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DNA methyltransferases have an essential role in female fecundity in brown planthopper, *Nilaparvata lugens*

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ABSTRACT

DNA methylation is an ancient epigenetic modification present in all three domains of life. However, the understanding of DNA methylation in insects is limited. Here, we amplified the full-length transcripts of the DNA methyltransferases *Nlu-Dnmt1* and *Nlu-Dnmt3*, indicating that a complete DNA methylation toolkit exists in the brown planthopper, *Nilaparvata lugens*, a destructive pest in rice production. Nlu-DNMT1 and Nlu-DNMT3 had the conserved motifs and domains of the DNA methyltransferase family. *Nlu-Dnmt1* and *Nlu-Dnmt3* were highly expressed in the mated and gravid female adults but weakly expressed in larvae, male adults, and virgin female adults. Silencing *Nlu-Dnmt1* and *Nlu-Dnmt3* in gravid brachypterous female adults led to fewer offspring, suggesting that DNA methylation regulates female fecundity in insects.

1. Introduction

DNA methylation, the addition of a methyl group to position 5 of cytosine bases in the genome, is an important epigenetic modification that is essential for gene regulation. It is associated with a number of key processes in Archaea, Bacteria, and Eukarya [1–5]. As a major epigenetic mechanism, DNA methylation has broad functions, participating in the gene expression regulation [6], genomic imprinting [7], histone modifications [8], X chromosome inactivation [9,10], alternative splicing [11,12], and repetitive elements suppression [13,14]. It is also essential for brain [15,16] and embryonic development [17,18]. Genes with CG islands tend to be hypermethylated. In contrast, the genes with infrequent CpG sites are often hypomethylated [3,6]. DNA methylation usually occurs in the promoter regions and normally induces gene silencing [19]. However, mounting evidence indicates that DNA methylation also appears in exons and maintains transcription integrity [9,20].

DNA methylation varies considerably across taxa, indicating that its role is not strictly conserved among organisms. There are several major differences in DNA methylation patterns between vertebrates and invertebrates. First, vertebrate genomes tend to show extensive levels of DNA methylation, whereas invertebrate genomes generally exhibit reduced levels. Second, unlike in vertebrates, DNA methylation is not required for transposon repression in invertebrates. Third, vertebrate genomes are globally methylated, whereas in invertebrates only intragenic regions are targeted by DNA methylation. Intergenic repetitive elements are largely unmethylated [2,4,21].

DNA methylation is accomplished by several evolutionarily conserved enzymes known as DNA methyltransferases (DNMT) [1]. A complete DNA methylation toolkit is found in almost all vertebrates and includes three types: *Dnmt1*, *Dnmt2*, and *Dnmt3*. *Dnmt1* is thought to function primarily as a maintenance DNMT responsible for copying the DNA methylation patterns from the parental to the daughter strand following replication [4]. *Dnmt1* mRNA is maternally provided to the embryo and is essential during early embryogenesis. *Dnmt2* was initially thought to be a DNMT but now is known to methylate tRNA [2]. Its function is poorly understood. *Dnmt3* is a *de novo* DNMT that forms new methylation sites and modifies DNA in response to environmental stimuli.

A complete DNA methylation toolkit is not always present in insects. Only Hymenoptera, such as the honeybee *Apis mellifera* [22] and wasp *Nasonia vitripennis* [13], and Hemiptera, such as the aphid *Acyrthosiphon pisum* [2], contain all three types of enzyme. The dipterans *Drosophila melanogaster* and *Anopheles gambiae* contain only *Dnmt2* [23], while Lepidoptera, such as *Bombyx mori*, and Coleoptera, such as *Tribolium castaneum*, have lost *Dnmt3* [22,24]. The presence of DNMT corresponds to the DNA methylation level.

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The genomes of honeybee, wasp, and aphid are moderately methylated because they have a complete set of DNMTs [13,21,22]. The invertebrate model organisms *D. melanogaster* and *Caenorhabditis elegans* have very low levels of DNA methylation because they have lost all DNMTs except *Dnmt2* [2]. Interestingly, the *Tribolium* genome is methylated at a moderate level although it does not have *Dnmt3* [4], suggesting an unknown DNA methylation mechanism in insects.

