Research

Identification and Function Analysis of *enolase* Gene *NlEno1* from *Nilaparvata lugens* (Stål) (Hemiptera:Delphacidae)

Wei-Xia Wang,[1] Kai-Long Li,[1] Yang Chen,[1] Feng-Xiang Lai,[1] and Qiang Fu[2]

[1]State Key Lab of Rice Biology, China National Rice Research Institute, Tiyuchang Road 359, Hangzhou, Zhejiang, 310006, China
[2]Corresponding author, e-mail: fuqiang@caas.cn

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ABSTRACT. The enolase [EC 4.2.1.11] is an essential enzyme in the glycolytic pathway catalyzing the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP). In this study, a full-length cDNA encoding α-enolase was cloned from rice brown planthopper (*Nilaparvata lugens*) and is provisionally designated as *NlEno1*. The cDNA sequence of *NlEno1* was 1,851 bp with an open reading frame (ORF) of 1,305 bp and encoding 434 amino acids. The deduced protein shares high identity of 80–87% with ENO1-like protein from Hemiptera, Diptera, and Lepidoptera speices. The *NlEno1* showed the highest mRNA expression level in hemolymph, followed by fat body, salivary gland, ovaries and egg, and showed trace mRNA levels in testis. The mRNA of *NlEno1* showed up-regulated level in virulent *N. lugens* population Mudgo, IR56 and IR42 when compared with TN1 population. Injection of double-stranded RNA (dsRNA) of *NlEno1* into the adults significantly down-regulated the *NlEno1* mRNA level along with decreased eggs and offspring. Moreover, injection of *NlEno1*-dsRNA decreased mRNA level of *Vitellogenin* (*Vg*) gene. These results showed that the *NlEno1*, as a key glycolytic enzyme, may play roles in regulation of fecundity and adaptation of *N. lugens* to resistant rice varieties.

Key Words: *Nilaparvata lugens*, enolase, RNAi, fecundity

The rice brown planthopper (*Nilaparvata lugens*) (Stål) (Hemiptera:Delphacidae) is one of the most destructive monophagous insect pest. It can cause huge yield losses every year in rice (*Oryza sativa*) grown throughout tropical, subtropical, and temperate areas in Asia (Park et al. 2008). The insects draw nutrients from the phloem of rice plants and can destroy a plant in a short period of time (Yang et al. 2002). In adult stage, *N. lugens* displays two wingforms: long (macropterous) and short (brachypterous). Macropterous adults fly long distances and invade rice-growing areas, whereas brachypterous adults are adapted for reproduction and produce numerous offspring in rice fields (Noda et al. 2008). Breeding and utilization of resistant rice varieties are the most economical and effective strategies in controlling rice planthoppers (Chen et al. 2009). Since 1969 many *N. lugens*-resistant rice varieties have been developed and cultivated, such as IR26 (with the resistance gene *Bph1*), IR36/42 (with the resistance gene *bph2*), and IR56 (with the resistance gene *Bph3*) (Horgan 2009). Up to now, 26 *N. lugens* resistance genes have been identified in wild species *O. australiensis*, *O. officinalis*, *O. glaberima*, *O. eichengiri*, *O. rufipogon*, *O. minuta* and Indian cultivars (Fujita et al. 2008; Zhang 2007). The ecological benefits associated with these resistant varieties have reduced insecticide applications and residues, and reduced mortality of beneficial arthropod populations. However, with the monoculture of *N. lugens*-resistant rice variety and chronic exposure *N. lugens* on resistant rice variety, new virulent strain of *N. lugens* (populations capable of feeding and/or reproducing on resistant rice varieties) generated (Rauscher 2001). Virulence to *Bph1* and *bph2* has been found in natural *N. lugens* populations (Tanaka and Matsumura 2000). Therefore, it is important to study the molecular mechanism of *N. lugens* adaptation to resistant rice.

Enolase (2-phospho-D-glycerate hydrolyase) [EC 4.2.1.11] is an essential enzyme catalyzing the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) (Brown and Doolittle 1997). In vertebrates, the enzyme occurs as three isoforms: α-enolase(Eno1) is found in almost all tissues, whereas β-enolase(Eno2) is predominatly found in muscle tissues, and γ-enolase (Eno3) is only found in neuron and neuroendocrine tissues. Besides the function of glycolytic enzyme, enolase showed many other important roles. It was reported that enolase functions as a cell associated stress protein involved in cellular protection during hypoxia (Aaronson et al. 1995) and as a Myc-binding protein acting in transcriptional regulation in human (Feo et al. 2000). Enolase has the ability to bind to polynucleotides and functions like a heat shock protein 48, and it plays an important role in thermal tolerance and growth control of yeast (Al-Giery and Brewer 1992; Iida and Yahara 1985). Recently, it has been reported that enolase is involved in both temperature and salt stress tolerance in algae (Ruan et al. 2009). In parasite *Fasciola hepatica*, *Onchocerca volvulus* and *Aphidius ervi*, enolase may be involved in degradation of host tissues, immune evasion, and oviposition in host (Bernal et al. 2004; Jolodara et al. 2003; Falabella et al. 2009; Nguyena et al. 2013). In parasite *Schistosoma bovis* and *Ornithodoros moubata*, the enzyme acted as a plasminogen receptor, most likely to avoid blood clot formation on their tegument (de la Torre-Escudero et al. 2010; Díaz-Martín et al. 2013). Futhermore, enolase expression is linked to the incidence of different stressors (density and infective agents) in the sea bream (Ribasa et al. 2004).It is concluded that enolase especially α-enolase is a multifunctional protein involved in sugar metabolism and many other processes in parasite and hematophagus insect. Whether enolase influences other processes or not in herbivorous insects is still unknown.

In this article, an *enolase* gene (*Eno1*) from *N. lugens* was isolated and characterized. Its expression patterns at different developmental stages, various tissues, different virulence population and wingforms were examined. With RNAi, a valuable tool for unveiling gene function in many model insects and studying the impact on down-regulated genes in Hemiptera (Li et al. 2013), we knocked down the expression of *NlEno1* and intended to provide basic information in fundamental biological phenomena and increases our understanding of the virulent mechanisms of *N. lugens* to rice.

