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ARTICLE

Characterization of *NlHox3*, an essential gene for embryonic development in *Nilaparvata lugens*

Ze-Wei Ren[1,2] | **Ji-Chong Zhuo**[2] | **Chuan-Xi Zhang**[2] | **Dun Wang**[1]

[1]College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China

[2]Institute of Insect Science, Zhejiang University, Hangzhou, Zhejiang, China

Correspondence
Dun Wang, Institute of Entomology, Northwest A&F University, Yangling, Shaanxi, 712100, P.R. China.
Email: wanghande@yahoo.com

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Abstract

Hox genes encode transcriptional regulatory proteins that control axial patterning in all bilaterians. The brown planthopper (BPH), *Nilaparvata lugens* (Hemiptera: Delphacidae), is a destructive insect pest of rice plants in Asian countries. During analysis of the *N. lugens* transcriptome, we identified a *Hox3*-like gene (*NlHox3*) that was highly and specifically expressed in the embryonic stage. We performed functional analysis on the gene to identify its roles in embryonic development and its potential use as a target in RNA interference (RNAi) based pest control. The sequence analysis showed that *NlHox3* was homologous to the *Hox3* gene and was most closely related with *zen* of *Drosophila*. There were no significant differences in oviposition between the treated and control females after injecting double-stranded RNA of *NlHox3* (ds*NlHox3*) into newly emerged female adult BPHs; however, there was a significant difference in the hatchability of those eggs laid, which no egg from the treated group hatched normally. Injecting female adult BPHs with ds*NlHox3* led to necrosis of these offspring embryos, with eye reversal and undeveloped organs, suggesting that *NlHox3* was an essential gene for embryonic development and might be a potential target for RNAi-based control of this insect pest.

KEYWORDS
embryonic development, *Hox* genes, *Nilaparvata lugens*, reversal, *zen*

1 | INTRODUCTION

Homeotic genes were first characterized in *Drosophila*, and play a major role in the expression and function of the arthropod (Pick, 2016). Homeotic genes are expressed in collinear patterns along the anteroposterior axis of the embryo and play vital roles in embryonic development (Lewis, 1978). In collinearity among homeotic genes, their linear organization along the chromosome correlates with the region of function along the anteroposterior axis of the animal (Garcia-Fernàndez, 2005). Homeodomain-containing proteins function as sequence-specific DNA-binding transcription factors that regulate development by activating or repressing the expression of downstream target genes (Pick, 2016). They are recognized mostly for their role in determining segment identity (Mallo, Wellik, & Deschamps, 2010).

Hox family members are different in mammals and insects though they were found to be highly conserved (Heffer & Pick, 2013). Mammals have four *HOX* clusters: *HOXA*, *HOXB*, *HOXC*, and *HOXD*. Therefore, at corresponding positions within the four clusters are genes with specific sequence similarity, that is, paralogous genes. The 39 mammalian *HOX* gene family members are thus subdivided into 13 paralogous groups (*HOX1–HOX13*). Most insects have only one *Hox* cluster whose gene composition is believed to be similar to the ancestral *Hox* complex in Urbilateria (Mallo et al., 2010). There are about 11 *Hox* genes in this cluster and they are distributed in 10 paralogous groups corresponding to mammalian systems. Not all insects have complete sequences for all 10 paralogous groups found in *Drosophila*; most insects are represented by data for some genes only (Cook, Smith, Telford, Bastianello, & Akam, 2001).

The functions of insect *Hox* genes have been studied via traditional mutant analysis in *Tribolium* (Beeman, Stuart, Brown, & Denell, 1993; Beeman, Stuart, Haas, & Denell, 1989; Zheng, Khoo, Fambrough, Garza, & Booker, 1999), *Manduca* (Kokubo, Ueno, Amanai, & Suzuki, 1997), and *Bombyx* (Hughes & Kaufman, 2000) and by RNA interference (RNAi) in *Tribolium* and *Oncopeltus* (Rogers & Kaufman, 1997). Mutants have also been found in the mosquito *Aedes* and the wasp *Nasonia*, and their functions are very *Hox*-like in determining segment identity (Quinn & Craig, 1971; Werren & Minnot, 1999).

