*Bulletin of Entomological Research* (2015) **105**, 390–398
doi:10.1017/S0007485315000231


# RNA interference-aided knockdown of a putative saccharopine dehydrogenase leads to abnormal ecdysis in the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae)

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## Abstract

The brown planthopper *Nilaparvata lugens* is a serious phloem-feeding pest of rice in China. The current study focuses on a saccharopine dehydrogenase (SDH) that catalyzes the penultimate reaction in biosynthesis of the amino acid lysine (Lys), which plays a role in insect growth and carnitine production (as a substrate). The protein, provisionally designated as NlylsSDH [a SDH derived from yeast-like symbiont (YLS) in *N. lugens*], had a higher transcript level in abdomens, compared with heads, wings, legs and thoraces, which agrees with YLS distribution in *N. lugens*. Ingestion of *Nlylssdh* targeted double-stranded RNA (ds*Nlylssdh*) for 5, 10 and 15 days decreased the mRNA abundance in the hoppers by 47, 70 and 31%, respectively, comparing with those ingesting normal or ds*egfp* diets. *Nlylssdh* knockdown slightly decreased the body weights, significantly delayed the development of females, and killed approximately 30% of the nymphs. Moreover, some surviving adults showed two apparent phenotypic defects: wing deformation and nymphal cuticles remained on tips of the legs and abdomens. The brachypterours/macropterours and sex ratios (female/male) of the adults on the dsRNA diet were lowered compared with the adults on diets without dsRNA. These results suggest that *Nlylssdh* encodes a functional SDH protein. The adverse effect of *Nlylssdh* knockdown on *N. lugens* implies the importance of Lys in hopper development. This study provides a proof of concept example that *Nlylssdh* could serve as a possible dsRNA-based pesticide for planthopper control.

**Keywords:** *Nilaparvata lugens*, saccharopine dehydrogenase, lysine, biosynthesis, ecdysis

(Accepted 27 February 2015; First published online 24 April 2015)

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The brown planthopper, *Nilaparvata lugens*, feeds on the phloem sap of rice plants and is a serious pest in paddy fields throughout Asia. Rice phloem sap contains high levels of glucose and starch but low levels of nitrogenous organic compounds such as free amino acids. Among these free amino acids, only asparagine, glutamate, glutamine, threonine and valine are abundant (Fukumorita & Chino, 1982). As *N. lugens*

Table 1. Primers of Nlylssdh used for RT-PCR, dsRNA synthesis and qRT-PCR.

| Prime name | Amplicon size (bp) | Forward sequence (5′–3′) | Reverse sequence (5′–3′) |
|---|---|---|---|
| *ORF verification* | | | |
| Nlylssdh | 1461 | GGACGCTGTCAATCATCTA | AATCTGCACTTTCCCTTCT |
| *dsRNA synthesis* | | | |
| dsegfp | 460 | CACAAGTTCAGCGTGTCCG | GTTCACCTTGATGCCGTTCT |
| dsNlylssdh | 368 | TACATCTTCCATGCCTCCGTT | TTTCGCAAAGCCAGCAATAC |
| *qRT-PCR* | | | |
| qNlylssdh | 185 | AATGCCTGCTCGTTGATAAC | ATGTCTTGCCCTAAGGTGCT |
| RPS15 | 150 | TAAAAATGGCAGACGAAGAGCCCAA | TTCCACGGTTGAAACGTCTGCG |
| TUB | 174 | ACTCGTTCGGAGGAGGCACC | GTTCCAGGGTGGTGTGGGTGGT |

feeds almost exclusively on rice, it must employ a strategy to compensate for the amino acid imbalance in its diet.

*N. lugens* harbors yeast-like symbionts (YLSs) mainly in abdominal fat bodies (Chen *et al*., 1981; Cheng & Hou, 2001; Dong *et al*., 2011). When YLS was experimentally removed, *N. lugens* nymphs lost weight, grew slowly, and had lower total protein concentrations (Fu *et al*., 2001*a*; Wilkinson & Ishikawa, 2001; Wang *et al*., 2005; Chen *et al*., 2011). It is suggested that YLSs may compensate for the imbalance in composition of amino acids in plant phloem (Lee & Hou, 1987). Our previous research indicated that YLSs may independently synthesize lysine (Lys), arginine, tryptophan, threonine, histidine and cysteine, and work collaboratively with *N. lugens* to biosynthesize proline, methionine, valine, leucine, isoleucine, phenylalanine and tyrosine. Moreover, YLSs independently synthesize Lys, arginine, tryptophan, threonine, histidine and cysteine (Wan *et al*., 2014).