Materials and Methods

Insects Rearing and Tissue Samplings. Four laboratory populations of *N. lugens* (TN1, Mudgo, IR56 and IR42) with different virulence

reared on rice varieties Taichung Native1 (a BPH-susceptible rice cultivar), Mudgo (carrying *Bph1* gene), IR56 (carrying *Bph3* gene), and IR42 (carrying *bph2* gene) in wire mesh cages under greenhouse conditions (28°C, 85% relative humidity (RH), and a photoperiod of 16:8 (L:D) h darkness) for more than 40 generations were used in this study. These laboratory populations are named after the host rice lines that they are reared on. Thirty newly emerged individuals from each *N. lugens* population were randomly selected, and 10 individuals were pooled into one group. The 30 brachypterous and 30 Macropterous female adults were selected from field in CNRRI (China National Rice Research Institute), and 10 individuals were pooled into one group, respectively. The field population in CNRRI is a mixture of *N. lugens* feeded on rice cultivars with or without resistant genes, *Bph1* or *bph2.* Only small amount of *N. lugens* can feed on rice cultivars with resistant gene *Bph3.*

Thirty individuals for each instar and 30 individuals at the day of 1, 3, 6, 9, 12, 15-d-old brachypterous female and male adults of TN1 population were randomly selected, and 10 individuals were pooled into one group. Tissues from 300 newly emerged 2-d-old adults (TN1 population) including fat body, gut, salivary gland, ovaries, hemolymph, testis, and wing were dissected and sampled, respectively. The samples were frozen in liquid nitrogen and stored at −80°C until use.

RNA Extraction. We employed RT-q Polymerase Chain Reaction (PCR) to detect and quantify the stage-, tissue- and sex-specific expression levels of *NlEno1*gene with total RNA. RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany). The potential genomic DNA contamination was eliminated by a treatment with DNase I kit (QIAGEN) after the RNA extraction procedure. RNA concentration and quality were determined using a Nanodrop spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized with ReverTra Ace qPCR RT Kit (Toyobo Co., Ltd., Japan). Each RNA sample was incubated at 65°C for 5 min before reverse transcription reaction. And each reaction contained: enzyme mix primer mix, RNA and DEPC water in a final volume of 20 µl accoding to manufacturer's protocol. The thermocycler was programed 37°C for 45 min, then maintained at 4°C. The 10× diluted first-strand cDNA (3.0 µl) was used as template for quantitative PCR.

Molecular Clonging and Phylogenetic Analysis of the NlEno1 in N. lugens. The full-length cDNA of *NlEno1* was cloned using the primers listed in Table 1. Amplification was carried out in a total reaction volume of 25 µl, containing 3.0 µl cDNA, 0.5 µl of each primer (10 µM), 2.0 µl dNTP (2.5 mM), 2.5 µl PCR buffer (10×), and 0.125 µl rTaq DNA polymerase (5 U/µl) (TaKaRa Bio Inc, Japan). PCRs were performed with the following cycles: initial denaturation at 95°C for 2 min; followed by 35 cycles of 1 min at 95°C, 30 s annealing at 58°C, 90 s extension at 72°C; and a final extension at 72°C for 10 min.

Amplified PCR products was purified with universal DNA purification kit (TIANGEN, Beijing, China) and ligated into the PCR2.1-TOPO cloning vector (Invitrogen, Shanghai, China). The resultant pTOP-NlEno1 plasmid was transformed into TOP10 chemically competent cell (Invitrogen). Positive clones were identified by the approaches of PCR detection. *NlEno1* cDNA insertion was further confirmed by DNA sequencing company (Shanghai Sunny Biotechnology, Co., Ltd.) using ABI3730 DNA analyzer.

The resulting sequence was submitted to GenBank (Accession number KF640639). The open reading frame (ORF) was predicted using DNASTAR software (DNASTAR Inc., Madison, USA). The sequence was also analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0) and http://www.cbs.dtu.dk/services/TMHMM/. A phylogenetic tree was constructed by MEGA version 5.2 (http://megasoftware.net/) using the neighbor-joining method. A Poisson-corrected distance was used, and the statistical significance of group in the neighbor-joining tree was assessed by the bootstrap probability with 1,000 replications.

Quantitative Real-Time PCR (RT-qPCR). The mRNA levels were measured by RT-qPCR using SYBR green realtime PCR Master Mix (Toyobo, Co., Ltd.). qRT-PCR was performed in a 20 µl: contained 3.0 µl cDNA, 0.1 µM each gene specific primers, and 10 µl SYBR Premix (Toyobo, Co., Ltd.). Nontemplate reactions (NTC) (total RNA was replaced with H_2O) and reverse transcriptase controls (PrimeScript RT Enzyme Mix was replaced with H_2O) were used as negative controls. Reactions were performed in a Real Time System ABI 7500 (Applied Biosystems). Reaction conditions were as follow: an initial incubation of 50°C for 2 min and 95°C for 30 s; 40 cycles of 95°C for 5 s, and 60°C for 30 s. A dissociation step cycled at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. And data analysis was performed by using the 7500 system SDS software. Two constitutively expressed 18 S rRNA gene (JN662398) and β-actin gene (EU179846) was used as the internal control (Liu et al. 2010; Xue et al. 2010). The primers of *NlEnol1*, *NlVg* [Vitellogenin (Vg) gene in *N. lugens*] and internal control used were listed in Table 1. The differences in the CT values of *NlEno1* or *NlVg* and the corresponding internal control (ΔCT) were calculated to normalize the difference in the amount of total RNA added to the cDNA reaction mixture. The ΔCT for the control sample was subtracted from the ΔCT of the challenged sample. The difference was expressed as a ΔΔCT value that allowed comparing the expressions of target genes in the challenged sample relative to the control. The expression level of *NlEnol1* or *NlVg* were calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

dsRNA Injection. pTOP-NlEno1 plasmid were used as the template for amplification of the target sequence by PCR using specific primers