However, *Hox* genes have higher evolutionary dynamics than other developmental genes (Casillas, Negre, Barbadilla, & Ruiz, 2006; Löhr, Yussa, & Pick, 2001). In winged insects, *Hox3* has lost its homeotic function, that is, the ability to transform the characteristics of one body part into those of another body part (Akam et al., 1994; Stauber, Jäckle, & Schmidt-Ott, 1999), and its expression domains are no longer arranged along the anteroposterior axis of the embryo. *Hox3* gained a novel extraembryonic function and underwent two consecutive duplications that gave rise to *bicoid* (*bcd*) and *zerknüllt* (*zen*) (Barker, Demuth, & Wade, 2005; Stauber, Prell, & Schmidt-Ott, 2002). In *Drosophila*, *zen* is zygotically expressed. *Zen* is not required for differentiation of the dorsoventral pattern and does not affect segmentation directly (Rushilow, Doyle, Hoey, & Levine, 1987). Later it was found that in *Tribolium*, the *zen* was expressed in extraembryonic tissues rather than in the embryo (Falciani et al., 1996). But Hox family members have not been found or studied in brown planthopper (BHP).

The BPH, *Nilaparvata lugens*, is the most destructive insect pest of rice. It has high fecundity and its populations can increase quickly and spread over long distances. The destructive influence of BPH on rice is strongly related to its numbers. Thus, regular and normal embryonic development is vital to BPH population expansion. In our analysis of the *N. lugens* transcriptome, we found a *Hox3*-like gene (*NlHox3*) that was highly expressed in the embryonic stage, but had nearly no expression during the other stages. We performed a functional analysis of this gene to determine its function and potential as a target for RNAi-based pest control.

2 | MATERIALS AND METHODS

2.1 | Insects

The BPH strain used in this study was collected from local rice fields at Zhejiang University, Hangzhou, Zhejiang, China. The insects were reared at 25 ± 1°C on fresh rice seedlings in 60–70% relative humidity under a 16-h light/8-h dark photoperiod.

2.2 | Gene sequence analyses and phylogeny

The sequence found in BPH transcriptome (MG641751) was used as a query to search for orthologs in the National Center for Biotechnology Information (NCBI) databases using the blastx algorithm (http://www.ncb.nlm.nih.gov/). Moreover, to investigate *NlHox3* and the whole *Hox* family in BPH further, we searched all genes containing the *Hox* domain from the BPH transcriptome and genome for further analysis. We also downloaded all *Hox* family gene sequences of *Drosophila melanogaster*, *Oncopeltus fasciatus*, *Anopheles gambiae*, *Nasonia vitripennis*, and *Tribolium castaneum* from NCBI. The *Hox* genes of these five insects have all been reported. All conserved domains of the above

sequences were found using NCBI blastx. The sequences were downloaded and aligned in multiple sequence alignment using MUSCLE. Phylogenetic analyses were carried out using MEGA 7.0 along with the maximum likelihood algorithm of the Jones–Thornton–Taylor (JTT) model and the gamma-distributed (G) model for amino acid substitution. Phylogenetic relationships were tested using the bootstrap method with 1,000 replications (Price, Dehal, & Arkin, 2009). Based on this phylogenetic analysis, we selected BPH genes that shared more than 50% identity with the *Hox* genes of the five insects. We then performed a multiple sequence alignment of these selected sequences and *NlHox3* using Clustal X.

2.3 | Developmental expression profile of *NlHox3*

To investigate the *NlHox3* developmental expression pattern, a sample set was collected from eggs at different stages laid by wild-type female BPH, and also from every nymphal instar as well as female and male adults. Both nymphs and adults were collected randomly. We dissected eggs from fresh rice seedlings at 24, 48, 72, 96, 120, and 144 h after they had been laid. To collect the eggs at the exact time point, we placed 20 adult female BPHs that had been mated with 10 adult male BPHs in a long tube, and placed five fresh rice seedlings in the tube with wet cotton to ensure the water supply. The tube was replaced with a new tube every 2 h. Conditions of every tube were identical, and all tubes were placed under the conditions described above. We then dissected the eggs collected at the stated time points. Each sample contained 150 dissected eggs. Total RNA was isolated using a Trizol Total RNA Isolation Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The First-strand cDNA Synthesis Kit (TIANGEN, Beijing, China) was used to synthesize first-strand complementary DNA (cDNA) according to the manufacturer's protocol.

To reveal the developmental expression pattern of the *NlHox3* gene, a real-time reverse transcription–polymerase chain reaction (real-time RT-PCR or qPCR) was conducted using gene-specific primer pairs designed using Primer Premier 6 software (Premier Biosoft International, Silicon Valley, USA). The cDNA was prepared as described above. The qPCR reactions were performed as described by Ye et al. (2017). The qPCR was repeated three times for each gene. Each replication was performed with independent RNA sample preparations and consisted of three technical replicates. The relative quantitative method [$2^{-\Delta\Delta Ct}$: 2^(- (Ct_{target} - Ct_{Nl18S})$_{time\ x}$ – (Ct_{target} -Ct_{Nl18S})$_{time\ 0}$)] was used, where time x is any time point, time 0 represents the 1 × expression of the target gene normalized to Nl18S and Ct is the cycle threshold used to evaluate the quantitative variation (Livak & Schmittgen, 2001). The sequences of the specific primer sets are as follows: *NlHox*3-sense 5′-GCCTACACGAACTCACAA-3′ and antisense 5′-GCATCATCTCACTCTG–GAA-3′; 18S rRNA gene sense 5′-CGCTACTACCGATTGAA-3′ and antisense 5′-GGAAACCTTGTTACGACTT-3′.