Lys plays a role in growth as an amino acid in protein biosynthesis. In particular, Lys is a substrate for carnitine production. During β-oxidation of long-chain fatty acids, carnitine transfers fatty acids from cytosol into mitochondria. In cytosol, fatty acids are attached with coenzyme A (CoA) to form acyl CoA. Under catalyzation of carnitine acyltransferase I, acyl CoA reacts with carnitine to generate acylcarnitine. Acylcarnitine is then transported across the outer mitochondrial membrane into the mitochondrial matrix by the action of carnitine:acyl-carnitine translocase. In the matrix, inner-mitochondrial carnitine acyltransferase II reconverts acylcarnitine into carnitine and acyl CoA. The latter is then ready for oxidative breakdown (Wolf, 2006). Dietary need of carnitine has been demonstrated in *Tenebrio molitor*, *Tenebrio obscurus* and *Palorus ratzeburgi*, where larvae grew slowly and started to die after 3–4 weeks on a carnitine-free artificial diet (Fraenkel *et al*., 1948; Fraenkel, 1951; Carter *et al*., 1952). In *Drosophila melanogaster*, the loss-of-function mutation of *withered* (*whd*) encoding carnitine palmitoyltransferase I (CPT I) resulted in a crinkled wing phenotype and hypersensitivity to oxidative stress (Strub *et al*., 2008).

It has been documented that Lys may be biosynthesized through the α-aminoadipate (AAA) pathway by *N. lugens*-symbiont (Wan *et al*., 2014) and *Laodelphax striatellus*-symbiont (Yang *et al*., 2012). This pathway is unique to euglenoids, fungi in the subkingdom Dikarya and a Eukaryota species *Capsaspora owczarzaki* (Urrestarazu *et al*., 1985; Kosuge & Hoshino, 1999; Velasco *et al*., 2002; Xu *et al*., 2006). Saccharopine dehydrogenase [SDH, N6-(glutaryl-2)-L-Lys: NAD oxidoreductase, EC 1.5.1.10] catalyzes the reversible reaction of AAA -δ-semialdehyde, L-glutamate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to generate saccharopine (Xu *et al*., 2006, 2007). A *sdh* gene was identified and designated as *Nlylssdh* (*sdh* derived from YLS) in *N. lugens* (Wan *et al*., 2014). In this study, the sequence of full-length cDNA of *Nlylssdh* was verified and analyzed phylogenetically. Furthermore, the temporal or spatial transcript profile of *Nlylssdh* and the effect of RNA interference (RNAi)-aided knockdown of *Nlylssdh* also were studied.

## Materials and methods

### *Sequence verification and phylogenetic analysis*

*N. lugens* were reared on fresh rice plants according to the method described by Wan *et al*. (2014). To verify the complete open reading frame (ORF) of a previously cloned putative *sdh* (*Nlylssdh*, JX125610), a pair of primers (table 1) were designed according to the 5′ and 3′ fragments of *Nlylssdh* (Wan *et al*., 2014). A cDNA sample derived from the third-instar nymphs was used as a template for polymerase chain reaction (PCR).

For phylogenetic analysis, SDH-like proteins from 16 fungi and a Eukaryota species *Capsaspora owczarzaki* were selected and aligned with *Nlylssdh* using ClustalW2.1 (Larkin *et al*., 2007). The alignments were used to construct maximum-likelihood (ML) trees using RAxML version 8 (Stamatakis, 2014) to select the best-fitting model (WAG + I + γ, with empirical frequencies) after estimation by ProtTest (Darriba *et al*., 2011). The reliability of the ML tree topology was evaluated by bootstrapping a sample of 1000 replicates.

### *dsRNA synthesis and bioassays*

Total RNA was isolated from the whole body of hoppers (including both *N. lugens* and YLS) following the manufacturer's standard protocol for TRIzol reagent (Invitrogen, Shanghai, China). First-strand cDNA was synthesized from 1 μg of the total RNA template using Moloney Murine Leukemia Virus reverse transcriptase (Takara Bio., Dalian, China) and an Oligo (dT) 18 primer. The cDNA and polyubiquitin-nuclear localizing sequence-EGFP (PUB-nls-EGFP: provided by Dr Handler, USDA) were used as templates for amplification of the target sequences by PCR, using the gene-specific primers (table 1) incorporating the T7 RNA polymerase promoter sequence (5′-taatacgactcactataggg-3′) (Jia *et al*., 2013*a*, *b*). The PCR products were purified with Wizard H SV Gel (Promega, Madison, WI, USA) and then cloned into pGEM-T easy vector (Promega). Several independent subclones were sequenced on an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) from both directions. The sequenced PCR products (*Nlylssdh*, 368 bp; *egfp*, 414 bp) were used as templates for dsRNA synthesis with the T7 Ribomax TM Express RNAi System (Promega) according to

