs were collected and snap frozen. All samples were stored at −80 °C. Each sample contained eight insect brains, with equal ratios of males and females. Four independent biological replicates were prepared for the expression profile analysis.

2.4. RNA extraction, cDNA synthesis, and qPCR

Total RNAs were isolated using a TRIzol kit (Life Technologies) following the manufacturer's instructions. RNase-free DNase (1 μl, 1 U/μl, Promega) was added to the RNA solution and incubated at 37 °C for 30 min to remove genomic DNA. The mixture was incubated at 65 °C for 10 min to inactivate DNase. The quantity and quality of total RNAs were determined by ultraviolet spectrophotometry. The first-strand cDNA was synthesized from 2 μg of total RNAs by using the M-MLV reverse transcriptase and oligo (dT)-primer (Promega) in accordance with the manufacturer's instructions.

Gene-specific primers used for qPCR were designed using Primer 5.0 software and listed in Table S1. *RP49* was used as the internal reference. Gene expression levels were detected by qPCR via the SYPR Green RealMasterMix (Roche) on a LightCycler 480 instrument (Roche). Melting curve analysis was used to confirm unique amplification. Data were analyzed by $2^{-\Delta CT}$ method to quantify relative mRNA expression levels.

2.5. Transcript knockdown by RNA interference

The dsRNAs of Dnmt3 (dsDnmt3) were prepared using T7 RiboMAX Express RNAi system (Promega) and then microinjected into the hemolymph of test insects at the second abdominal segment toward the head (18 μg/locust). dsRNA of GFP (dsGFP) were used as the control in RNAi experiments. The behavior of locust nymphs was observed 72 h after injection as described below. Both dsRNA injection and behavior observation were performed in the fourth instar locusts.

2.6. Behavioral assay

The behavioral assay was performed in a rectangular Perspex arena (40 cm × 30 cm × 10 cm) that contains three chambers as described in our previous study (Hou et al., 2017). The left chamber contained 30 fourth-instar gregarious locusts as the stimulus group, and the right chamber was empty. Locust behavior was recorded for 300 s by EthoVision video tracking system and analyzed in accordance with the binary logistic regression model constructed in our previous work (Guo et al., 2011). Attraction index (AI) represents the extent to which the tested animals are attracted by the gregarious locusts. Total duration of movement (TDMV) and total distance moved (TDM) indicate the

locomotor activity. In the behavioral assay, 30–50 locusts were tested for each treatment according to the sample size reported in a previous work (Ma et al., 2011). Locusts that did not move during the behavioral test were excluded.

2.7. Transcriptome analysis

The brains of the fourth-instar nymphs of gregarious locusts were collected 72 h after injection of dsDnmt3 or dsGFP. Three independent biological replicates were performed for each treatment. Each sample contained eight brains (four males and four females). Total RNAs were isolated as previously described, and RNA quality was confirmed by agarose gel electrophoresis. cDNA libraries were prepared according to Illumina's protocols and sequenced on Illumina Hiseq2000. Raw reads with low quality and adaptor sequences were filtered, and the clean data were mapped to the locust genome sequences by using Tophat2 (Wang et al., 2014). The number of unique mapped reads to each gene model was counted using HTseq (statistics of raw data and unique mapped reads were shown in Table S2). The number of total reads was normalized by multiple normalization factors. The gene transcript levels were measured as reads per kilobase per million reads (RPKM). Differentially expressed genes (DEGs) were detected using edgeR package (Robinson et al., 2010), defined as fold change $>$ 2 and FDR $<$ 0.1. Unsupervised hierarchical clustering was performed by using Clustal 3.0 software with uncentered Pearson correlation and average linkage methods. Hot map was presented by Java TreeView software. Enrichment analysis of the Gene Ontology (GO) for DEGs was performed based on an algorithm presented by GOstat (Beissbarth and Speed, 2004). Only GO terms with $p < 0.01$ were considered. The RNA-seq data were deposited in the Sequence Read Archive database of the National Center for Biotechnology Information (NCBI) (BioProject ID: PRJNA597376).