2.4 | Double-stranded RNA preparation and injection

The *NlHox3* sequence was cloned into PMD-19T vectors (Takara, Dalian, China). PCR-generated DNA templates were used to synthesize double-stranded RNA (dsRNA) containing T7 promoter sequences at each end. The primers used to synthesize the *NlHox3* dsRNA were as follows: 5′-TAATACGACTCACTATAGGGAGACTATGTGCCCGAGGAAGC-3′ (sense) and 5′-TAATACGACTCACTATAGGGAGAGGTGTAGCGGATGAAAGC-3′ (antisense).

We used the MEGAscript T7 transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions to produce the dsRNA. Then, we quantified the dsRNA spectrophotometrically using a NanoDrop unit (Thermo Scientific, Wilmington, DE, USA). The quality and size of the dsRNA products were verified using 1% agarose gel electrophoresis.

The dsRNA injection was performed as described by Xu et al. (2015). We injected female adult BPHs within 2 h after eclosion. About 25 nl dsRNA (10 $\mu g/\mu l$) was injected into the mesothorax of each insect using FemtoJet (Eppendorf-Nethler-Hinz, Hamburg, Germany). We repeated this experiment more than three times. Subsequently, we separated the female and male adult BPHs into different tubes containing fresh rice seedlings for the following experiments.

2.5 | Oviposition and hatching rate experiments

Three days after being injected with ds*NlHox3*, a female adult BPH was mated with a wild-type male adult BPH; the control was a female adult BPH injected with green fluorescent protein (ds*GFP*) mated with a wild-type male adult BPH.

The experiment was also carried out in a long tube with rice seedlings and wet cotton. Only three rice seedlings were needed per tube in the oviposition experiment, and we replaced the tubes every 24 h five times; five repetitions were performed. Then, we counted the eggs and photographed the developmental stages of the eggs. For the hatching rate experiment, each tube contained 20 rice seedlings, and we replaced the tubes every 5 days two times; 10 repetitions were performed. After 8 days, the hatched nymphs were counted.

2.6 | Fluorescence staining of BPH eggs

After the female adult BPHs injected with ds*GFP* or ds*NlHox3* had laid their eggs, we dissected the eggs at 120 h. The eggs were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at 4°C. Then, samples were blocked with Jung Tissue Freezing Medium (Leica Microsystems, Wetzlar, Germany) at −20°C; the blocks were cut into 7-μm sections using a Leica CM1900 cryotome (Leica Microsystems, Nussloch, Germany) at −20°C. Steps providing frozen sections were followed as described by Xue et al. (2013). The samples were then stained with phalloidin for 40 min, followed by washing in PBST three times at 5 min per wash. Finally, diamidinophenylindole (DAPI) was added for 2 min and the samples were washed with PBST as above. Fluorescence images were acquired using a Zeiss LSM 780 confocal microscope (Carl Zeiss MicroImaging, Göttingen, Germany).

2.7 | Statistical analysis

All data were analyzed using SPSS 20.0. As for the oviposition and hatching rate, differences between ds*GFP* and ds*NlHox3* were tested by Student's *t*-test. All graphs were mapped by Origin 8.6.0 (OriginLab, Northhampton, MA).

3 | RESULTS

3.1 | Sequence analysis and phylogeny

When we used the *NlHox3* nucleotide sequence as the query to search GenBank using blastn and blastx, respectively, we found that *Cimex lectularius* was the only insect that matched both sequences, with 72 and 64% identity, respectively. Other matched sequences were found in noninsect species in the result of blastx, including fish, mammals, and birds, where *Callorhinchus milii*, *Monodelphis domestica*, and *Falco cherruq* shared 71, 70, and 69% similarity with *NlHox3*, respectively. At the same time, all matched sequences belonged to *HOXD3* and *HOXB3*, two members of the paralogous *HOX3* (including *HOXA3*, *HOXB3*, *HOXC3*, *HOXD3*) of mammals. *NlHox3* contained a conserved *Hox* domain and shared about 70% similarity with class 3 *Hox* genes, suggesting it was a member of the homeobox family and most likely to be *Hox3*.