DNA methylation has not been well studied in invertebrates because the model organism *D. melanogaster* and *C. elegans* lack this type of epigenetic modification. A breakthrough was reported in honeybee. Two *Dnmt1* and one *Dnmt3a/b* were identified from its genome, the first reported complete DNA methylation toolkit in invertebrates [25]. Silencing of *Dnmt3* in newly hatched larvae had a royal jelly-like effect; RNAi-treated individuals emerged as queens with fully developed ovaries, suggesting the DNA methylation participates in honeybee caste determination [26]. Since then, mounting evidence has demonstrated that DNA methylation also has important regulatory roles in insects. It functions in sex determination in *Nasonia* [13], in memory processing in honeybee [27], in developmental plasticity in horned beetles [28], and in caste differentiation in ant, termite, and aphid [29–31]. Here, we present evidence that rice brown planthopper (BPH), *Nilaparvata lugens*, one of the most destructive insect pests causing huge yield lose in rice production in Asia, has a complete DNA methylation toolkit. Silencing of *Dnmt1* in female adults led to fewer offspring, suggesting an essential role in regulating female fecundity.

2. Materials and methods

2.1. Insects

BPH were collected from a rice field in Nanjing, Jiangsu Province, China and maintained in the laboratory on rice seedlings at 27 ± 1 °C under a 16-h light/8-h dark photoperiod and 70–80% relative humidity. The BPH were maintained in beakers (15 cm in diameter, 20 cm in height) and were transferred to fresh seedlings every 5–10 days to guarantee sufficient nutrition.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using the SV Total RNA Isolation System according to the manufacturer's protocol (Promega, Madison, WI, USA). RNA integrity was examined by 1.2% agarose gel. RNA quality was measured using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The cDNA synthesis was carried out using the PrimeScript™ RT Reagent Kit with gDNA Eraser following the manufacturer's instructions (Takara, Otsu, Japan). To generate RACE-ready cDNA, the SMARTer™ RACE cDNA Amplification Kit was used following the user manual (Clontech, Mountain View, CA, USA). Possible genomic DNA contamination was removed by using the gDNA Eraser in the RT Reagent Kit (Takara).

2.3. RT-PCR and RACE

Dnmt1 and *Dnmt3* fragments were obtained from a BPH transcriptome (SRX023419) and then searched in the BPH genome [32]. Reverse transcription (RT) PCR was conducted to verify the fragments. All primer sequences are listed in Table S1. The PCR conditions were: 94 °C for 3 min; followed by five cycles of 94 °C for 30 s, 54 °C–46 °C (−2°C/cycle) for 30 s, and 72 °C for 1 min; then 30 cycles of 94 °C for 30 s, 46 °C for 30 s, and 72 °C for 1 min. The last step was followed by a final extension at 72 °C for 10 min.

To obtain the full-length sequences of *Nlu-Dnmt1* and *Nlu-Dnmt3*, RACE reactions were performed using the SMARTer™ RACE cDNA Amplification kit (Clontech), according to the user manual. Gene-specific primers (GSP) and nested gene-specific primers (NGSP) used for the 5′-RACE and 3′-RACE reactions were designed based on the *Dnmt1* and *Dnmt3* fragments using Primer Premier 5.0 (Table S1). The first-step PCRs were performed with the GSPs and universal primer mix. The PCR conditions were: incubation at 94 °C for 3 min; five cycles at 94 °C for 30 s, 72 °C for 3 min; five cycles at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min; and 25 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. The final extension was 72 °C for 10 min. In the nested PCRs, the first-round PCR products were 100 × diluted and then used as the templates with the NGSPs and nested universal primer mix. The PCR program was: incubation at 94 °C for 3 min; followed by 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; with a final extension at 72 °C for 10 min.

The PCR products were separated on an agarose gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Purified cDNA was ligated into the pGEM-T Easy Vector (Promega) and then bi-directionally sequenced (GenScript, Nanjing, China).