2.8. Statistical analysis

All data were analyzed using SPSS 15 software. The gene expression levels were presented as mean ± s.e.m. Student's *t*-test was used for two-group comparison, and one-way ANOVA followed by Tukey's post-hoc test was used for multigroup comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. DNA methyltransferases in the locust genome

We obtained three genes encoding DNA methyltransferases (*Dnmt1*, *Dnmt2*, and *Dnmt3*) from the locust genome and transcriptome. Based on homologous searching, the sequences of DNA methyltransferases from 12 insect species were used to construct the phylogenetic tree. DNMT1, DNMT2, and DNMT3 from the migratory locust and other insect species were grouped to a monophyletic clade. The homologs of *Locusta* DNMT1 and DNMT2 displayed a close evolutionary relationship with those of the desert locust *Schistocerca gregaria*. *Locusta* DNMT3 showed a close relationship with its homolog in *Zootermopsis nevadensis* due to the absence of *Schistocerca* DNMT3 (Fig. 1A).

By predicting domain structures of DNMTs proteins, we found that all three DNMT enzymes contained a site-specific DNA-cytosine methylase domain (MTase), which is responsible for cytosine methylation (Wu and Zhang, 2014). A PWWP domain was also observed in DNMT3 (Fig. 1B). Unlike DNMT1, DNMT3 lacks the bromo-adjacent homology domains (BAH), the replication foci domain (RFD), and the CXXC zinc finger domain (zf-C) (Fig. 1B).

3.2. Phase-related expression patterns of DNA methyltransferase genes

We compared expression levels of three methyltransferase genes in the brains of the fourth instar nymphs between gregarious and solitarious locusts via qPCR to explore whether DNA methylation was related to phase change. The three *Dnmt* genes in the brains of gregarious locusts showed more abundant mRNA levels than those of solitarious locusts (Fig. 2).

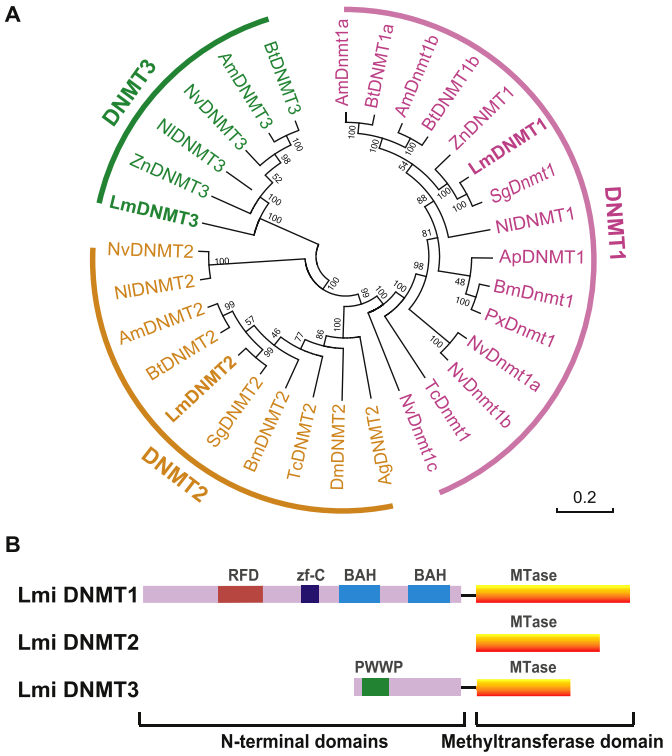

Fig. 1. Phylogenetic analysis and protein structures of DNA methyltransferases. (**A**) Phylogenetic tree based on amino acid sequences of DNMT1, DNMT2, and DNMT3 from locusts and other insect species. Proteins were from Am, *A. mellifera*; Bt, *B. terrestris*; Ag, *A. gambiae*; Ap, *A. pisum*; Bm, *B. mori*; Cf, *C. floridanus*; Dm, *D. melanogaster*; Px, *P. xylostella*; Tc, *T. castaneum*; Nv, *N.vitripennis*; Zn, *Z. nevadensis*, and *S. gregaria*. (**B**) Predicted protein domains of three DNA methyltransferases in locusts. RFD: cytosine specific DNA methyltransferase replication foci domain; zf-C: CXXC zinc finger domain; BAH: bromo-adjacent homology domain; MTase: site-specific DNA-cytosine methylase domain; PWWP: domain with conserved PWWP motif.