Table 1. Primers used in this study

Primer	Primer sequence	Length (bp)	NCBI accession no.	Reference
For full-length cDNA amplification				
NlEno1-F	GCCTATAAATTCCTTTGTCC	1,852	KF640639	This study
NlEno1-R	ATCCAACTTTCTTGGATGGTG			
For RT-qPCR				
18 S rRNA-F	CGCTACTACCGATTGAA	131	JN662398	Xue et al. 2010
18 S rRNA-R	GGAAACCTTGTTACGACTT			
β-ACT-F	TGGACTTCGAGCAGGAAATGG	200	EU179846	Liu et al. 2010
β-ACT-R	ACGTCGCACTTCATGATCGAG			
qNlEno1-F	CGCTGTCCCGTCCGGAGCTT	274	KF640639	This study
qNlEno1-R	GGCAGCTCCCGCCTTGCATAC			
qNlVg-F	AGTCAACTACAAGCAGGGAGCAGTG	235	AB353856	Gamalath et al. 2012
qNlVg-R	GCTCATCAACATCGTAGTGGGTCTC			
For dsRNA synthesis				
dsNlEno1-F	TAATACGACTCACTATAGGGATTCCGTGCCGCTGTCCCGTC	391	KF640639	This study
dsNlEno1-R	TAATACGACTCACTATAGGGTACCGGCGTGGGAACCTCCA			
dsGFP-F	TAATACGACTCACTATAGGGCCTGAAGTTCATCTGCACCAC	355	AB608314	This study
dsGFP-R	TAATACGACTCACTATAGGGTGATGCCGTTCTTCTGCTTGT			

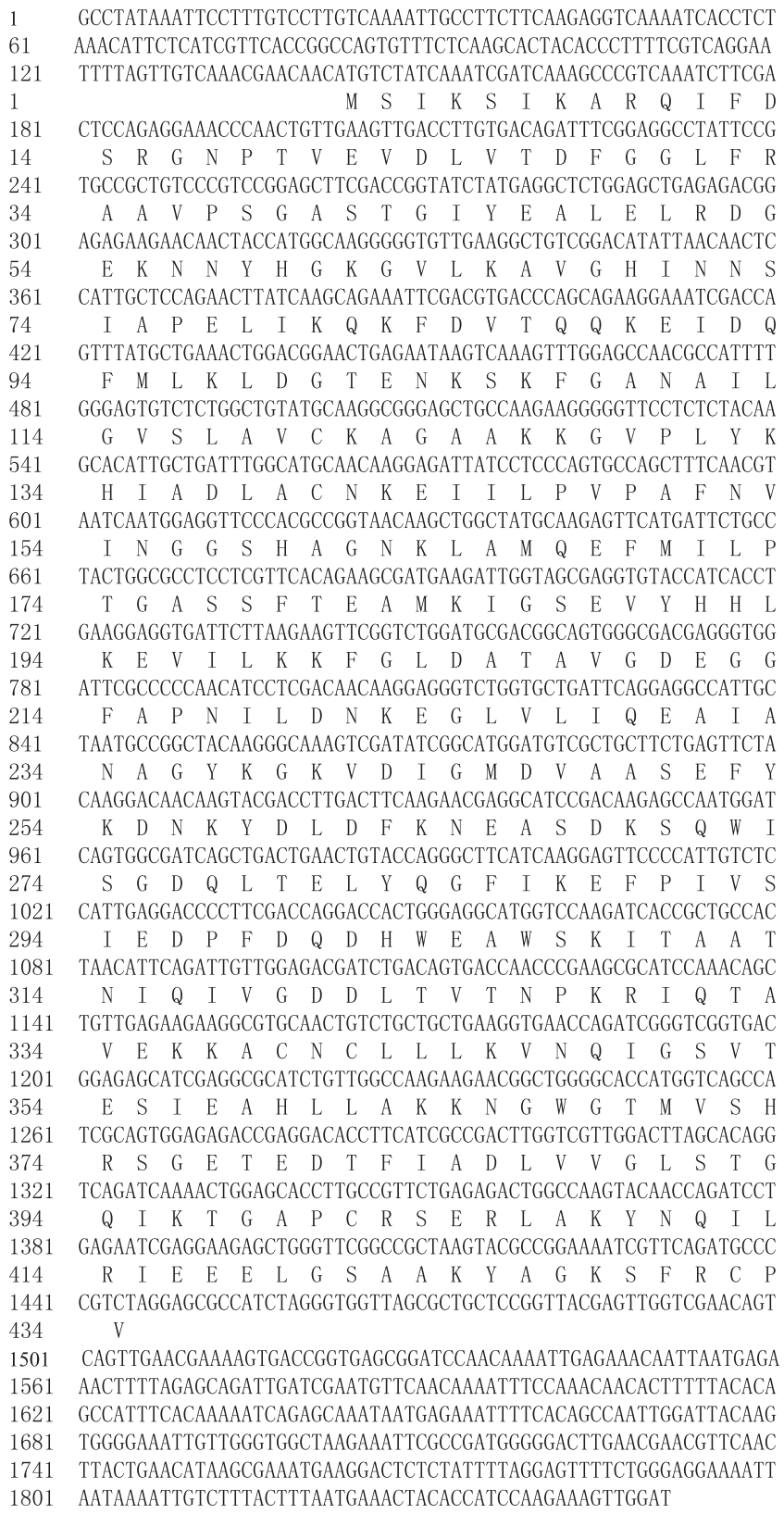

Fig. 1. Nucleotide sequence and flanking regions, predicted amino sequence of the *NlEno1* (KF640639).

dsEno1-F/dsEno1-R conjugated with the T7 RNA polymerase promoter (TAATACGACTCACTATAGGG) (Table1). PCR amplification was performed in 35 cycles of 94°C for 40 s; 58°C for 30 s and 72°C for 40 s; with final extension step of 72°C for 7 min. PCR products were examined on 1% agarose gel for verification and then purified using Tiangen Midi Purification Kit (TIANGEN). A dsRNA that targets *Nleno1* expression was then in vitro generated using MEGAscript RNAi Kit (Life Technologies). Purified *NlEno1* dsRNA was stored at

−80°C until use. A dsRNA that targets the green fluorescent protein (GFP) gene (AB608314) expression was also produced as described earlier and used as control.