2.4. Quantitative real-time PCR

The mRNA abundances were determined by quantitative real-time PCR (qPCR) using an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq™ (Takara) according to the manufacturer's instruction. For qPCR, 50 ng cDNA was used as the template in 20-μL reactions. The qPCR conditions were: one cycle of 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Primers are listed in Table S1. The housekeeping gene β-actin was used as an internal control for data normalization. Primer pair efficiencies were calculated by a series of 10-fold logarithmic dilutions of the cDNA template. All experiments were repeated in triplicate. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.5. Sequence analysis

Compute pI/Mw software (http://web.expasy.org/compute_pi/) was used to predict the molecular weight (Mw) and isoelectric point (pI). DNMT sequences of other species were downloaded from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The GenBank accession numbers are shown in Table S2. Protein domains were analyzed by searching the Pfam database (http://pfam.janelia.org/search). Pfam accession numbers and descriptions of these domains are shown in Table S3. Multiple sequence alignments were carried out using GeneDoc software (http://www.nrbsc.org/gfx/genedoc/). Gene structures were shown with Exon–Intron Graphic Maker (http://wormweb.org/exonintron) with default parameters.

2.6. Construction of a phylogenetic tree

A phylogenetic tree was constructed using the neighbor-joining method in MEGA 6.0 [33]. The multiple protein sequences were aligned with ClustalX [34]. The phylogenetic relationships were tested using 1000 bootstrap replications. The DNMT sequences of *Plutella xylostella, Linepithema humile*, and *Pogonomyrmex barbatus* were downloaded from the Diamondback moth Genome Database and Ant Genomes Portal [35]. Data for the rice stripped borer, *Chilo suppressalis*, were downloaded from ChiloDB [36]. The DNMT sequences of other species were retrieved from NCBI. All accession numbers are listed in Table S2.

2.7. RNA interference

The double-strand RNAs (dsRNAs) *dsDnmt1* (447 bp) and *dsDnmt3* (124 bp) were designed according to the full-length

sequences of *Nlu-Dnmt1* and *Nlu-Dnmt3*. T7 polymerase promoter was fused with GSPs at the 5′-end. DsRNA was synthesized using the T7 RiboMax Express RNAi System (Promega) according to the manufacturer's instructions. The concentration of dsRNA was quantified with a NanoDrop 2000 (ThermoFisher, Wilmington, DE, USA). The quality and size of the dsRNAs were verified by 1% agarose gel electrophoresis. The DEPC-treated H_2O and the dsRNA of green fluorescent protein (GFP) were used as the negative control.

Female adults on the fourth day after eclosion were collected from the culture beaker and anaesthetized with CO_2 for 30 s. They were immobilized on 2% agarose plates upside down. Two microliters solution containing 300 ng dsRNA (either ds*Dnmt1*, ds*Dnmt3*, ds*GFP*, or H_2O), were injected into the thorax between the mesocoxa and hind coxa using a nanoliter injector (World Precision Instruments, Sarasota, Florida, USA). One hundred adults were used for each experiment, and all experiments were done in triplicate. After injection, plentiful fresh rice seedlings were provided and renewed every 3 days. Each beaker contained the same number of RNAi-treated female adults and untreated male adults. To examine RNAi efficiency, total RNA was extracted from five individuals that were collected at 24, 48, and 72 h after injection. Phenotype changes were observed, and the number of offspring was calculated after each RNAi treatment.