We further quantified the expression levels of *Dnmt1*, *Dnmt2*, and *Dnmt3* during two time-course processes of locust phase change, namely, crowding (crowding of solitarious locusts) and isolation (isolation of gregarious locusts). The mRNA level of *Dnmt3* rapidly responded to changes in population density, with a significant decrease (~1.5-fold) 16 h after the isolation of gregarious locusts; however, a dramatic increase (> two-fold) was observed 64 h after the crowding of solitarious locusts (Fig. 3C). By contrast, *Dnmt1* and *Dnmt2* showed

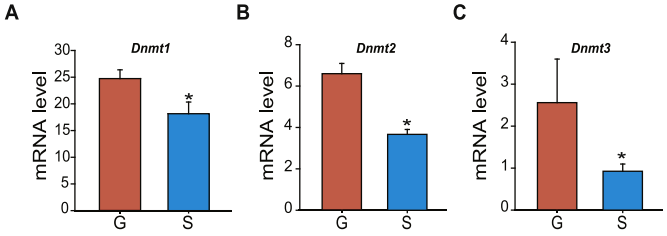

Fig. 2. Expression levels of *Dnmt1*, *Dnmt2*, and *Dnmt3* in brains of gregarious and solitarious locusts. qPCR analysis revealed that all three *Dnmt* genes were highly expressed in the fourth-instar gregarious locust brains. G, gregarious; S, solitarious. The data are presented as mean ± s.e.m. (n = 4/treatment, 8 animals/sample, Student's *t*-test, *$p < 0.05$).

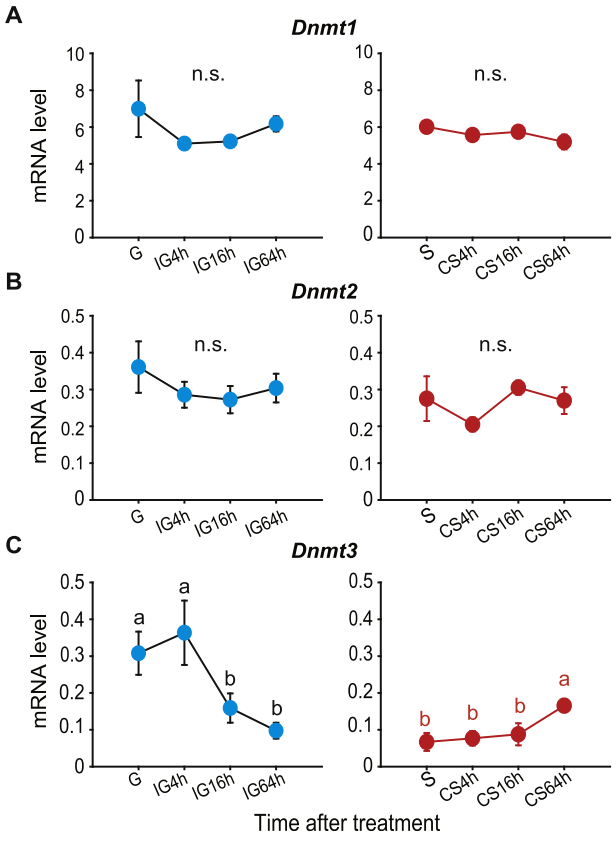

Fig. 3. Expression levels of *Dnmt1*, *Dnmt2*, and *Dnmt3* during locust phase transition. The mRNA levels of (A) *Dnmt1,* (B) *Dnmt2*, and (C) *Dnmt3* during the two time-course processes of phase change were detected by qPCR. IG indicates the isolation treatment of gregarious locusts; CS indicates the crowding treatment of solitarious locusts. The X-axis means time after the treatment of IG and CS, respectively. The data are presented as mean ± s.e.m. Significant differences at different times are denoted by letters. Data points labelled with b were statistically significantly different to data points labelled with a. n.s. indicates no statistical significance (n = 4/treatment, 8 animals/sample, one-way ANOVA, $p < 0.05$).

significant expression changes neither in the isolation nor the crowding processes (Fig. 3A and B). These results imply that *Dnmt3* transcription might play an important role in the regulation of rapid behavioral transition of locusts in response to density changes.