In other insects, Hox3 is only located on one cluster in the genome with only one or two members. To know more about NlHox3, we analyzed the Hox gene family in the BPH genome and transcriptome. In total, we identified 58 genes whose protein contained a Hox domain in BPH. These genes were in 51 different scaffolds in the BPH genome assembly. Among the 51 scaffolds, one contained three different genes and four contained two different genes, with the remaining scaffolds containing only one Hox gene. Phylogenetic analysis using the Hox genes from *N. lugens*, *D. melanogaster*, *O. fasciatus*, *A. gambiae*, *N. vitripennis*, and *T. castaneum* showed that the Hox genes are highly diverse. NlHox3 was most closely related to the zen gene of other insects, although they were not wholly identical (Figure 1). We also observed that five BPH genes shared more than 50% identity in the Hox domain with the reported Hox genes of the five insects (Table 1). The multiple alignment analysis of these domains also revealed that they are closely related. Among the genes, NlHox3 shared the closest relationship with the zen gene, and most closely to zen of *O. fasciatus* (Figure 2). These results all suggest that NlHox3 is a member of the Hox3 subfamily and that it is most likely a zen homolog.

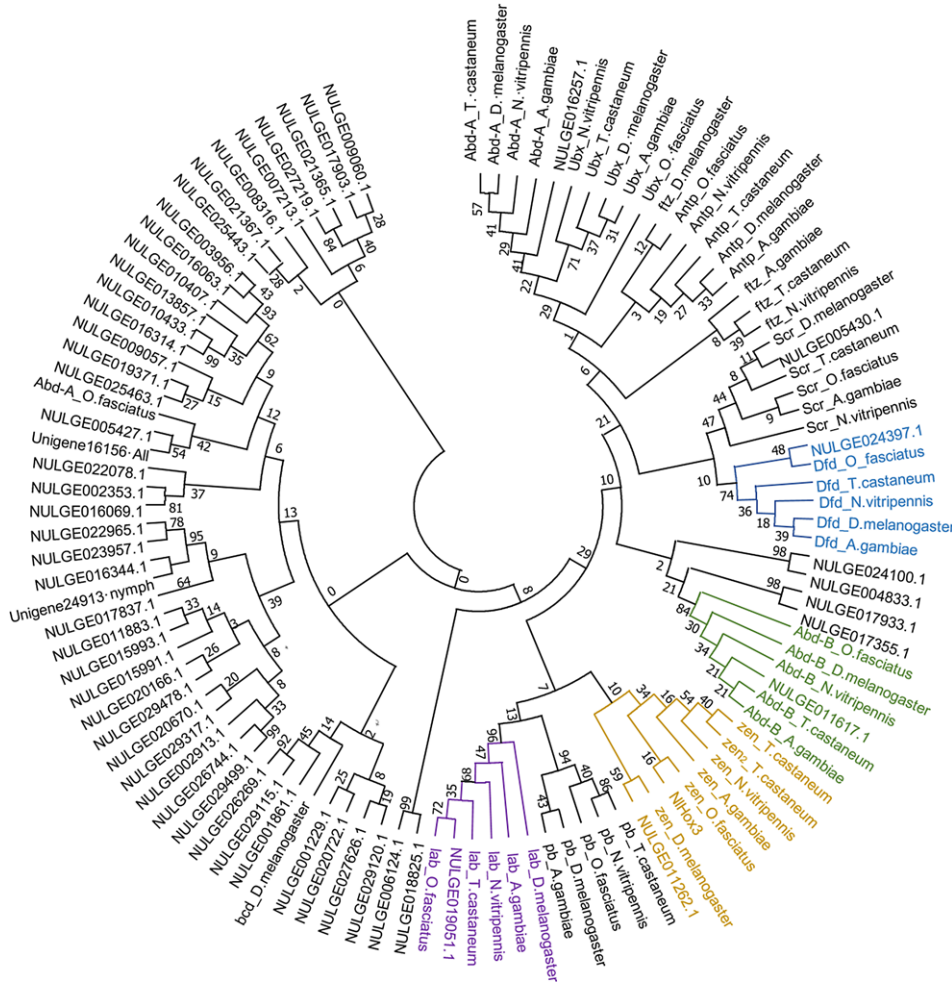

FIGURE 1 Phylogenetic tree of hox family genes from *Nilaparvata lugens*, *Drosophila melanogaster*, *Oncopeltus fasciatus*, *Anopheles gambiae*, *Nasonia vitripennis*, and *Tribolium castaneum*

Note: The tree was constructed using the MEGA 7.0 software with maximum likelihood phylogeny using the Jones–Thornton–Taylor (JTT) and distributed (G) models. This phylogenetic relationship was tested by the bootstrap method with 1000 replications; bootstrap values (%) are shown in the cladogram. Abd-B, abdominal-A; Dfd, deformed; lab, labial; zen, zerknüllt; Abd-A, abdominal-A; Scr, sex combs reduced; bcd, bicoid; pb, proboscipedia; Ubx, ultrabithorax; Antp, antennapedia.