The dsRNA injection was performed according to reference (Liu et al. 2010) with small modification. *N. lugens* was injected with 0.1 μl dsRNA (1 μg/μl) using eppendorf microingection system TransferMan NK2. The injection site was the second thorax surface of their exoskeleton. To study whether *NlEno1* affects the survival rate of insect, the third instar were used for injection. At least 30 individual nymphs were injected and reared on 30- to 35-day-old rice of Taichung Native1 in one cage at 28°C, 85% RH, and 16:8 (L:D) h darkness with three cages as parallel replication as separated samples. After injection, three individuals were taken from each cage at day of 2, 4, 6, 8, 10, and 12 as separated samples for RNA extraction. To study whether *NlEno1* affects reproduction of insect, the fifth instar were used for injection. Once adult emerge, each female was matched with one male and each pair was put into one cage. Five newly emerged 4-d-old females were collected for RNA extraction. GFP dsRNA or water were used for injection in control treatments.

Gene expression knockdown was confirmed by the determination of mRNA expression levels of the *NlEno1* using SYBR RT-PCR kit and the ABI7500 system (Applied Biosystems). The *NlEno1* and *NlVg* mRNA levels were normalized against 18 S rRNA and actin using comparative Ct method by comparison of the mRNA levels in NlEno1-dsRNA- and GFP-dsRNA- injected *N. lugens* using Duncan's multiple range test. Values of $P < 0.05$ were considered significant.

Bioassay and Data Analysis. The survival rates of the third instar after injection were observed at 24 h intervals with duration of 12 d. To study the fecundity and egg hatching rate, a total of 15 pairs per group were matched successfully. The number of newly hatched nymphs was recorded and removed every day. At the 10th d after the female parent die, the unhatched eggs in rice were recorded through dissecting leaves under stereo microscope. The data was summarized as means ± standard errors. The differences between *N. lugens* injected with NlEno1-dsRNA and those injected with GFP-dsRNA or water were assessed by Duncan's multiple range test. Values of $P < 0.05$ were considered significant.

Result Identification, Isolation and Phylogenetic Analysis of the NlEno1. Based on our *N. lugens* transcriptome data and EST data (http://bphest.dna.affrc.go.jp/), the cDNA sequence of 1,851 bp (GenBank accession, KF640639) with 1,304 bp of ORF predicted to code enolase (NlENO1) was assembled and amplified with PCR method (Fig. 1). The predicted NlENO1 contains 434 amino acids with the isoelectric point of 6.00 and molecular weight of 47KD. Protein analysis revealed putative enolase N (2-135) and enolase C domains (143-434) in NlENO1. In N-terminal, hydrophobic domain (AAVPSGASTGI) located at position 31−41 was found. Seven amino acids for substrate (2-PGE) binding pocket (H^{159}, E^{211}, K^{345}, $HRS^{373-375}$, and K^{396}), metal-binding site (S^{38}, D^{246}, E^{295}, and D^{320}) of the enolase family and the enolase signature (LLLKVNQIGSVTES) from position 342 − 355 were identified in C-terminal domain of NlENO (Fig. 2). No putative signal peptide and transmembrane helices was found. The NlENO1 showed high homology to those from *Oncometopia nigricans* and *Anopheles darlingi*, with amino acids identities of 87%, 85% respectively. To clarify the evolutionary relationship of NlENO1, we used a neighbor-joining tree construction method based on distances of 28 enolase sequences from 14 arthropod including 10 insecta, 3 nematoda, 1 arachnida and nonarthropod. The dendrogram obtained places the NlEno1 with other insects and particularly with hemipteran as a distinct cluster (Fig. 3).The tree also clearly divides the NlEno1 from insect, arachnids, nematods, vertebrates, plants, fungi, and bacteria into separate groups. The insect enolase proteins are more