3.3. Effects of Dnmt3 knockdown on phase-related behavior

To validate whether *Dnmt3* participates in the regulation of phase-related behaviors, we performed arena assay to measure the phase-related behavioral parameters of locusts injected with double-strand RNA (dsRNA) of *Dnmt3*. The knockdown of *Dnmt3* in gregarious locusts resulted in a 22% decrease of its gene expression level in the brain tissue relative to that in dsGFP-injected locusts (Fig. S1). Meanwhile, two parameters representing locomotor activity, TDM and TDMV, significantly decreased in gregarious locusts and the crowded-solitarious locusts after *Dnmt3* knockdown (Fig. 4B, C, E, and F). However, the AI was not affected by the RNA interference of *Dnmt3* in gregarious locusts as well as in the crowded-solitarious locusts (Fig. 4A and D). These results indicated that the presumed de novo methyltransferase, Dnmt3, is essential in controlling phase-related locomotor plasticity in locusts.

3.4. Effects of Dnmt3 knockdown on the gene expression profiles of gregarious locusts

To explore potential mechanisms through which DNMT3 are

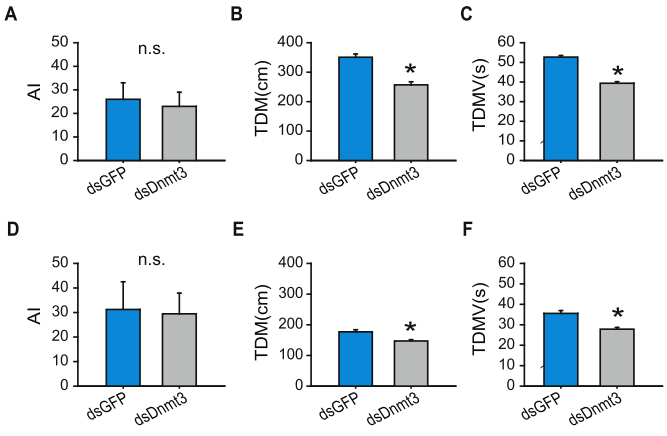

Fig. 4. Behavioral analysis after *Dnmt3* knockdown in the locust. (**A**) AI, (**B**) TMD, and (**C**) TDMV in gregarious locusts after *Dnmt3* knockdown (n > 30 locusts). (**D**) AI, (**E**) TDM, and (**F**) TDMV in crowded solitarious locusts after *Dnmt3* knockdown of *Dnmt3* (n > 30 locusts). AI indicates attraction index, TMD indicates total distance moved, and TDMV indicates total duration of movement. n.s. indicates no statistical significance. Student's *t*-test, *$p < 0.05$.

involved in the plasticity of phase-related locomotion, we analyzed the changes in the gene expression profiles in the brains of gregarious locusts under the injection of ds *DNMT3*. A total of 346 differentially expressed genes (DEGs), including 259 downregulated genes (64 annotated genes) and 87 upregulated genes (49 annotated genes), were identified after *Dnmt3* knockdown (FDR < 0.1, fold-change > 2) (Fig. S2). The Gene Ontology analysis of these DEGs indicated that the downregulated genes were mainly enriched in terms of peroxidase activity, response to oxidative stress, chitin binding, chitin metabolic process, aminoglycan metabolic process, and serine-type endopeptidase activity. However, the upregulated genes mainly refer to oxidation–reduction, transmembrane transport, oxidoreductase activity, single-organism metabolic process, and catalytic activity (Fig. 5).

Among these DEGs, many genes encoding serine protease, peroxidase precursors, and cytochrome P450 members (cytochrome P450 4G49 and cytochrome P450 4C1) displayed significantly reduced expression levels. The transcript levels of several important signal transduction molecules, such as protein spaetzle, nuclear hormone receptor HR3, GTP-binding protein 5, and secretory phospholipase A2-like, were strongly inhibited by *Dnmt3* knockdown. By contrast, *Dnmt3* knockdown resulted in the remarkable upregulation of the lipid metabolism-associated genes, including mitochondrial pyruvate carrier 1-like, medium-chain specific acyl-CoA dehydrogenase, cyclohex-1-ene-1-carboxyl-coA hydratase, mitochondrial import inner membrane translocase subunit Tim8, and hormone synthesis, such as juvenile hormone acid methyltransferase and juvenile hormone binding protein (Fig. 6). These DEGs may be involved in Dnmt3 regulation in phase-related behaviors in locusts.