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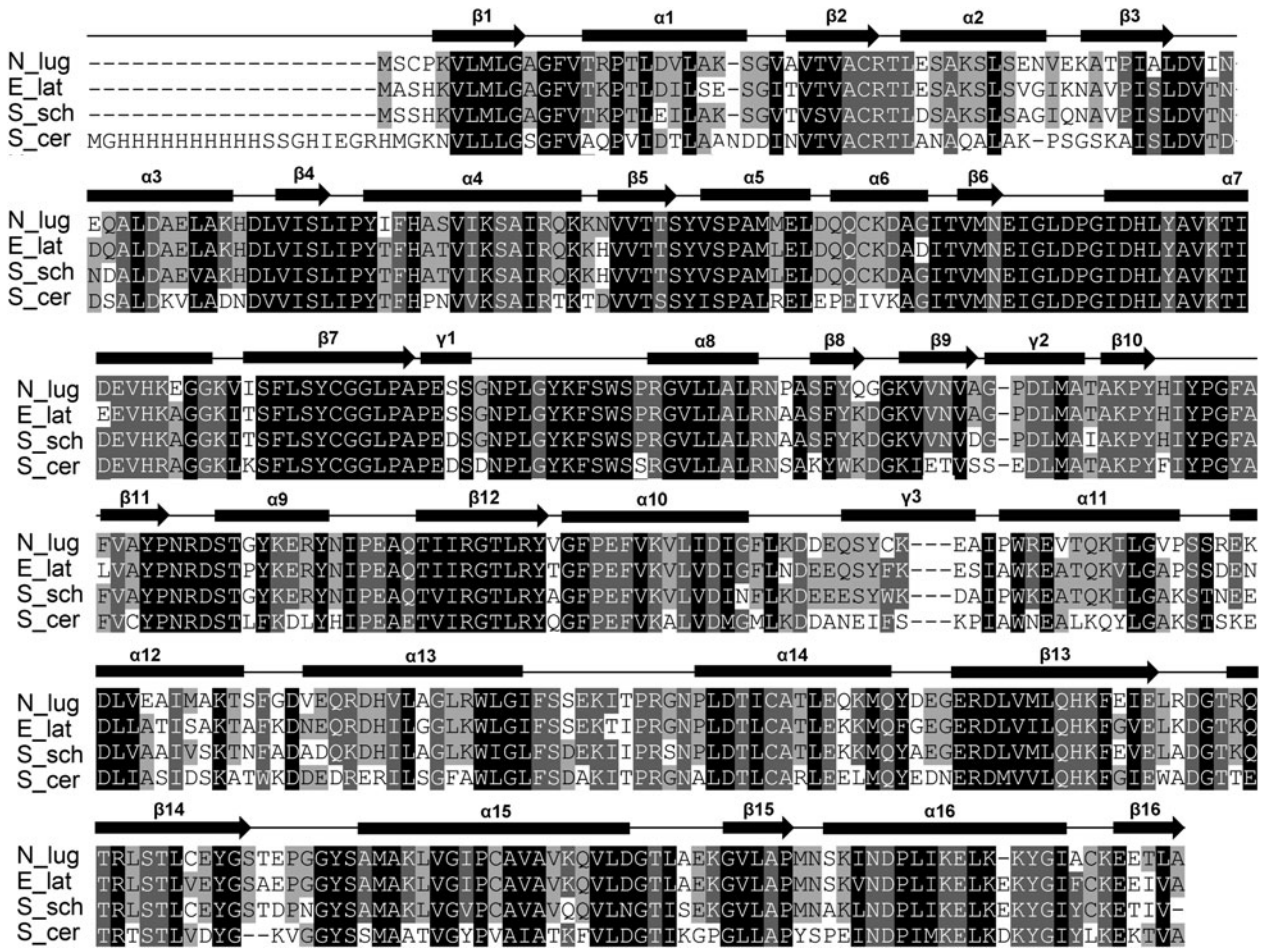


Fig. 1. Sequence alignment of SDH-like proteins. SDH proteins originate from *N. lugens* (N_lug), *Eutypa lata* UCREL1 (E_lat, XP_007795925), *S. schenckii* ATCC 58251 (S_sch, ERS99505) and *Saccharomyces cerevisiae* (S_cer, 2AXQ_A). Secondary-structure elements are marked with arrows (β strands) and filled rectangles (helices). Amino acids with 100, >80, and >60% conservation are shaded in black, dark gray and light gray, respectively. Gaps have been introduced to permit alignment.

the manufacturer's instructions. The synthesized dsRNAs were isopropanol-precipitated, resuspended in nuclease-free water, quantified by a spectrophotometry (NanoDrop TM 1000, Thermo Fisher Scientific, USA) at 260 nm, and kept at −70°C until use.

For bioassays, a dietary dsRNA-delivering procedure (Jia *et al.*, 2013*a*, *b*) was used. Briefly, planthoppers were confined in glass cylinders (12 cm in length and 2.8 cm in internal diameter) and fed with Lys-free artificial diet D-97 (Fu *et al.*, 2001*a*, *b*). The three dietary treatments were non-dsRNA diet (blank control), diet containing ds*egfp* at the concentration of 0.50 mg $ml^{-1}$ (negative control), and diet containing ds*Nlylssdh* at the concentration of 0.50 mg $ml^{-1}$. Twenty *N. lugens* nymphs (3 days old) were carefully transferred into each glass cylinder, with one end covered by a mesh screen, and the other end covered with two layers of stretched Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL, USA) sandwiching a designed liquid diet treatment. All diets were changed daily while dead nymphs were removed. In this experiment, all treatments were replicated 15 times (15 glass cylinders) with a total of 300 nymphs in each treatment (six replicates for biological observation and nine replicates for quantitation of mRNA levels at 5, 10 and 15 days of feeding with three replicates each). The biological observations were made continuously until the adult stage. Mortality was recorded daily. Development duration, sex and brachypterours/macropterours ratios of the surviving adults were determined. Three independent biological replicates were carried out.