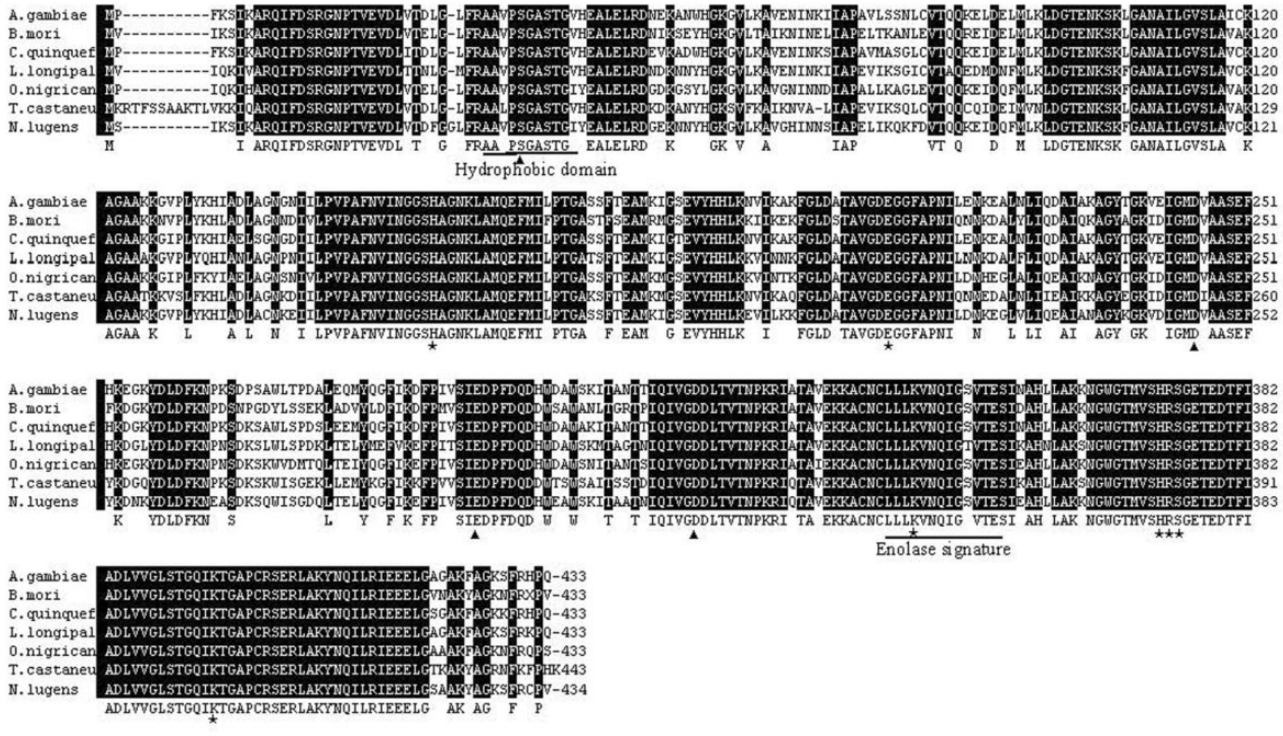

Fig. 2. Comparison of the *Nilaparvata lugens* enolase amino acid sequence with other insects. *Anopheles gambiae str* (XP_317672.2), *Bombyx mori* (NP_001091831), *Culex quinquefasciatus* (XP-001842618), *Lutzomyia longipalpis* (ABV60328), *Oncometopia nigricans* (AAU95200), and *Tribolium castaneum* (XP_975266). The residus that matched *N.lugens* exactly was shaded with solid black. The following motifs are conserved in all these sequences: (i) the four amino acids involved in Mg^{2+} binding (S^{38}, D^{246},E^{295}, and D^{320}) are indicated with▲; (ii) the seven amino acids for substrate (2-PGE) binding (H^{159}, E^{211}, K^{345}, $HRS^{373-375}$, and K^{396}) are indicated with ★; (iii) the 14 amino acids constituting the so-called enolase signature (LLLKVNQIGSVTES) located at positions 342–355; and (iv) hydrophobic domain (AAVPSGASTGI) located at position 31–41.