3. Results and discussion

3.1. BPH has a complete DNA methylation toolkit

Bioinformatics analysis of a BPH transcriptome found fragments of *Dnmt1*, *Dnmt2*, and *Dnmt3*, indicating that BPH may have a complete DNA methylation toolkit. This interesting finding prompted us to amplify their full-length transcripts. We focused on *Nlu-Dnmt1* and *Nlu-Dnmt3* because they are known to methylate genomic DNA. Using RACE, we obtained the complete cDNA sequences of *Nlu-Dnmt1* and *Nlu-Dnmt3*. The full-length *Dnmt1* cDNA

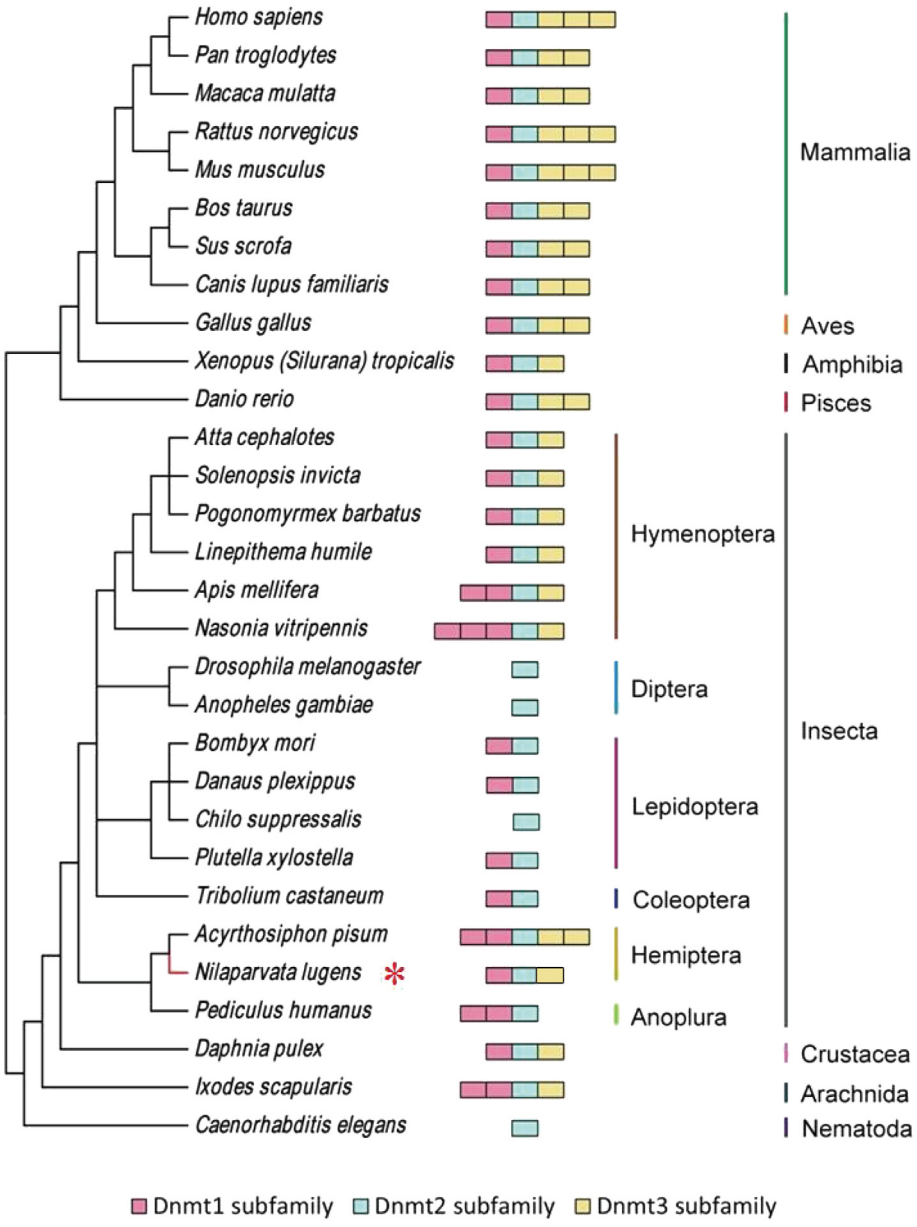

Fig. 1. The DNA methylation toolkit in diverse taxa. Left: phylogenetic tree of 30 species. Right: presence of DNA methyltransferases.