### *Quantitative real-time PCR (qRT-PCR)*

To characterize the temporal or spatial transcript profile of *Nlylssdh*, total RNA samples were prepared from the whole bodies of the first through fifth larval instar (I1, I2, I3, I4 and I5) nymphs and sexually mature adults, from (Promega, USA) of sexually mature females, using the SV Total RNA Isolation System Kit (Promega). Samples from the adult survivors of the dsRNA treatment were also prepared. Putative mRNA abundance of *Nlylssdh* in each sample was estimated by qRT-PCR (primers are listed in table 1), using *ribosomal protein S15e* (*RPS15*) and *α2-tubulin* (*TUB*) as internal reference genes (Yuan *et al.*, 2014). All experiments were repeated in technical triplicate. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, using the geometric mean of *RPS15* and *TUB* for

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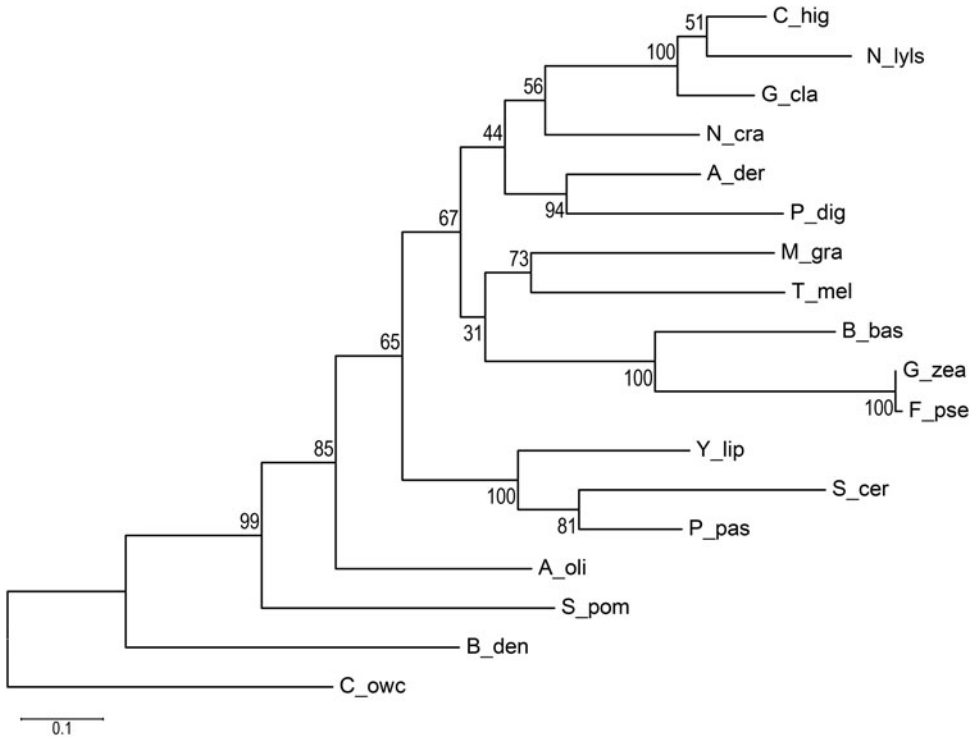


Fig. 2. A phylogenic tree of SDH-like proteins. An unrooted phylogenetic tree was constructed by the ML method (the best-fitting model, WAG + I + γ, with empirical frequency) based on the protein sequence alignments. The SDH-like sequences originated from *N. lugens* YLS (N_lyls), *Colletotrichum higginsianum* strain IMI 349063 (C_hig, H1VZP2), *Grosmannia clavigera* strain kw1407 (G_cla, F0XLZ3), *Neurospora crassa* strain ATCC 24698 (N_cra, F5HDY3), *Ajellomyces dermatitidis* strain SLH14081 (A_der, C5JXA5), *Penicillium digitatum* strain Pd1 (P_dig, K9GUP3), *Mycosphaerella graminicola* strain CBS 115943 (M_gra, F9XHQ0), *Tuber melanosporum* strain Mel28 (T_mel, D5GD57), *Beauveria bassiana* strain ARSEF 2860 (B_bas, J5JS46), *Gibberella zeae* strain PH-1 (G_zea, I1RA28), *Fusarium pseudograminearum strain CS3096* (F_pse, K3VVD3), *Yarrowia lipolytica* strain CLIB 122 (Y_lip, Q6C844), *Saccharomyces cerevisiae* strain RM11-1a (S_cer, B3LPJ3), *Pichia pastoris* strain GS115 (P_pas, C4R4U1), *Arthrobotrys oligospora* strain ATCC 24927 (A_oli, G1XUE7), *Schizosaccharomyces pombe* strain 972 (S_pom, LYS9), *Batrachochytrium dendrobatidis* strain JAM81 (B_den, F4NTD1), *Capsaspora owczarzaki* ATCC 30864 (C_owc, EPH53197). The percentiles of bootstrap values (1000 replicates) are indicated. The scale bar represents the amino acid divergence.

normalization according to the strategy described previously (Yuan *et al*., 2014).

### *Data analysis*

All data from the three independent biological replicates were pooled, expressed as mean ± SE, and analyzed by one-way ANOVA followed by the Tukey–Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA).