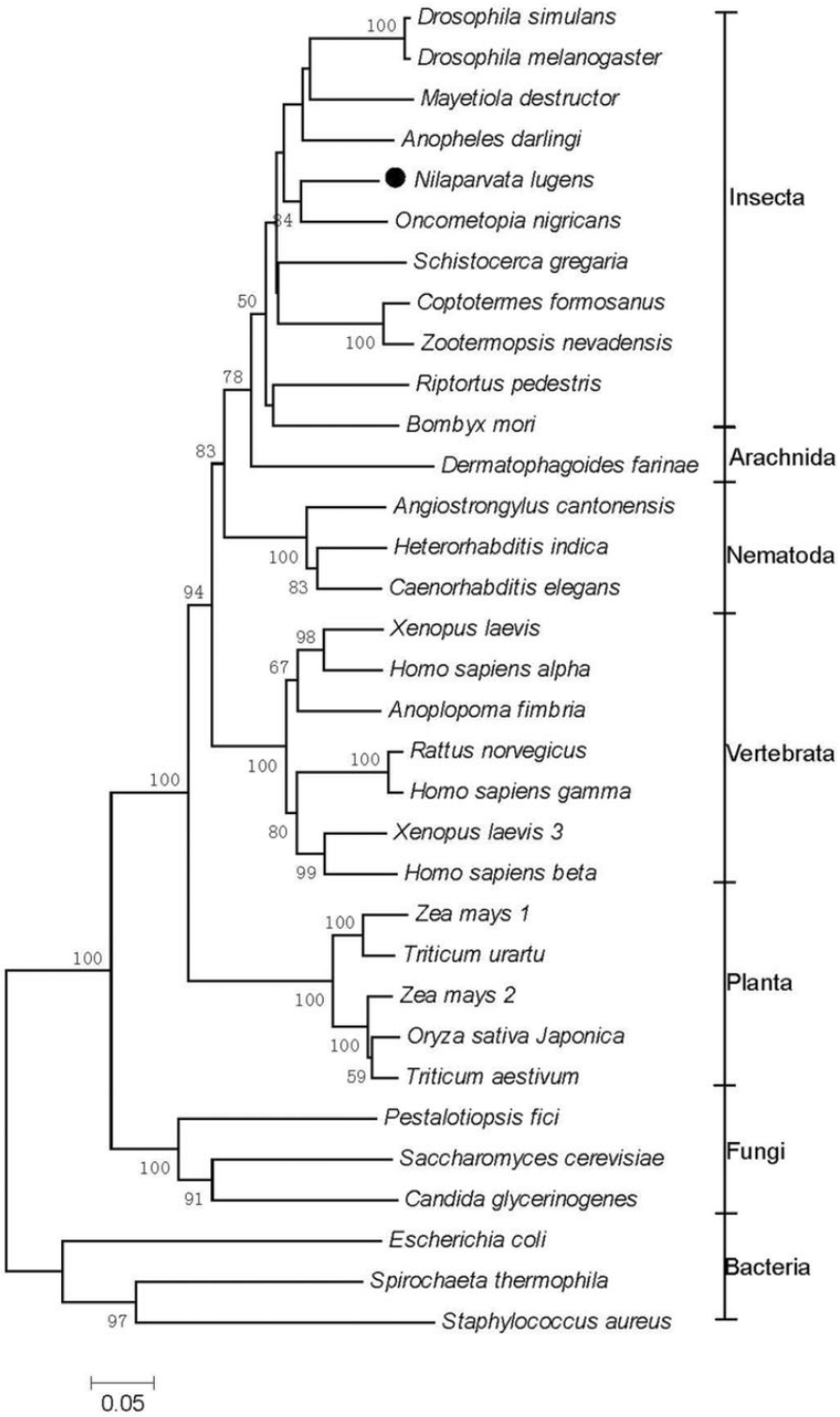

Fig. 3. A distance analysis of amino acid of enolase from different species was performed using a neighbor-joining tree construction program Mega 5. Evolutionary distances were computed using Poisson correction method. Branch support values (1000 bootstraps) for nodes are indicated only support values $>$ 50% are shown. All sequences were obtained from Gen Bank protein sequence data with accession numbers as mentioned in brackets. *Drosophila simulans* (ABH06849), *Drosophila melanogaster* (NP477421), *Mayetiola destructor* (AHB50485), *Anopheles darlingi* (ETN65833), *Nilipavata.Lμgens* (AHB33499), *Oncometopia nigricans* (AAU95200), *Schistocerca gregaria* (AEV89757), *Coptotermes formosanus* (AGM32397), *Zootermopsis nevadensis* (KDR20985), *Riptortus pedestris* (BAN20388), *Bombyx mori* (NP_001091831), *Dermatophagoides farinae* (AHV90299), *Angiostrongylus cantonensis* (AGO81688), *Heterorhabditis indica* (ADH95415), *Caenorhabditis elegans* (NP_495900), *Xenopus laevis* (NP_001080606), *Homo sapiens*αa enolase (NP_001419), *Homo sapiens*βenolase (NP_001967), *Homo sapiens*γ*enolase* (CAA36215), *Anoplopoma fimbria* (ACQ58328), *Rattus norvegicus* (AAB72088), *Xenopus laevis* (NP_001080346) *Spirochaeta thermophila* (AEJ62315), *Staphylococcus aureus* (ABQ48603), *Escherichia coli* (BAE76853), *Zea mays* enolase1 (NP_001105371), *Zea mays* enolase2 (NP_001105896), *Saccharomyces cerevisiae* (AAA88713), Candida glycerinogenes (ABO28523), *Pestalotiopsis fici* (XP007828338), *Oryza sativa* (AAC49173), *Triticum* (AGH20061), *Triticum urartu* (EMS66544).

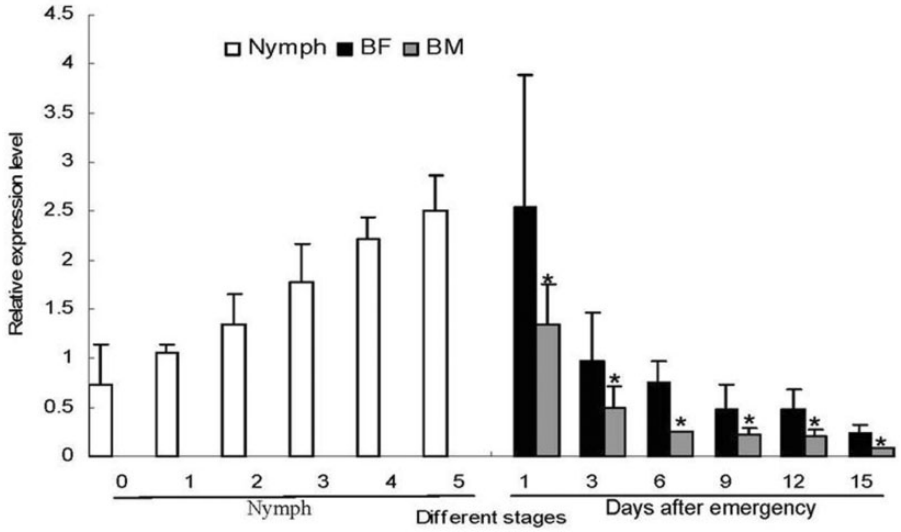

Fig. 4. Expression of *NlEno1* gene at different development stages in *N. lugens* was determined by RT-qPCR. mRNA level was normalized relative to the actin and 18 S rRNA transcript. Each point represent mean value ± SD of three independent experiments with 10 individuals in each replicate. BF brachypterous female adults; BM, brachypterous male adults. "*" means statistically significant difference in expression levels between male and female adult by *t*-test ($P < 0.05$).

close to arachnida and nematode enolase than to vertebrate, plant, and bacteria.

Developmental and Tissue-Specific Expression of NlEno1 in N. lugens. RT-qPCR experiments revealed that *NlEno1* was transcribed in all the life stages of *N. lugens*. Expression level of mRNA increased gradually from the first to fifth instars and reached the maximum at newly emerged adult, then decreased gradually. The expression of *NlEno1* was higher in female adult than in male adult (Fig. 4). A high level of *NlEno1* expression was detected in hemolymph, fat body, gut, ovaries, salivary glands, wings, and eggs but trace mRNA levles was detected in testis (Fig. 5).

In order to examine the influence of resistant rice and wingforms on *Nleno1* gene expression in *N. lugens*, total RNA extracted from newly emerged adult was used as template. We found that the mRNA expression level of *NlEno1* gene in Mudgo, IR56 and IR42 laboratory population was significantly up-regulated when compared with the TN1 population and higher in brochypterous adult than in macropterous adult (Fig. 6).

Injection of dsRNA on Expression of NlEno1 and NlVg. The injection of *NlEno1*-dsRNA caused a significant reduction in *NlEno1* mRNA levels by 97.2% from 4th to 12th d after injection (Fig. 7). This indicated that the RNAi-mediated knockdown of *NlEno1* was successful.

Since the expression of *NlEno1* gene was higher in brochypterous adult than in macropterous adult. We proposed that higher expression of *NlEno1* gene will be helpful for protein synthesis especially for yolk protein, Vg are the major yolk protein precursors of vitellins and play important role in the reproduction of *N. lugens*. The possible effect of *NlEno1* knockdown on the transcript level of NlVg gene was examined at 4 d after emergency (12 d after injection). The result showed that the injection of *NlEno1*-dsRNA in third instar nymphs, Vg expression levels decreased by 37.8% at 4 d after adult emergence compared with the dsGFP injection (Fig. 8).

Negative Effect of dsRNA on the Survival Rates and Fecundity of N. lugens.. There was no difference in nymphs survival rate between *NlEno1*-dsRNA (97.0%) and GFP -dsRNA (98.0%) at the 5th d after injection. At the 6th d, the survival rate of *NlEno1*-dsRNA decreased slightly to 92% compared with GFP-dsRNA to 94%. Once insects emerged, we successfully allocated them into 15 pairs per group. The number of egg and offspring from every individual female adult was

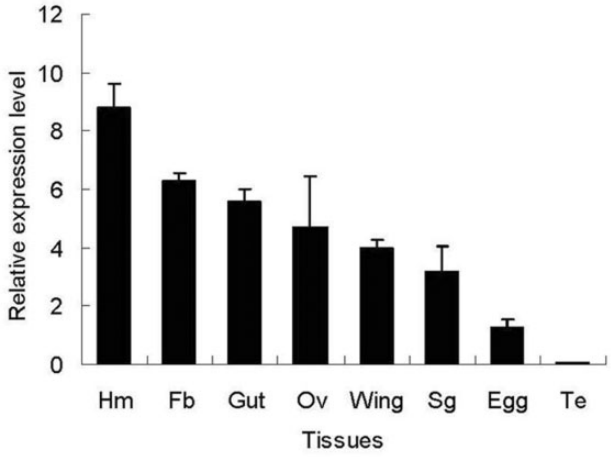

Fig. 5. Expression of *Nleno1* gene in various tissues of brachypterous adults *N. lugens* was determined by RT-qPCR. Hm, Hemolymph; Ov, ovaries; Fb, fat body; Sg, Salivary glands;Te, Testis. The mRNA level was normalized relative to the 18 S rRNA and β-actin transcript. Each point represents mean value ± SD of three independent experiments with 100 individuals in each replicate.