is 5729 bp with a 4563-bp open reading frame (ORF), a 119-bp 5′-untranslated region (UTR), and a 1047-bp 3′-UTR. The ORF encodes 1520 amino acids with a predicted molecular weight of 171.6 kDa and a pI of 6.18 (Fig. S1). The full-length *Dnmt3* cDNA is 2040 bp, comprising 316 bp of 5′-UTR, 1230 bp of ORF, and 494 bp of 3′-UTR. The ORF encodes 409 amino acids with a molecular weight of 46 kDa and pI of 8.62 (Fig. S2). Compared with other insect DNMT3s, the protein encoded by *Nlu-Dnmt3* lacks a short fragment at the 5′-end. We repeated the 5′-RACE experiments and obtained the same product, indicating that this transcript is authentic. The full-length sequences of *Nlu-Dnmt1* and *Nlu-Dnmt3* were submitted to GenBank with accession numbers KF294265 and KP890855, respectively (Table S2).

The common ancestor of vertebrates and invertebrates likely had all three *Dnmt* genes, but there have been various duplications and deletions within the invertebrates. All vertebrate species harbor the full toolkit for DNA methylation with distinguishing duplications of *Dnmt3*. Three *Dnmt3* copies (*Dnmt3a*, *Dnmt3b*, and *Dnmt3L*) are found in humans and mice, but only *Dnmt3a* and *Dnmt3b* exist in other species. Amphibians have only one copy of *Dnmt3*. Both *Dnmt1* and *Dnmt2* exist as a single copy (Fig. 1). In invertebrates, the distribution of *Dnmt* genes varies among organisms. Crustaceans, arachnids, and hymenopterans possess a complete DNA methylation toolkit with different duplications of *Dnmt1*. *Dnmt3* was lost in Lepidoptera, Coleoptera, and Anoplura. Both *Dnmt1* and *Dnmt3* were lost in the lepidopteran *C. suppressalis*. Only *Dnmt2* is found in flies, mosquitos and nematodes. Here, we found one copy *Dnmt1* and *Dnmt3* in the BPH genome and amplified their full-length transcripts. Another hemipteran, pea aphid, has two copies of *Dnmt1* and *Dnmt3* in the genome. These results suggested that Hemipteran could have a complete DNA methylation toolkit, providing new insights into the importance of DNA methylation in insects.

3.2. *Nlu-Dnmt1 and Nlu-Dnmt3 have conserved motifs but few exons*

The gene structures of *Dnmt1* and *Dnmt3* were determined by comparing putative cDNA and genomic sequences. The length of *Nlu-Dnmt1* is 29,932 bp, much longer than in other insects. Compared with their vertebrate counterparts, insect *Dnmt1* genes have fewer but longer exons; there are 39–41 exons in mammals, 33 in fish and amphibians, and only 8–22 exons in insects (Fig. 2A, Table S6). Pfam Domains analysis indicated that *Nlu-Dnmt1* consists of a DMAP binding domain, a zf-CXXC domain, two BAH domains, and a DNA methylase domain (Fig. S3), the same as in *Homo sapiens*, *Mus musculus*, *Xenopus tropicalis*, and *Danio rerio*, but there are slight differences from other insects. The DMAP-binding domain was not found in other insects. The DNA methylase catalytic domain, the key domain of *Dnmt* genes, shared >70% sequence similarity among all studied species, with an extremely conserved active site and six highly conserved motifs (Fig. S4).

Nlu-Dnmt3 is 8806 bp long. Insect *Dnmt3* genes also have fewer and longer exons than in vertebrates. Interestingly, *D. rerio Dnmt3* has a similar gene structure to insects (Fig. 2B, Table S7). All DNMT3 have conserved domains of linker, DNA-binding loop L1, and DNA binding loop L2 (Fig. S5, Fig. S6). This evidence suggests that DNMTs are highly conserved among diverse taxa, suggesting that DNA methylation is an ancient regulatory mechanism.