## Results

### *Sequence analysis of the putative Nlylssdh*

A full-length cDNA encoding a putative SDH protein was previously identified (Wan *et al*., 2014). Sequence analysis showed that the cDNA consisted of 1528 bp, with an ORF of 1347 bp and encoding 448 amino acids. The predicted protein shares the greatest similarity (91%) with SDH-like proteins from *Sporothrix schenckii*, *Ophiostoma piceae*, *Colletotrichum higginsianum*, *Colletotrichum sublineola* and *Eutypa lata*. Similarly, it has 89% of similarity with those from *Grosmannia clavigera* and *Aspergillus terreus*; 88% of similarity with that from *Gaeumannomyces graminis*; 87% of similarity with those from *Byssochlamys spectabilis* and *Magnaporthe oryzae*. Moreover, it shows 84% of similarity with that from *Trichoderma virens*; 83% of similarity with that from *Chaetomium globosum*; 82% of similarity with those from *Togninia minima*, *Neurospora crassa*, *Thielavia terrestris*, *Myceliophthora thermophile* and *Podospora anserine*. It has 81% of similarity with that from *Chaetomium thermophilum*; 80% of similarity with those from *Macrophomina phaseolina* and *Neofusicoccum parvum*; 77% of similarity with that from *Sordaria macrospora*; and 75% of similarity with that from *Saccharomyces cerevisiae*.

According to the three-dimensional structures of the three domains of ScSDH and MgSDH enzymes (SDH enzymes from *S. cerevisiae* and *Magnaporthe grisea* previously determined by Andi *et al*., 2006 and Johansson *et al*., 2000, respectively), NlylsSDH domain I contains a variant of the Rossmann fold that binds NADPH. It consists of a seven-stranded β-sheet (β1–6 and β15), sandwiched between four helices (α1, 2, 15, 16) on one side and four helices (α3–6) on the other side. NADPH binds at the carboxy end of this β sheet, containing a conserved consensus sequence Gly–Ser–Gly–Phe–Val–Thr at the amino acid residues 10–15 (fig. 1).

Comparative fold alignment with the ScSDH and MgSDH enzymes showed that NlylsSDH domain II contains a central seven-stranded mixed β-sheet (β7–9, β12–14 and β16). This

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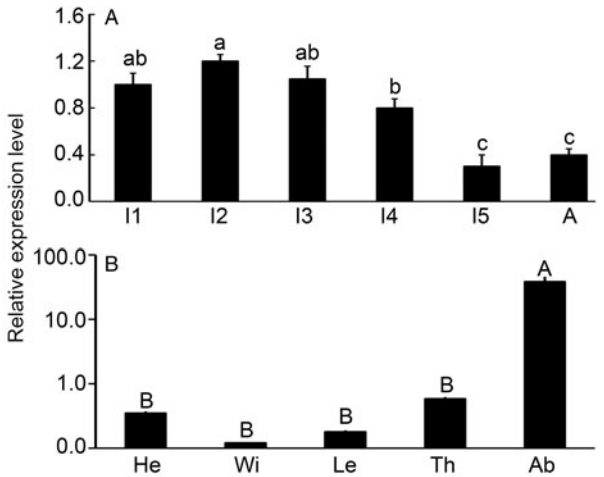


Fig. 3. Temporal and spatial expression patterns of *Nlylssdh*. (A) Relative expression levels of *N. lugens* first-, second-, third-, fourth- and fifth-instar nymphs (I1, I2, I3, I4 and I5, respectively), and sexually mature adults; (B) relative expression levels of heads (He), wings (Wi), legs (Le), thoraces (Th) and abdomens (Ab) of sexually mature females. For each sample, three independent pools of five to ten individuals were measured in technical triplicate using qRT-PCR. The bars represent $2^{-\Delta\Delta CT}$ values (±SE) normalized to the geometrical mean of housekeeping gene expression. Different lowercase or uppercase letters indicate significant difference at $P$ value <0.05 or <0.01, respectively.

mixed β-sheet is surrounded by helices α7 and α9 on one side and helices α8, γ1 and γ2, and strands β10–11 on the other side. Domain II is involved in substrate binding and dimer formation. The loop connecting the β9 and β10 strands is involved in dimer interaction. NlylsSDH domain III is inserted between strands β12 and β13 in the domain II and is composed of helices α10–14 and γ3. Domain III is involved in closing the active site of the enzyme once substrates are bound (fig. 1).

The phylogenetic tree showed that the SDH-like proteins of the fungi species formed a large cluster, and SDH of *C. owczarzaki* formed a separate cluster. These two clusters were well segregated from each other. Among the fungi cluster, 16 proteins from Ascomycota phylum were well segregated from that from Chytridiomycota phylum species *Batrachochytrium dendrobatidis*. Within the Ascomycota phylum subcluster, NlylsSDH was the closest to those from *Colletotrichum higginsianum* and *G. clavigera* (fig. 2), similar to YLS phylogenetic position based on partial 18s rDNA sequences (Noda *et al.*, 1995).

### *Expression patterns of Nlylssdh*

qRT-PCR revealed that *Nlylssdh* showed a high expression peak in the second-instar nymphs, followed by those in the first-, third- and fourth-instar nymphs. However, there were no significant differences among the first four nymph stages. *Nlylssdh* expression level in the fifth-instar nymphs was significantly lower than that of the four other instars. The *Nlylssdh* mRNA level in adults was slightly higher than that of the fifth-instar nymphs with no statistical difference (fig. 3A).

