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

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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 species. 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

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reared on rice varieties Taichung Native 1 (a BPH-susceptible rice cultivar), Mudgo (carrying Bph1 gene), IR56 (carrying Bph3 gene), and IR24 (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 fed on rice cultivars with or without resistant genes, Bph1 and 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 the RNeasy Mini Kit (QIAGEN, Germany). The potential genomic DNA contamination was eliminated by a treatment with DNase 1 kit (QIAGEN) after the RNA extraction procedure. RNA concentration and quality were determined using a Nanodrop spectrophotometer (QIAGEN). The potential genomic DNA contamination was removed by incubation 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 10 min. The 10× diluted first-strand cDNA (3.0 μl) was used as template for quantitative PCR.

**Molecular Cloning 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 Taq DNA polymerase (5 U/μl) (TaKaRa Bio Inc, Japan). PCR reactions 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 μl each gene specific primers, and 10 μl SYBR Premix (Toyobo, Co., Ltd.). Non-template reactions (NTC) (total RNA was replaced with H2O) and reverse transcriptase controls (PrimeScript RT Enzyme Mix was replaced with H2O) 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 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 NlEno1, Vtg [Vitellogenin (Vtg) gene in N. lugens] and internal control used were listed in Table 1. The differences in the CT values of NlEno1 or Vtg and the corresponding internal control (ACT) 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 expression of target genes in the challenged sample relative to the control. The expression level of NlEno1 or Vtg was calculated by 2−ΔΔCT (Livak and Schmittgen 2001).

**dsRNA injection.** pTOP-NlEno1 plasmid was 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               |
|------------|----------------------------------------|-------------|--------------------|-------------------------|
| NlEno1-F   | GCCATAAAATCTTTCTTGCC                   | 1,852       | KF640639           | This study             |
| NlEno1-R   | ATCCAATCTTCCTGGATGTTG                  |             |                    |                         |
| For RT-qPCR|                                        |             |                    |                         |
| Bph1 rRNA-F| CGCTACTACCGATTGAA                      | 131         | JN662398           | Xue et al. 2010        |
| Bph1 rRNA-R| GGAAACCTGGTACGACCT                    | 200         | EU179846           | Liu et al. 2010        |
| β-act-F    | TGGACCTCGAGAGAATGG                    | 274         | KF640639           | This study             |
| β-act-R    | ACCTGGAGACCTTACGTGAG                   | 235         | AB353856           | Gamaliath et al. 2012  |
| qNlEno1-F  | CGGTGCCCGCTGGCGAGCTT                  |             |                    |                         |
| qNlEno1-R  | GSGACCTCCGGCTCCGATAC                  |             |                    |                         |
| qNlVtg-F   | AGTCACACCAAGAAGAAGAGAGAGT             |             |                    |                         |
| qNlVtg-R   | GCTCATCAACATCTGATGCTTC                |             |                    |                         |
| For dsRNA synthesis |                             |             |                    |                         |
| dsNlEno1-F | TAATAGCAGTACATAATAGGATTGTCGCCGGCTTCC  | 391         | KF640639           | This study             |
| dsNlEno1-R | TAATAGCAGTACATAATAGGATTGTCGCCGTTCCT  | 355         | AB6808314          | This study             |
| dsGFP-F    | TAATAGCAGTACATAATAGGATTGTCGCCGTTCCT  |             |                    |                         |
| dsGFP-R    | TAATAGCAGTACATAATAGGATTGTCGCCGTTCCT  |             |                    |                         |
dsEno1-F/dsEno1-R conjugated with the T7 RNA polymerase promoter (TAATACGACTCACTATAGGG) (Table 1). 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

Fig. 1. Nucleotide sequence and flanking regions, predicted amino sequence of the NlEno1 (KF640639).
constituting the so-called enolase signature (LLLKVNQIGSVTES) located at positions 342–355; and (iv) hydrophobic domain (AAVPSGASTGI) located at position 31–41.

Comparison of the enolase amino acid sequence with other insects.

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://bhpest.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 47 KD. 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 (H159, E211, K345, HRS373–375, and K396), metal-binding site (S38, D246, E295, and D320) of the enolase family and the enolase signature (LLLKVNQIGSVTES) located at positions 342–355 were identified in C-terminal domain of NlENO1 (Fig. 2). The 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. 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), Nilapavata lugens (AHB33499), Oncometopia nigricans (AAU95200), Schistocerca gregaria (AEV9757), Coptotermes formosanus (AGM32397), Zoctermopsis 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 enolase (NP_001419), Homo sapiens enolase (NP_001967), Homo sapiens enolase (CAA36215), Anoplophora fimbriata (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 (AAA8713), Candida glycerinogenes (ABO28523), Pestalotiopsis fici (XP007828338), Oryza sativa (AAC49173), Triticum (AGH20061), Triticum urartu (EMS66544).
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 levels 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 brochtypterous 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 brochtypterous 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 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 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
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 overexpression 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 ovaries, 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.
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 NIEN01 (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, Ëœ84% 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 (Tufial et al. 2010). The amount of Vg mRNA 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, NIVg 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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