3.3. *Phylogenetic analysis of insect Dnmt genes*

Phylogenetic trees were constructed based on the amino acid sequences of *Dnmt1* and *Dnmt3* from 20 species including Mammalia, Osteichthyes, Amphibia, Insecta, and Crustacea. The DNMT

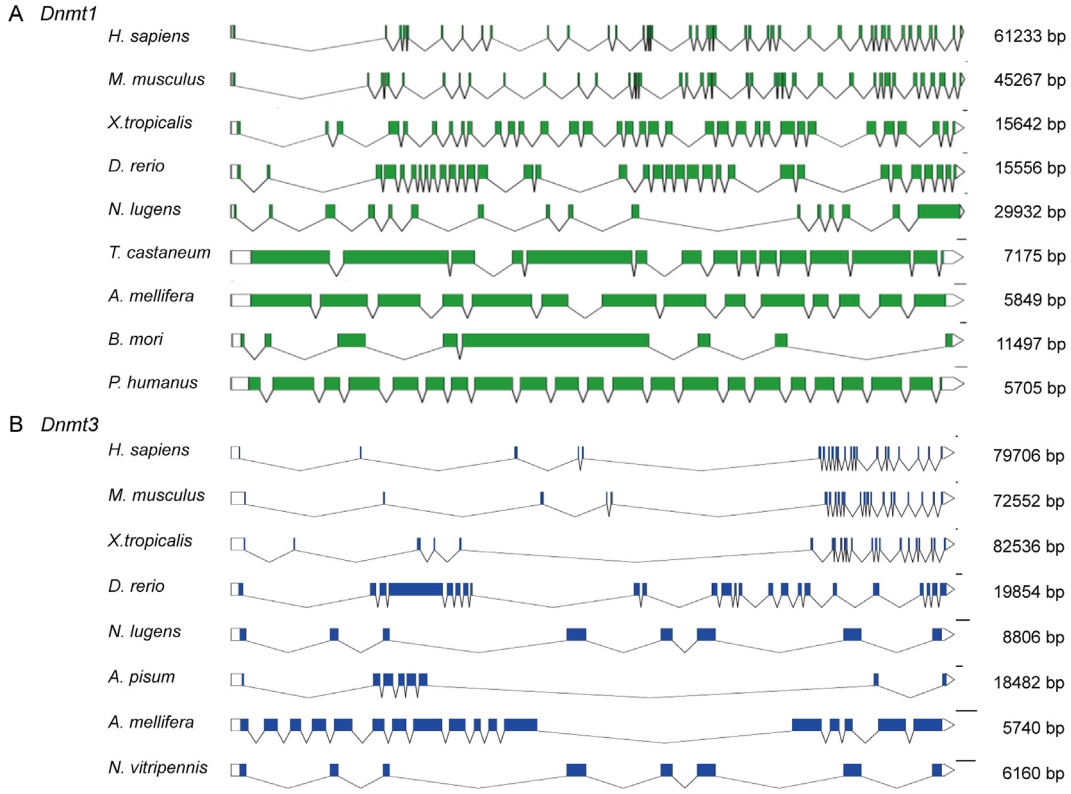

Fig. 2. Gene structures of *Nlu-Dnmt1* and *Nlu-Dnmt3*. (A) *Dnmt1* gene structures in nine species. The lengths of each exon and intron are given in Table S4. (B) *Dnmt3* gene structures of eight species. The lengths of each exon and intron are given in Table S5. Exons: green boxes. Introns: black lines. Short black lines above each structure represent 100 bp.

sequences were aligned with ClustalX and analyzed with MEGA 6.0 (Table S8). DNMT1 and DNMT2 were grouped into two different branches (Fig. S7), suggesting different ancestors. Interestingly, Nlu-DNMT1 is evolutionally closer to the Hymenoptera sequence than to that of pea aphid (Fig. S8). In contrast, Nlu-DNMT3 and Api-DNMT3 formed a subgroup (Fig. S9). Phylogenetic analysis indicated that DNA methylation undergoes rapid evolution in BPH.