The spatial mRNA level of *Nlylssdh* in sexually mature female adults showed that *Nlylssdh* clearly had a higher transcript level in the abdomens where YLSs were located, in comparison with the heads, wings, legs and thoraces (fig. 3B). The lower levels detected in heads, wings, legs and thoraces were likely resulted from fat body contaminations during dissection.

### *Effect of dsRNA on the expression of Nlylssdh mRNA level and planthopper development*

*Nlylssdh* mRNA abundance in the surviving planthoppers after ds*Nlylssdh* exposure for 5, 10 and 15 days were decreased by 47, 70 and 31%, respectively, comparing with the blank control. As expected, *Nlylssdh* mRNA level in the negative control was not significantly different from that in the blank control (fig. 4A).

Continuous ingestion of ds*Nlylssdh* reduced the average body weights of male and female adults by 19 and 15%, respectively, comparing with the blank control. However, the ANOVA analysis revealed that there were no significant differences among planthoppers fed with normal, ds*egfp*-, or ds*Nlylssdh*-containing diets (fig. 4B).

Male planthoppers ingesting normal, ds*egfp*- or ds*Nlylssdh*-containing diets had the average nymphal development durations of 26.1, 26.4 and 26.6 days, respectively. No significant differences were found among them. Female planthoppers having exposed to normal, ds*egfp*- or ds*Nlylssdh*-containing diets had the average nymphal development stages of 28.0, 28.3 and 30.0 days, respectively. The ds*Nlylssdh*-containing diet significantly delayed the development of female planthoppers compared with that of the blank or negative controls (fig. 4C).

Ingestion of the ds*Nlylssdh*-containing diet for 22, 24 and 26 days (with nymphal stages of 25, 27 and 29 days, respectively), caused higher nymphal mortalities (19, 24 and 27%, respectively), comparing with the normal or ds*egfp*-containing diet treatments (fig. 4D). Since the average nymphal durations for males and females were 26 and 28 days, respectively, many of the deaths occurred during nymph–adult ecdysis. Moreover, some adult individuals showed two apparent phenotypic defects. One is that the wings were wrinkled, puckered and/or bent, and not extended properly during adult ecdysis. The other is that the nymphal cuticles remained on the tips of legs and abdomens and were not completely shed (fig. 5).

The sex ratios (female/male) of the adults that reared on normal, ds*egfp*- and ds*Nlylssdh*-containing diets were 1.39, 1.41 and 1.12, respectively (fig. 4E). The ratios of brachypterours/macropterours individuals were 1.05, 1.06 and 0.81, respectively (fig. 4F).

## Discussion

In this study, a full-length cDNA encoding a putative SDH protein (previously identified and designated as *Nlylssdh*) was characterized in *N. lugens*. We provided three independent lines of evidence to support that the *Nlylssdh* was from the YLSs in *N. lugens*. Firstly, SDH catalyzes a reaction in the AAA pathway which is unique to fungi in the subkingdom Dikarya, the euglenids, and a Eukaryota species *C. owczarzaki* (Urrestarazu *et al.*, 1985; Kosuge & Hoshino, 1999; Velasco *et al.*, 2002; Xu *et al.*, 2006). The fact that the *sdh* gene identified in *N. lugens* strongly suggests that the gene comes from YLS