TABLE 1 Five BPH *Hox* genes with reported homologs in other insects

	Homologous genes reported in other insects				
Hox gene in BPH	***Drosophila melanogaster***	***Anopheles gambiae***	***Nasonia vitripennis***	***Tribolium castaneum***	***Oncopeltus fasciatus***
NULGE 011617.1(Nl Abd-B)	Abd-B	Abd-B	Abd-B	Abd-B	Abd-B
NULGE 019051.1(Nl lab)	Lab	Lab	Lab	Lab	Lab
NULGE024397.1(Nl Dfd)	Dfd	Dfd	Dfd	Dfd	Dfd
NULGE011262.1 (Nlzen1)	Zen				
NlHox3 (Nlzen2)	zen				zen

Abd-B, abdominal-A; Dfd, deformed; lab, labial; zen, zerknüllt.

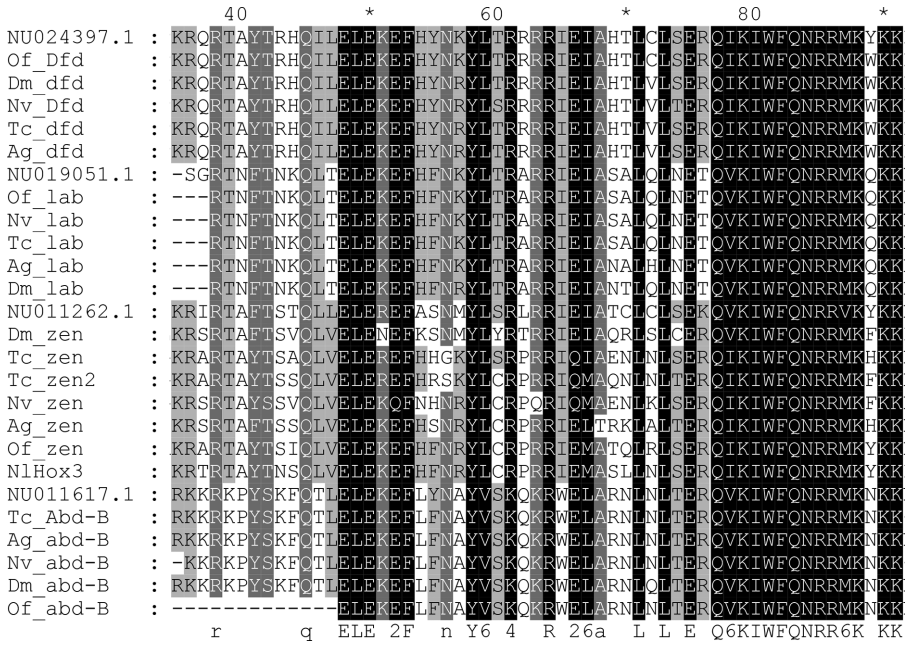

FIGURE 2 Multiple hox domain sequence alignment of five hox genes from BPH and their homologs that have already been reported in five other insects

Notes. These alignments were performed using the CLUSTALX program. Ag, *Anopheles gambiae*; Dm, *Drosophila melanogaster*; Nv, *Nasonia vitripennis*; Of, *Oncopeltus fasciatus*; Tc, *Tribolium castaneum*; NU, NULGE.

3.2 | Developmental expression of *NlHox3*

In the BPHs, we analyzed the developmental expression of *NlHox3* on different days in the BPH embryonic stage and in every nymphal instar and female and male adult using qRT-PCR. The qRT-PCR showed that *NlHox3* transcripts could be detected in all embryonic stages, and expression was highest at 72 h. However, almost no expression was detected in the nymph and adult stages (Figure 3), suggesting that *NlHox3* is specifically expressed in the embryonic stage and might mainly play roles in the early and intermediate stages of embryonic development.

3.3 | RNAi efficiency and effects on hatching rate and fecundity

Maternal RNAi has been reported to be an efficient method to study gene function in BPH (Xu et al., 2013; Zhuo et al., 2017). To determine the function of *NlHox3*, we injected dsRNA targeting *NlHox3* into virgin female adult BPHs within 2 h after emergence. The qRT-PCR showed that *NlHox3* transcript levels in the eggs were efficiently suppressed. As indicated in Figure 4, we observed an 89.3% reduction in the expression of *NlHox3* in the 72th hour embryos laid by females injected with *dsNlHox3*.