As a down-regulated gene affected by *Dnmt3* knockdown, HR3 was shown to be a phase-core transcription factor and there were 135 down-regulated genes and 36 up-regulated genes after the knockdown of *HR3* (Yang et al., 2019). We thus performed comparative analysis of transcriptome data of brain samples after *Dnmt3* RNAi and *HR3* RNAi. We indeed found that there was significant overlap in the DEGs between *Dnmt3* RNAi and *HR3* RNAi data sets, especially in the down-regulated gene sets (Fig. 7). About 30.5% (79 genes) down-regulated gene in *Dnmt3* knockdown group were inhibited by *HR3* knockdown as well, whereas only 2 genes were overlapped in the up-related gene sets of *Dnmt3* RNAi and *HR3* RNAi samples (Fig. 7). Notably, several antioxidant genes (chorion peroxidase precursors, Homocysteine S-methyltransferase) and signal transduction molecules (secretory phospholipase A2, Serine proteinases, Protein spaetzle and HR3) were suppressed by both *Dnmt3* RNAi and *HR3* RNAi treatments (Table S3),

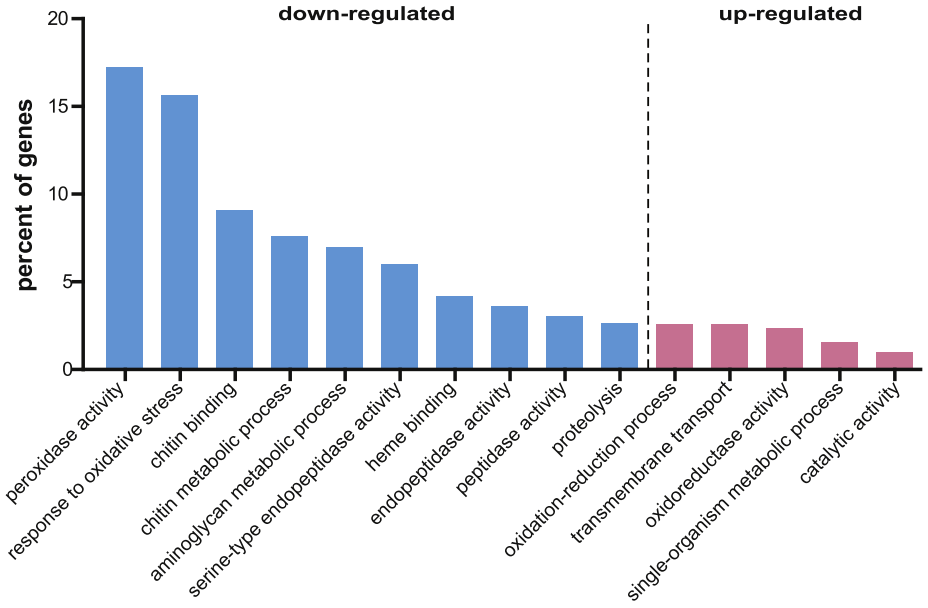

Fig. 5. GO enrichment of DEGs in the transcriptome after *Dnmt3* knockdown in gregarious locusts. Enrichment analysis of the GO was performed based on an algorithm presented by GOstat. GO terms were defined as $p < 0.01$.

suggesting HR3 may act as a key mediator in Dnmt3-regulated phase change through modifying global gene expressions.

4. Discussion

In this study, we identified all three genes encoding DNA methyltransferases, thereby confirming the existence of Dnmt3, and analyzed their phase-related expression patterns in the brains of the migratory locusts. We provided the experimental evidence for the regulation of *Dnmt3* in fine-tuning of phase-related locomotor plasticity by RNAi-mediated gene knockdown. This study lays the molecular foundation for further uncovering epigenetic mechanisms underlying locust phase change.

Our study proves that the migratory locust possesses an integral DNA methylation system that is composed of three methyltransferases, namely, DNMT1, DNMT2, and DNMT3. On the basis of the transcriptome or expression sequence tag (EST) sequences, DNMT1 and DNMT2 are identified in *L. moratoria* and *S. gregaria* (Robinson et al., 2011, 2016). The present work provided the first molecular characterization of DNMT3 in locusts. DNA methylation is primarily carried out by DNMT1 and DNMT3 (Jones, 2012). However, *Dnmt1* and *Dnmt3* were selectively lost in the genome of some insect species (Li et al., 2018). For example, both *Dnmt1* and *Dnmt3* were lost in the dipteral insects *Dosophila* and *Anophele,* while only *Dnmt3* was lost in the lepidopterans *Bombyx*, *Danaus*, and *Plutella*, and the coleopteran *Tribolium*. All three methyltransferases were retained in the genome of Hemiptera *Nilaparvata*, *Acyrthosiphon*, as well as the hymenopteran insects, including *Apis*, *Bombus*, and *Nasonia*. It seems that the complete functional DNA methylation toolkit was largely maintained in insect species that display striking phenotypical plasticity and sociality.