3.4. *Nlu-Dnmt1 and Nlu-Dnmt3 were highly expressed in mated female adults*

The developmental expression profile of *Nlu-Dnmt1* and *Nlu-Dnmt3* in different developmental stages were examined using qPCR. *Nlu-Dnmt1* was highly expressed in the both gravid brachypterous and macropterous female adults (Fig. 3). However, only gravid female adults showed high expression of *Nlu-Dnmt1*. The newly emerged female insects (within 24 h of molting) had a low-level expression of *Nlu-Dnmt1* (t-test, $P < 0.05$). The abundance of the *Nlu-Dnmt1* transcript was also low in both larvae and male adults (Fig. 3A, t-test, $P < 0.01$). The *Nlu-Dnmt3* was highly expressed in gravid brachypterous female adults (t-test, $P < 0.01$). Unlike *Nlu-Dnmt1*, *Nlu-Dnmt3* showed only moderate expression in gravid macropterous female adults. However, like *Nlu-Dnmt1*, *Nlu-Dnmt3* was weakly expressed in larvae, male adults, and virgin female adults (Fig. 3B). In general, *Nlu-Dnmt1* and *Nlu-Dnmt3* were highly expressed in mated and gravid female adults but weakly in virgin female adults, suggesting that mating induce their expressions. High expression in female adults compared with male adults indicated that DNA methylation might function in BPH reproduction.

3.5. *Silencing of Nlu-Dnmt1 and Nlu-Dnmt3 led to low fecundity*

We knocked down the expression of *Nlu-Dnmt1* and *Nlu-Dnmt3* in female adults by injecting dsRNA designed from the full-length transcript sequences. Female adults on the fourth day after eclosion were selected for RNAi experiments, because *Nlu-Dnmt1* and *Nlu-Dnmt3* were highly expressed in gravid female adults. Compared with the control levels in the dsGFP group, the mRNA levels of *Nlu-Dnmt1* and *Nlu-Dnmt3* were reduced to 49% at 24 h, to 38% at 48 h, and to 50% at 72 h after RNAi treatment. The *Nlu-Dnmt3* mRNA levels were reduced to 41% at 24 h, to 42% at 48 h, and to 26% at 72 h after dsRNA injection (Fig. 4A). These results indicated that both *Nlu-Dnmt1* and *Nlu-Dnmt3* were successfully inhibited (t-test, $P < 0.05$).

After injecting the dsRNA, neither brachypterous nor macropterous female adults showed any apparent phenotype change in the F1 generation or in their offspring. Interestingly, we found that silencing of *Nlu-Dnmt1* or *Nlu-Dnmt3* in brachypterous female adults led to fewer offspring (Fig. 4B). In the control group (treated with dsGFP), the average was 327 ± 35. However, the dsDnmt1 group had significantly fewer offspring, 51 ± 17 (t-test, $P < 0.01$), and the number was 218 ± 28 in the dsDnmt3 group (t-test, $P < 0.05$). These results suggested that *Dnmt*, especially *Nlu-Dnmt1*, regulates female fecundity in BPH. DNA methylation has been reported to be associated with healthy embryonic development [17]. Target mutation of murine *Dnmt* in stem cells was embryonic lethal [18]. When women undergo ovarian stimulation for *in vitro* fertilization, DNA methylation changes in whole blood are highly associated with exposure to environmental contaminants [37]. In BPH, the female adults transfer the DNA methylation patterns to their