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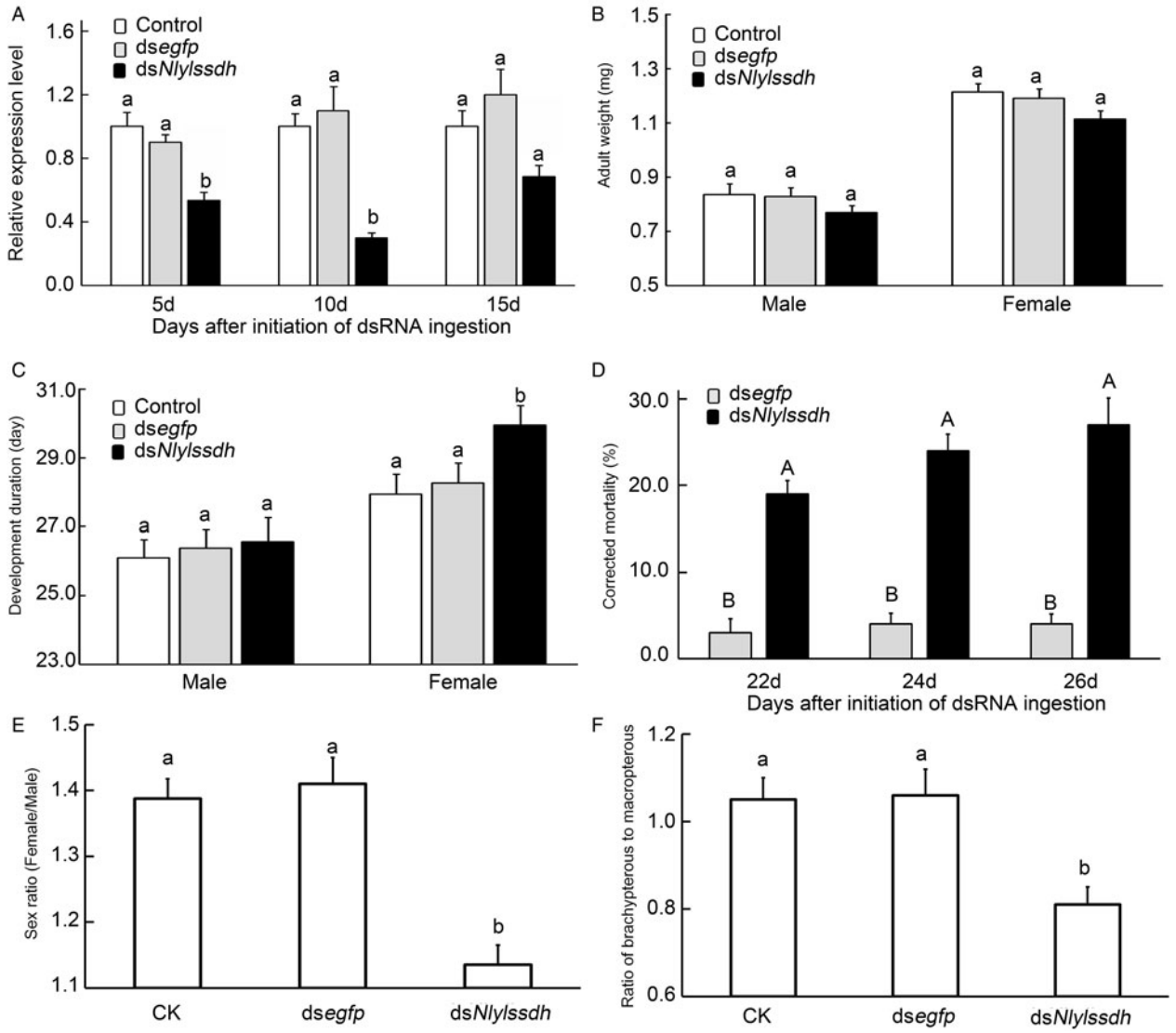


Fig. 4. The performance of *N. lugens* subjected to dietary dsRNA exposure. (A) Transcript levels of *Nlylssdh* in nymphs after fed on the dsRNA or normal diets. The bars represent $2^{-\Delta\Delta CT}$ values (±SE) normalized to the geometrical mean of housekeeping gene expression. (B) Adult body weight (mg); (C) nymphal stage duration; (D) accumulative mortalities; (E) sex ratio of adults; and (F) the ratio of brachypterous to macropterous adults. The bars represent means with vertical lines indicating SE. Bars topped with the same letters are not statistically different at $P < 0.05$.

genome rather than *N. lugens*. Secondly, phylogenetic analysis showed that NlylsSDH was closest to those from *Colletotrichum higginsianum* and *Grosmannia clavigera*, similar to YLS phylogenetic position based on partial 18s rDNA sequences (Noda *et al*., 1995). Finally, *Nlylssdh* transcript level was clearly much higher in the abdomens compared with other tested tissues. YLSs reside intracellularly in planthopper's mycetocytes formed by abdominal fat body cells and propagated by asexual budding (Chen *et al*., 1981). Beside abdominal fat body cells, YLSs are distributed in ovaries and eggs (in abdomens), but not in heads, legs, thoraces and wings (Chen *et al*., 1981; Cheng & Hou, 2001; Dong *et al*., 2011), and are vertically transmitted to the next generation through female ovaries (Cheng & Hou, 2001). Thus, a fungal uniqueness pathway participated by SDH, phylogenetic closeness to fungi and abdominal dominated spatial expression pattern strongly suggested that *Nlylssdh* is from the YLSs in *N. lugens* rather than *N. lugens* itself.

Previous studies showed that lack of carnitine delayed larval growth and caused larval death in several beetle species (Fraenkel *et al*., 1948; Fraenkel, 1951; Carter *et al*., 1952). Moreover, in *D. melanogaster* a mutation of *yellow* gene caused elevated saccharopine and Lys, and had negative effect on larval development (Bratty *et al*., 2012). Thus, a lack or excess of saccharopine can negatively affect insect development. In this study, ingestion of dsRNAs targeting the *Nlylssdh* slightly decreased the body weights, delayed nymphal development, and killed approximately 30% of planthoppers. Similar

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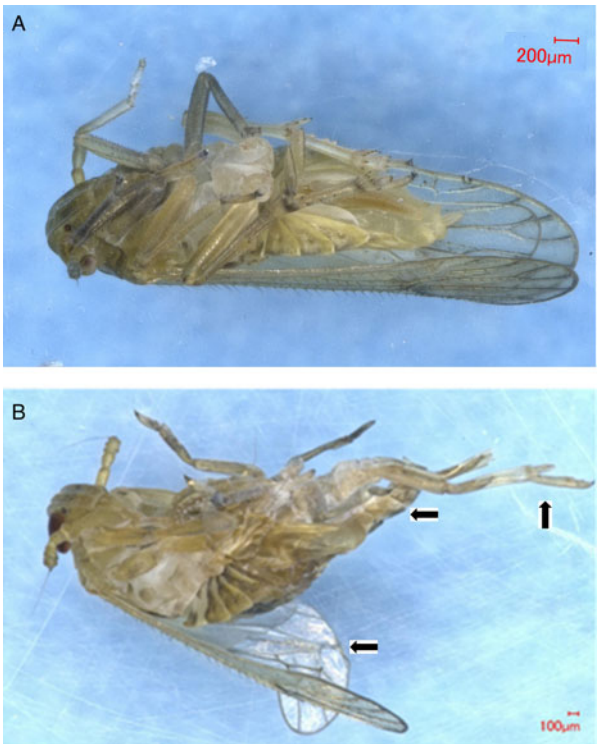