Obvious gregarious-abundant expression was observed for all three *Dnmt* genes in the fourth-instar nymphs, indicating a high turnover rates of DNA methylation in the brain cells of gregarious locusts. The brain expression levels of *Dnmt1* and *Dnmt2* did not change during the isolation or the crowding process, but the expression levels of *Dnmt3* in the brain rapidly responded to the time-course treatments of locust

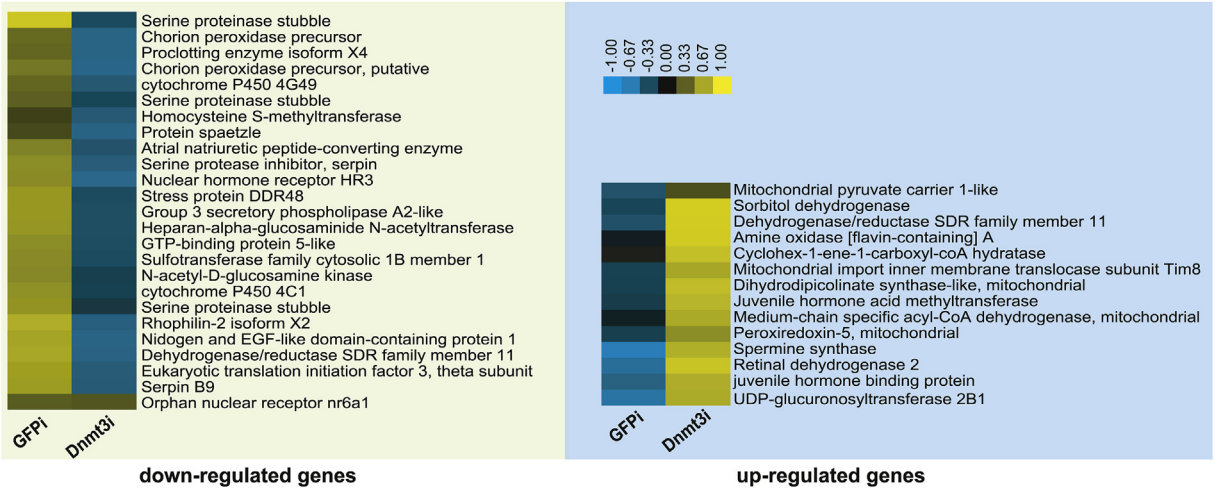

Fig. 6. Cluster analysis of DEGs in the transcriptome after *Dnmt3* knockdown in gregarious locusts. Logarithmic fold alteration of dsDnmt3 versus dsGFP is shown in the heat map. Yellow and blue colors indicate upregulation and downregulation, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

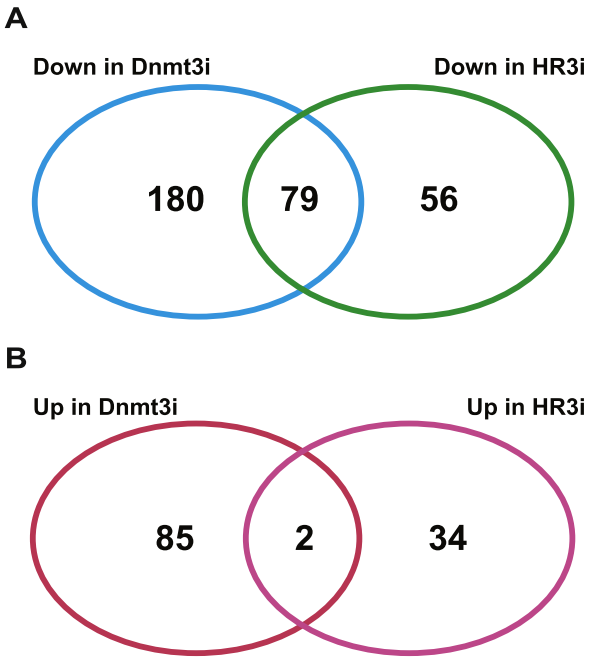

Fig. 7. Comparative analysis of DEGs in *Dnmt3* knockdown and *HR3* knockdown RNA-seq data. Knockdown of *HR3* resulted in 135 down-regulated genes and 36 up-regulated genes (Yang et al., 2019). About 30.5% (79 genes) down-regulated genes caused by *Dnmt3* knockdown were also inhibited by *HR3* knockdown, whereas only 2 gene were overlapped in the up-related gene sets of *Dnmt3* knockdown and *HR3* knockdown.