According to data, we gained from the mating experiment, none of the eggs laid by the ds*NlHox3*-treated female BPHs hatched normally. We observed highly significant differences in hatching rates between ds*GFP* and ds*NlHox3*, suggesting that *NlHox3* was an essential gene for growth and development for embryos of *N. lugens* (Figure 5A). However, we found no significant difference in fecundity between the ds*GFP*- and ds*NlHox3*-treated female BPHs (Figure 5B).

3.4 | Embryo phenotypes after RNAi of *NlHox3*

After maternal RNAi of ds*NlHox3*, we observed the phenotypes of eggs laid by ds*NlHox3*-treated female BPHs in different stages (Figure 6). Typically, eyespots can be observed at day 5 of the embryonic stage in BPHs. At around

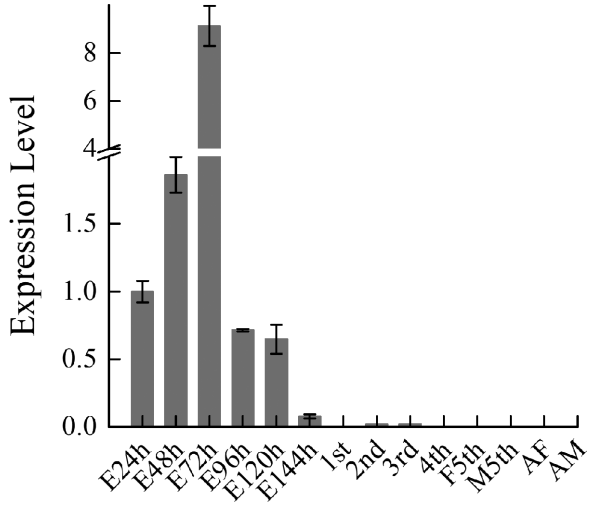

FIGURE 3 Development expression profiles of Gene NlHox3
Note: Total RNA was extracted from different stages of eggs (n =150–170), every instar and adults. The 18S ribosomal RNA gene was used as an internal control. The real-time qPCR results were analyzed by the △△Ct (cycle threshold) method. Three biological replicates based on independent RNA sample preparations were performed for each gene. Bars: ±standard errors (SE). Additional: E24h, egg for 24th hour; E48h, egg for 48th hour; E72h, egg for 72th hour; E96h, egg for 96th hour; E120h, egg for 120th hour; E144h, egg for 144th hour; 1st, larva of the first instar; 2nd, larva of the second instar; 3rd, larva of the third instar; 4th, larva of the fourth instar; F5th, female larva of the fifth instar; M5th, male larva of the fifth instar; AF, female adult; AM, male adult.

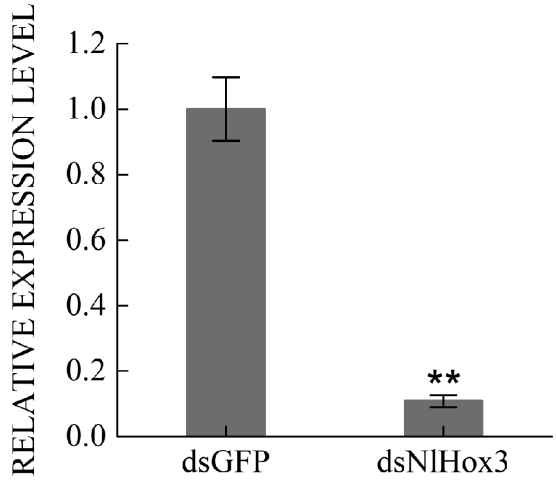

FIGURE 4 Efficiency of maternal RNAi of *NlHox3* on the gene expression level in laid eggs (n = 150–170)
Note: The eggs were dissected at the 72th hour after been laid. Three biological replicates based on independent RNA sample preparations were performed for each gene. Bars: ±standard errors (SE). The mean expression of NlHox3 in RNAi eggs at the 72th hour is shown as a fold change compared with the mean expression in the control (dsGFP), which was ascribed an arbitrary value of 1. A significant difference was observed using a t-test (*$P < 0.05$ and **$P < 0.01$).