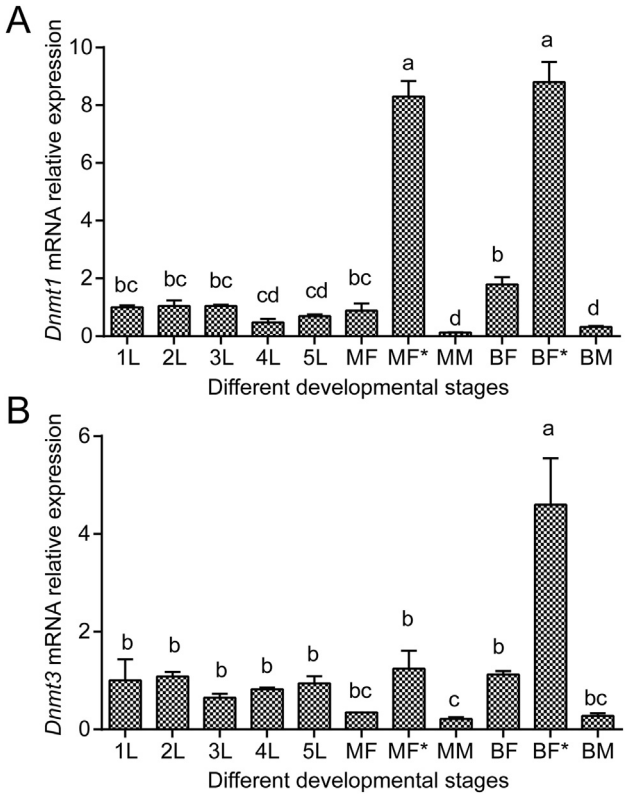

Fig. 3. Gene expressions of *Nlu-Dnmt1* and *Nlu-Dnmt3* in different developmental stages of rice planthopper. (A) Expression of *Nlu-Dnmt1*. *Nlu-Dnmt1* was highly expressed in gravid brachypterous and macropterous female adults. (B) Expression of *Nlu-Dnmt3*. *Nlu-Dnmt3* was highly expressed in gravid brachypterous female adults.

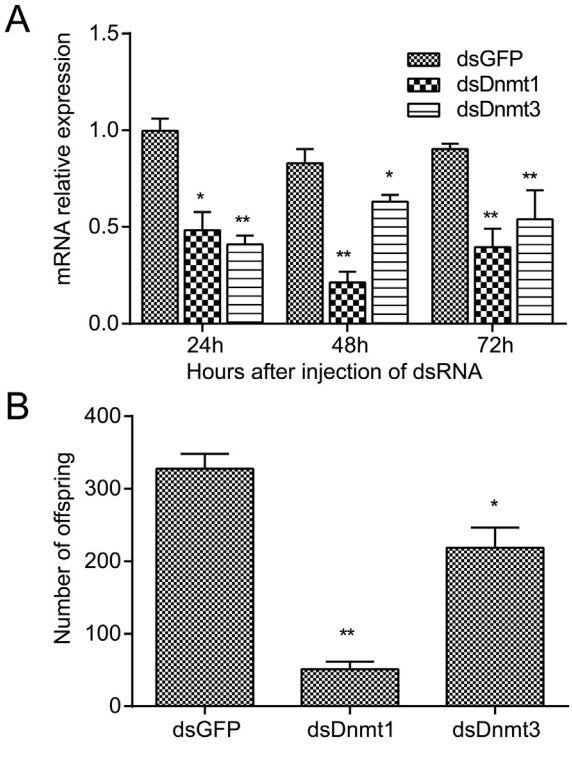

Fig. 4. Silencing of *Nlu-Dnmt1* and *Nlu-Dnmt3* resulted in fewer offspring. Brachypterous female adults on the fourth day after eclosion were injected with dsDnmt1, dsGFP, and DEPC-treated H_2O. (A) *Nlu-Dnmt1* and *Nlu-Dnmt3* were successfully knocked down by RNA interference. (B) Numbers of offspring in the RNAi-treated group were significantly reduced.

offspring via the maintenance DNMT *Nlu-Dnmt1*. The environmental stimuli and stresses were sensed by female adults and then transferred to the offspring by the *de novo* DNMT *Nlu-Dnmt3*. Therefore, silencing *Nlu-Dnmt1* and *Nlu-Dnmt3* resulted in low fecundity in BPH, suggesting that regulating DNA methylation could be an alternative pest control method.

In summary, we presented evidence that a complete DNA methylation toolkit exists in BPH, a destructive pest in rice production. Both *Nlu-Dnmt1* and *Nlu-Dnmt3* were amplified with full-length transcripts. Two genes were highly expressed in the mated and gravid female adults. Knockdown of *Nlu-Dnmt1* or *Nlu-Dnmt3* led to fewer offspring, suggesting that DNA methylation participates in reproductive regulation in BPH.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.114.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.114.

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