phase change, suggesting that the brain *Dnmt3* transcription plays a role in regulating the behavioral transition of locusts in response to density changes. Previous studies revealed that *Dnmt1* did not display a phase-related expression pattern, but *Dnmt2* showed solitarious-abundant transcript level in the nervous system of fifth-instar nymphs (Robinson et al., 2016); this result is different from that of the phase-related expression patterns of *Dnmt1* and *Dnmt2* of the fourth-instar nymphs in our experiments. Actually, one possible explanation from our previous transcriptome analysis is that the global phase-related gene expression profiles of the fourth-instar were different in some content from that of the fifth-instar nymphs of the locust (Chen et al., 2010). The abundant expression levels of three *Dnmt* genes in the gregarious brains of fourth-instar nymphs suggest their potential significance in the formation of gregarious-specific characters. Moreover, the tissue-dependent gene expression patterns of *Dnmts* between two phases were reported in another locust species. For example, *Dnmt1* and *Dnmt2* showed gregarious-abundant expressions in the testes but solitarious-abundant expressions in the brains of desert locusts (Boerjan et al., 2011). The distinct expression profiles of *Dnmt* genes suggest that they may be associated with certain types of phase polyphenism in locusts. As a maintainer of DNA methylation, *Dnmt1* has been shown to play essential roles in gonadogenesis and offspring survival in insects (Zhang et al., 2015; Bewick et al., 2019). Thus, regulation in transgenerational transfer of phase state may be proposed for *Dnmt1*. Although *Dnmt2* is not required for DNA methylation in mammals, this enzyme has been shown to play important roles in epigenetic regulation in insects by modifying the methylation of transfer RNA (tRNA) (Kunert et al., 2003; Schaefer et al., 2008; Phalke et al., 2009). In *Drosophila*, loss-function of *Dnmt2* lead to dsRNA accumulation upon heat shock through affecting siRNA pathways (Durdevic et al., 2013), indicating a regulatory role of *Dnmt2* in siRNA homeostasis and stress response. The higher *Dnmt2* expression in the fourth-instar gregarious locusts may contribute to the regulation of global gene expression profiles at this developmental stage that is essential for the formation of phase traits through a siRNA-dependent mechanism. Further studies are needed to determine whether Dnmt2 participates in the dynamic shift of phase-dependent gene expression profiles between the fourth and the fifth instar.

The expression levels of *Dnmt3* significantly changed in response to the crowding and isolation treatments. As a de novo methyltransferase, DNMT3 is primarily responsible for the new methylation generation of unmethylated DNA. The rapid changes in *Dnmt3* expression levels may reflect highly dynamic DNA methylation modification that occurred in locust phase transition. *Dnmt3*-mediated DNA methylation plays important roles in regulating gene expression and global transcriptional state in mammals (Holliday and Pugh, 1975). In fact, thousands of genes exhibit differential expression changes in locust brains, mediating a sequential behavioral shift toward the opposite phase (Guo et al., 2011; Ma et al., 2011). A reasonable assumption is that *Dnmt3* may participate in the regulation of phase-related brain plasticity by influencing the overall DNA methylation pattern of these genes. We verified this function of *Dnmt3* in phase-related locomotion through an RNAi experiment. This finding is in line with a recent study that uncovered the significant role of DNMT3a in promoting voluntary exercise in mice (MacKay et al., 2019), suggesting the possible conserved roles of DNMT3 in regulating locomotor plasticity. The potential roles of DNA methylation in insect phenotypic plasticity have been extensively studied, particularly in social insects. However, the results remain controversial. Based on multi-omics systematic analysis of individual brains, Patalano et al. have revealed that DNA methylation shows few differences between different phenotypes in an ant (Dinoponera quadriceps) and a wasp (Polistes canadensis) (Patalano et al., 2015). A study in the fire ant *Solenopsis invicta* showed that *Dnmt1* and *Dnmt3* displayed caste-independent expression patterns among heads of behaviorally distinct adults (Kay et al., 2018). In contrast, it has been suggested that DNA methylation participates in the regulation of caste specialization in *Camponotus* (Bonasio et al., 2012) and *Formica exsecta* (Morandin et al., 2019). So far, there is limited direct experimental evidence validating the roles of Dnmts in phenotypic plasticity. One of the evidences from the honey bees reveals that silencing of *Dnmt3* gene expression in worker bees induces queen-like phenotypes (Kucharski et al., 2008). Thus, our findings provide additional evidence for the significance of *Dnmt3* in fine-tuning phenotypic plasticity in insects.