120 h, we observed that the phenotypes of eggs laid by the ds*GFP*- and ds*NlHox3*-treated female BPHs were quite different. The red eyespots of ds*GFP* embryos were near the micropylar cap of the egg, whereas those of *dsNlHox3* embryos were reversed, that is, at the posterior of the egg. These eggs remained abnormal, did not hatch, and the embryos eventually died, whereas all eggs laid by the ds*GFP*-treated female BPHs hatched at day 8. In the frozen tissue sections, we were able to observe the embryonic development more clearly in the phalloidin and DAPI-stained

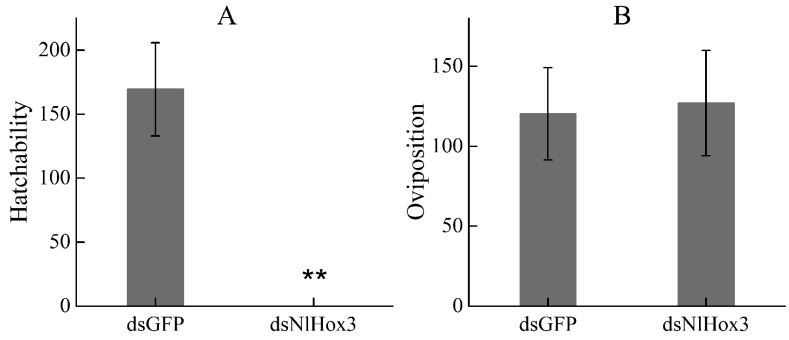

FIGURE 5 Effect of NlHox3 RNAi on hatchability and oviposition
Note: (A) The hatchability of the laid eggs after injection of ds *NlHox3* or dsGFP ($n = 5$). The dsRNA was injected into the virgin female adults. Three days later, the injected female BPH was mated with wild male BPH. Seven days later the number of first instar larva was counted every day for 8 days. (B) The fecundity (amount of eggs laid) per female after injection of ds *NlHox3* or dsGFP ($n = 5$). The tubes with fresh seedlings were changed every 24 h for five times. A significant difference was observed using a *t*-test ($^*P < 0.05$ and $^{**}P < 0.01$).

sections than under fluorescence microscopy (Figure 7). The embryos of the ds*GFP* group had developed normally at 120 h, with head, thorax, abdomen, and legs intact, whereas embryo development in the ds*NlHox3* group appeared to halt before somite and appendage formation.

4. | DISCUSSION

BPH genome and transcriptome analysis showed that BPH have a much greater quantity of *Hox* genes than do other insects, with 58 genes in total. Blastx showed that *NlHox3* was most similar to *HoxB3* and *HoxD3*, indicating that it is a member of *Hox3*. Phylogenetic analysis and multiple alignment analysis show that *NlHox3* was more similar to *zen*, a rapidly evolving *Hox3* gene in insects. This is consistent with previous studies showing that *zen* is derived from *hox3* due to the duplication and evolution of *hox3* (Rushlow, Doyle, Hoey, & Levine, 1987). The *zen* allele is required in embryogenesis. The mutant phenotype is characterized by the absence of the optic lobe, defects in involution of the head segments, and in some cases, failure of germ band elongation (Wakimoto, Turner, & Kaufman, 1984).

In our study, knocking down *NlHox3* reversed the eyespot position of the BPH embryo. Other related studies report embryo eversion. In *O. fasciatus*, RNAi knockdown of *zen* resulted in inside-out embryos, but the embryos were well developed and the organs were complete despite the eversion (Panfilio, Liu, Akam, & Kaufman, 2006). The same phenotypes have been observed in *Tribolium*, where everted embryos and head and abdominal segments were observed (Van Der Zee, Berns, & Roth, 2005). However, when *NlHox3* was knocked down in BPH, the development of all segments and most organs, including the eyes, was abnormal; this is quite different from *O. fasciatus* and *Tribolium*, suggesting that the functions of *zen* may have interspecific differences.

Parental RNAi has been widely used in many insects including coleopteran, orthopteran, lepidopteran, and hemipteran species (Bettencourt, Terenius, & Faye, 2002; Bucher, Scholten, & Klingler, 2002; He et al., 2006). There were some specific studies into the role of parental RNAi in hemipteran insects. It was reported that parental RNAi of *Dctra-2* has a great influence on female fecundity and egg hatchability in *Diaphorina citri* (Yu & Killiny, 2017). It was also found that offspring wing morphs can be influenced by the maternal RNAi of the *NlTra-2* gene in a sex-specific manner in *N. lugens* (Zhuo et al., 2017). Parental RNAi can last for seven generations in *Sitobion avenae* (Abdellatef et al., 2015). This effect could contribute to the limitation of infestations caused by migrating aphids. In our study, embryonic development of the planthopper was seriously hindered by the parental RNAi of *NlHox3*. All the reports demonstrated that parental RNAi was not only an effective tool for research on functions of genes but held great potential to use an RNAi strategy for controlling some pests.