counted. The average number of egg and offspring in the group treated with 1 μg/μl *NlEno1*-dsRNA was 139.8 and 120.5, respectively, significantly less than those in the GFP-dsRNA treated groups (Fig. 9). The result demonstrated that *NlEno1*-dsRNA injection caused a significant reduction in egg production (36.0%), offspring (40.6%), and hatching rate (5.59%) as compared with the treatment with GFP-dsRNA. So knockdown of *NlEno1* resulted in lower Vg gene expression and reduced fecundity.

Discussion

Rice brown planthopper is one of the most serious insect pests in Asia, it has strong adaptability to environmental variation and result in new virulent strains occurred and overcome the rice resistance. The mechanism underlying this problem remains unknown. Coping with

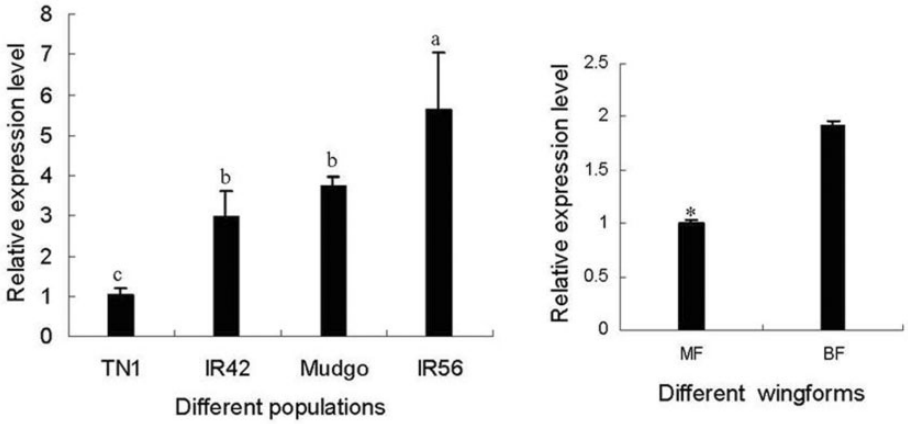

Fig. 6. Expression of *NlEno1* gene between four laboratory populations and two different wingforms of *N. lugens*. The data represent the mean values ± SD of three replicate with 10 individuals in each replicate. Different letters or "*" means significant difference by Duncan's multiple range test or *t*-test ($P < 0.05$).

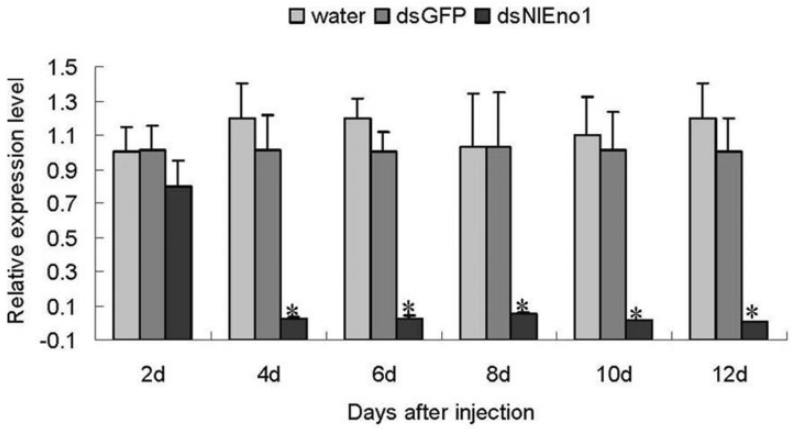

Fig. 7. Detection of efficiency of injection-based RNAi and impact on *NlEno1* mRNA level by RT-qPCR. dsNlEno1,1 μg/μl *NlEno1*-dsRNA; dsGFP,1 μg/μl GFP-dsRNA; water, injection with water. The data represent the mean values ± SD of three replicate with three individuals in each replicate. "*" means statistically significant difference in expression levels between CK and dsNlEno1 (*t*-test $P < 0.05$).

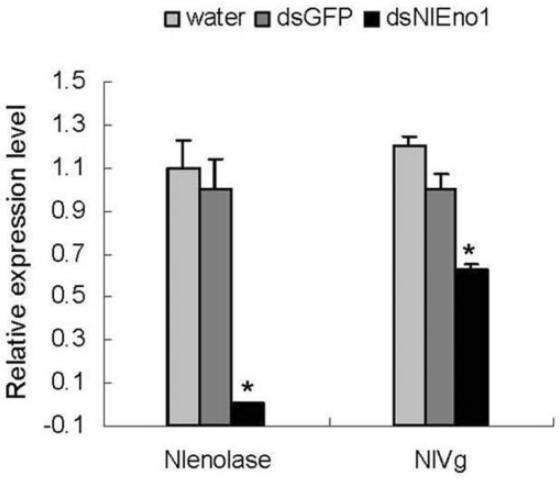

Fig. 8. Detection of efficiency of injection-based RNAi and impact on *NlEno1* and *NlVg* mRNA level by RT-qPCR at 4 d after adult emergency (day 12th injection). dsNlEno1,1 μg/μl *NlEno1*-dsRNA; dsGFP, 1 μg/μl GFP-dsRNA; water, injection with water. The data represent the mean values ± SD of three replicate with five individuals in each replicate. "*" means statistically significant difference in expression levels between CK and dsNlEno1 (*t*-test $P < 0.05$).