Fig. 5. Normal adult (A) and adult with phenotypic defects (B) caused by dsRNA ingestion during nymphal stage. Two phenotypic defects are apparent: wing deformation and old nymphal cuticles on the tips of legs and abdomen that were not shed off (marked with arrows).

phenotypical observations were reported when YLSs were experimentally removed in *N. lugens*, i.e., aposymbiotic nymphs lost weight, grew slowly, and had lower total protein concentrations (Fu *et al.*, 2001*a*; Wilkinson & Ishikawa, 2001; Wang *et al.*, 2005; Chen *et al.*, 2011). *Tribolium confusum* larvae fed on barleys with higher Lys concentrations developed faster than that on barleys with lower Lys concentrations (Loschiavo, 1980). An addition of carnitine significantly increased the yields of *D. melanogaster* S2 cells (Gingle, 1985). A mutation of the gene encoding CPT I resulted in a crinkled wing phenotype in *D. melanogaster* (Strub *et al.*, 2008). We found that the knockdown of *Nlylssdh* caused two phenotypic defects: wing deformation and the inability to shed old nymphal cuticles on the tips of legs and abdomens. All these results suggest that NlylsSDH plays a role in Lys biosynthesis in *N. lugens* and that Lys is important for insect development.

The current study also showed that the brachypterours/macropterours ratio was decreased by dsRNA exposure. In *N. lugens*, wing dimorphism is nutrition dependent (Zou *et al.*, 1982). The rate of brachypterours is positively correlated with the amounts of total nitrogenous substances and free amino acid content, and negatively correlated with the sugar content in rice plants (Zhang, 1983). Moreover, the brachypterours morph occurs at low population densities, whereas the macropterours morph often emerges at high population density (Kishimoto, 1956; Morooka & Tojo, 1992). Population density can affect nutritional status and consequently change wing dimorphism in *N. lugens*. Therefore, the effect of *Nlylssdh* knockdown on wing dimorphism might be a result of poor nutritional status due to the lower Lys biosynthesis.

In this study, we found that *Nlylssdh* knockdown had little influence on male development duration, but significantly delayed female development. Moreover, the sex ratio (female/male) was lower in the ds*Nlylssdh*-ingesting planthoppers, suggesting that more females than males were dead due to *Nlylssdh* knockdown. These results suggest that female planthoppers were more susceptible to *Nlylssdh* knockdown. Similarly, in *Choristoneura fumiferana*, nutritional stress affected sex ratio of pupae and adults. Low quality food resulted in fewer females (Quezada-Garcia *et al.*, 2014). Female insects are often heavier (Charnov *et al.*, 1981; Caswell & Weeks, 1986), and need more nutrients, especially nitrogenous compounds, to ensure egg production, when compared with their male partners (Isaac *et al.*, 2010). In some species such as *Lymantria dispar*, females undergo an additional larval instar in order to accumulate nutrients (Telang *et al.*, 2001). Alternately, females may obtain more nutrients by ingesting food at a greater rate than males to accumulate more nitrogenous compounds (Nakano & Monsi, 1968; Mathavan & Bhaskaran, 1975; Horie *et al.*, 1976; Bhat & Bhattacharya, 1978). For example, *L. dispar* terminal-instar female larvae ate a high-protein and low-lipid content diet, whereas male larvae ate more of a low-protein/high-lipid diet (Stockhoff, 1993). Similarly, *Heliothis virescens* larvae accumulate proteins by regulating both intake and post-ingestive processing with high-protein foods (Telang *et al.*, 2001). Therefore, *N. lugens* females might be more sensitive to deficiencies of nitrogenous nutrients or amino acids.

Finally, our results provide a proof of concept example that the RNAi-induced effects in *N. lugens* could serve as a possible method for pest control. Off-target silencing of gene homologs by dsRNA in non-target organisms is a major road block in successful commercialization of dsRNA-based insecticides because it could adversely affect non-target organisms including parasites, predators and pollinators (Palli, 2014). In this perspective, knockdown of a gene that comes from YLS genome could avoid unwanted effect on non-target organisms. Since insect genomes contain no SDH genes, dsRNA that targets *Nlylssdh* can indirectly affect pests that harbor YLSs (Chen *et al.*, 1981; Cheng & Hou, 2001; Dong *et al.*, 2011), and be safe for non-target organisms with no YLSs.

## Acknowledgements

This research was supported by the National Natural Science Foundation of China (31371939) and the Rice Pest Management Research Group of the Agricultural Science and Technology Innovation Program of China Academy of Agricultural Science.

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