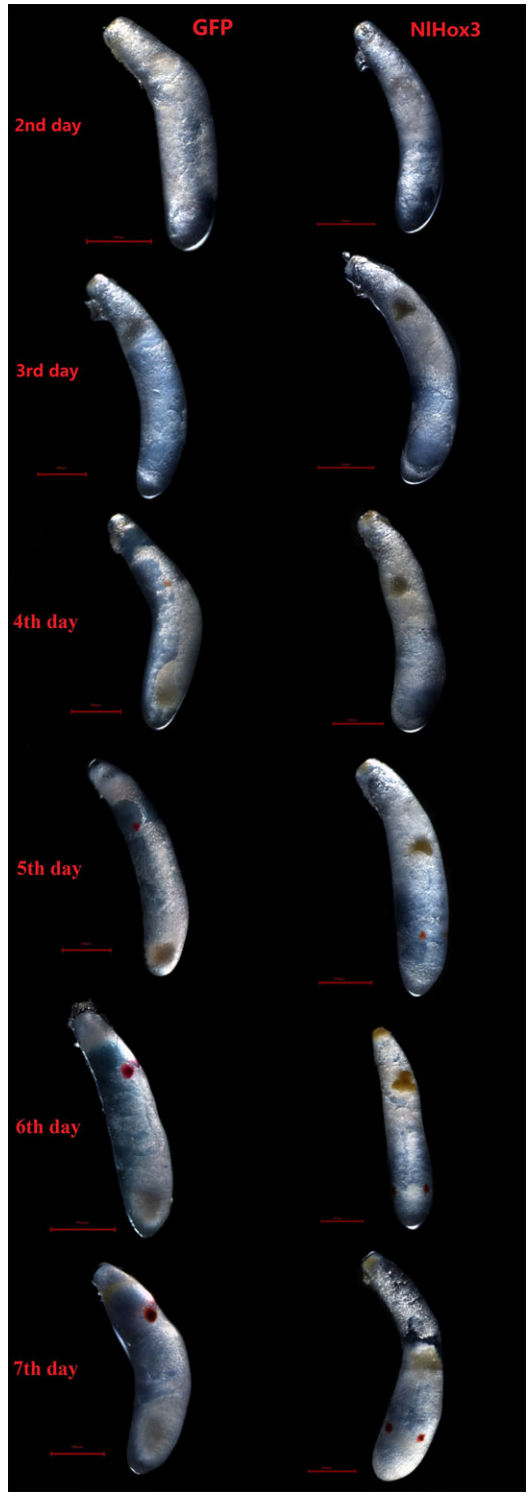

FIGURE 6 Phenotypes of eggs in different days

Note: On the left are eggs laid by virgin BPH injected with dsGFP, at the second day, third day, fourth day, fifth day, sixth day, and seventh day (the 48th, 72nd, 96th, 120th, 144th, 168th hours), respectively. In contrast, on the right are eggs showing the corresponding phenotypes under ds*NlHox3* treatment, respectively.

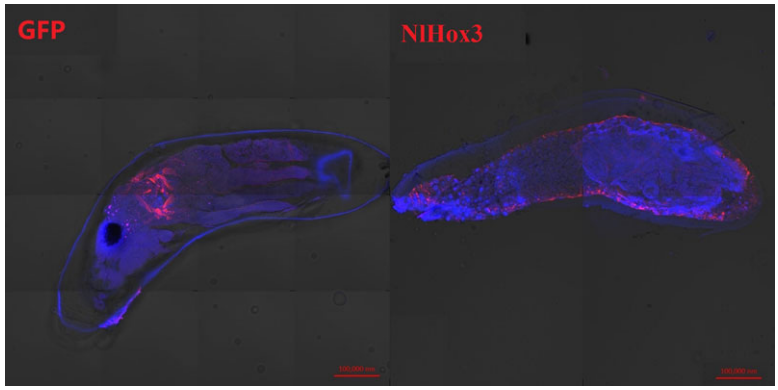

FIGURE 7 Phenotypes of *NlHox3* maternal RNAi in their embryonic development
Note: Frozen tissue sections of eggs at the fifth day (120th hour) after eggs were laid. The whole egg of dsGFP is on the left, while the frozen section of dsNLHox3 is on the right, both stained with phalloidine and DAPI. The fluorescence images were acquired using a Zeiss LSM 780 confocal microscope. Nuclear (DAPI, blue) and cell skeleton (phalloidine, red).

In conclusion, phylogenetic analysis shows that *NlHox3* belongs to the cluster *Hox3* and shares more similarities with *zen*, whether in phylogeny or function. The RNAi phenotypes in our experiment demonstrate that *NlHox3* plays an important role in BPH embryo development in both the formation of segments and eyespots. *NlHox3* knockdown can lead to embryo death. Our findings identify a potential target for RNAi-based pest management. The functions and properties of other genes containing the *Hox* domain in BPH require further study.

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CONFLICT OF INTEREST

The authors have no conflict of interests.

ORCID

Dun Wang http://orcid.org/0000-0001-5762-5560

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