the adverse environment can be costly and requires energy and resource allocation for adaptation and survival. Several behavioral, physiological, and genetic mechanisms are used by insects to handle adverse environment, such as insecticide resistance by constitutive over expression of detoxification enzymes or inducing mutations in the target sites (Kliot and Ghanim 2012). House sparrows challenged with phytohaemagglutinin significantly elevated their resting metabolic rate relative to controls (Martin et al. 2003). Such actions are costly and may affect reproduction, impair dispersal ability and have several other effects on the insect's fitness (Kliot and Ghanim 2012). Carbohydrates, especially sucrose, are the main chemical components in the phloem sap of rice and are essential for the phloem-sucking insects as phagostimulants as well as nutrients. In this study, we cloned the enolase gene and compared the expression difference between different virulent populations of *N. lugens*. The comparison of NlENO1 protein sequences with other insect enolases revealed a high degree of conservation (from 74 to 82% sequence identity), including full conservation of the metal- and substrate-binding motifs and the enolase signature (LLLKVNQIGSVTES). A hydrophobic domain in haematophagous arthropods enolase (AAVPSGASTGI) suggested to play a role in its membrane association (Pancholi 2001) is existed in the *N. lugens* (Fig. 2). Because of high expression of *NlEno1* in salivary glands and ovaies, it is tempting to speculate that this motif may be helpful for drawing nutrients from rice tissue and ovipositing on rice. This requires experimental demonstration.

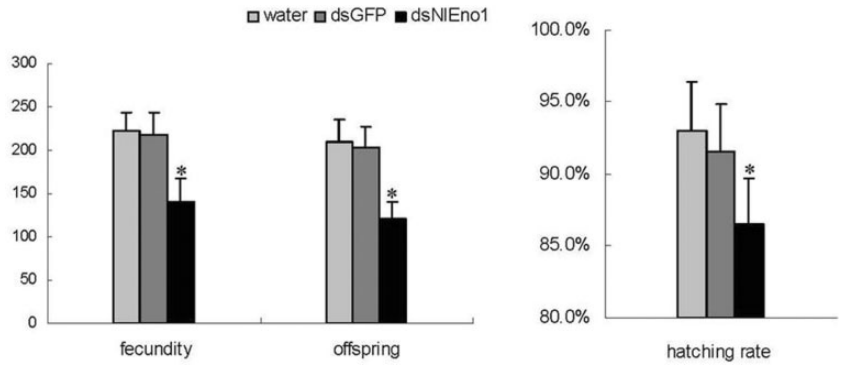

Fig. 9. RNAi-mediated knockdown of *NlEno1* gene decrease fecundity, offspring and hatching rate (15 pairs were analyzed per group). dsNlEno1, 1 μg/μl Nleno1-dsRNA; dsGFP, 1 μg/μl GFP-dsRNA; water, injection with water "*" means statistically significant difference in expression levels between CK and dsNlEno1 (*t*-test $P < 0.05$).

Enolase is an essential enzyme catalyzing the conversion of 2-PGE to PEP, the only dehydration step in the glycolytic pathway. Although it is expressed in most of the cells, the gene that encodes enolase is not considered a housekeeping gene since its expression varies according to the pathophysiological, metabolic, or developmental conditions of cells (McAlister and Holland 1982). Enolase expression which is primarily under developmental control is significantly up-regulated during cellular growth and practically undetectable during quiescent phases (Giallongo et al. 1990; Holland et al. 1983). Our results showed that the expression of *NlEno1* gradually increased during *N. lugens* development, expression in female is higher than in male, and expression in brachypterous higher than in macropterous. Female and brachypterous adults are responsible for reproduction and produce numerous offspring, so more nutrition and energy are required. The up-regulated expression of *NlEno1* was beneficial to high metabolic activity in *N. lugens*. However, the injection of NlEno1-ds RNA did not affect *N. lugens* mortality. This result indicated that there is large excess *NlEno1* over the amount required to support primary demand as reported in *Drosophila melanogaster* (Eanes et al. 2006) and in human erythrocytes(Salvador and Michael 2003).

Our result showed that the expression level of mRNA is significantly higher in Mudgo, IR56 and IR42 population compared with TN1 population. We also found 10 protein spots was up-regulated in Mudgo population than in TN1 population with method 2D-PAGE, one protein spot was as same as NlENO1 (unpublished results). This means the *N. lugens* adaptation to resistant host requires a balance between maintaining cellular energy production and reducing the associated damage caused by resistant rice. In fat body of *N. lugens*, 37 genes, ∼84% in glycolysis had higher transcript levels in the Mudgo population than in the TN1 population (Yu et al. 2014). As the total carbohydrates, especially the soluble sugar content, in resistant rice varieties is lower than in susceptible ones (Shang and Hua 2012; Sogawa and Pathak 1970), a high expression level of *NlEno1* in Mudgo, IR56 and IR42 population of *N. lugens* might compensate for insufficient nutrients in the phloem sap of resistant rice varieties and is beneficial to their adaptation to resistant varieties.

Wing dimorphism of *N. lugens* was not only dominated by genes but influenced by environmental factors as well Syobu et al. (2002). The brachypterous adults with reduced wings and flight muscles, is unable to fly and are adapted for reproduction and produce numerous offspring in rice fields. Macropterous adults fly long distances and invade rice-growing areas. Lipids and triglyceride are main flight fuels. The energy used to construct wings and flight muscles is simply not available for reproductive investment (Zera and Denno 1997). Our result showed the brachypterous female adults have higher *NlEno1* expression than the macropterous female adults. It was indicated that *NlEno1* could be involved in the regulation of *N. lugens* reproduction. Knockdown of *NlEno1* caused a significant reduction in egg production, offspring and hatching rate, as compared with the treatment with GFP dsRNA. The reproductive success of *N. lugens* depends on Vg (the major yolk protein precursor) biosynthesis and its accumulation in the developing oocytes (Tufail et al. 2010). The amount of Vg mRMA started to accumulate at day 3 and increased to high levels at day 4 after adult emergence in brachypterous females (Gamalath et al. 2012). Our result showed the injection of NlEno1-dsRNA in third instar nymphs, *NlVg* expression levels decrease by 37.8% at 4 d after adult emergence compared with the GFP-dsRNA injection. Different expression of Vg mRNA can be the result of either direct interaction or a secondary response. The reduction of Vg mRNA may be caused by lack of sufficient energy reserves after NlEno1-dsRNA injection.

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