We demonstrated that the genes related to oxidative stress response were significantly inhibited, but the genes related to oxidation–reduction were strongly activated after *Dnmt3* knockdown. The fourth-instar gregarious locusts might invest more on the interaction with other conspecifics by upregulating signal transduction and anti-oxidation activity; solitarious locusts are active in metabolism and biosynthesis by enhancing oxidation–reduction (Chen et al., 2010; Wang et al., 2014). The global transcriptome profiles resulting from *Dnmt3* silencing in gregarious locusts seem close to that of solitarious locusts, which is consistent with the solitarious-like locomotion performance caused by the same treatment. The antioxidant genes, including peroxidases precursors, cytochrome P450 members, and Glutathione-S-transferase, are essential in protecting against the damage of reactive oxygen species caused by high motor activity (Magwere et al., 2006). Therefore, the expressional decrease in these genes reflects the adaptation for decreased locomotor activity caused by the RNAi of *Dnmt3*. Although most cytochrome P450 genes show distinct tissue-specific expression profiles and have multiple biological roles in insects, the brain-derived cytochrome P450 plays key roles in the neuroendocrine and behavioral regulation in *Drosophila* (Kang et al., 2011), supporting the potential involvement of this gene family in behavioral phase change in locusts. Moreover, cytochrome P450 4G has been shown to participate in the resistance to biological toxification in *Chironomus riparius* (Martinez-Paz et al., 2012). The reduced gene expression of cytochrome P450 genes may also indicate a decrease in detoxification function in *Dnmt3* knockdown locusts. However, whether the expression changes of these antioxidant genes were directly controlled by Dnmt3-mediated DNA

methylation need to be further validated. In addition to genes associated with metabolism, JH-related genes, including juvenile hormone (JH) acid methyltransferase and juvenile hormone binding protein, were significantly upregulated by the interference of *Dnmt3*. JH can induce several phase-related characteristics, including body color (Tanaka, 2000) and metamorphosis. Further work will make sense to examine the long-term effects of Dnmt3 on JH-regulated phenotypes (e.g. body color and ecdysis) and other phase-related features (e.g. morphological traits).

In this work, *Dnmt3* knockdown significantly affected the locomotor activity instead of the attraction index during phase change. Among these DEGs, a phase-core transcription factor named hormone receptor 3 (*HR3* also called *HR46*) was significantly downregulated by the knockdown of *Dnmt3*. Our previous studies have shown that the RNAi of *HR3* in gregarious phase locusts induced significantly transcriptional changes in the locust brain, and caused behavioral phase transition toward the solitarious state by suppressing locomotor activity (Yang et al., 2019). Moreover, many DEGs affected by *HR3* knockdown and *Dnmt3* knockdown were overlapped. As such, HR3 may serve as a key molecular switch that mediates the behavioral effects caused by *Dnmt3*. Usually, DNA methylation in the transcriptional sites or promoter region blocks target gene expression, but the methylation in the gene body region may stimulate gene transcription (Jones, 1999; Hellman and Chess, 2007). In mammals, higher levels of promoter methylation strongly correlate with hormone receptor positive status of breast tumors, e.g. estrogen receptor (ER) and progesterone receptor (PR) (Benevolenskaya et al., 2016). Estrogen receptor 1 gene expression could be elevated by estrogen bisphenol A through Dnmt-mediated hypermethylation of its promoter (Bhandari et al., 2019). These findings suggest a key role of Dnmt in regulating the expressions of hormone receptor genes. Future work is needed to understand how Dnmt3 directs *HR3* gene expression to control phase-related locomotor plasticity.

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Appendix A. Supplementary data

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