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Feeding-based RNA interference of a *trehalose phosphate synthase* gene in the brown planthopper, *Nilaparvata lugens*

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Abstract

The brown planthopper, *Nilaparvata lugens*, is the most devastating rice insect pest to have given rise to an outbreak in recent years. RNA interference (RNAi) is a technological breakthrough that has been developed as a powerful tool for studying gene function and for the highly targeted control of insect pests. Here, we examined the effects of using a feeding-based RNAi technique to target the gene *trehalose phosphate synthase* (*TPS*) in *N. lugens*. The full-length cDNA of *N. lugens TPS (NITPS)* is 3235 bp and has an open reading frame of 2424 bp, encoding a protein of 807 amino acids. *NITPS* was expressed in the fat body, midgut and ovary. Quantitative real-time PCR (qRT-PCR) analysis revealed that *NITPS* mRNA is expressed continuously with little change during the life of the insect. Efficient silencing of the *TPS* gene through double-stranded RNA (dsRNA) feeding led to rapid and significant reduction levels of *TPS* mRNA and enzymatic activity. Additionally, the development of *N. lugens* larvae that had been fed with the dsRNA was disturbed, resulting in lethality, and the cumulative survival rates dropped to 75.56, 64.44, 55.56 and 40.00% after continuous ingestion of 0.5 μg/μl dsRNA for 2, 4, 7 and 10 days, respectively. These values were significantly lower than those of the insects in the control group, suggesting that *NITPS* dsRNA may be useful as a means of insect pest control.

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Introduction

The brown planthopper, *Nilaparvata lugens*, is a member of the Hemiptera order and a serious rice pest in many parts of Asia. In China, *N. lugens* occurred over areas totalling 9.3 million square hectometer (hm^2) in 1974 and in 2005, this area had risen to 23.23 million hm^2, causing huge economic losses (Wang, 2007). It migrates long distances every year to invade the rice growing areas of China, Japan and Korea (Rosenberg & Magor, 1983; Akira *et al*., 2005; Riley *et al*., 2008). Huge numbers of offspring in rice fields produced by female adults of the immigrants attack rice plants and suck fluid from the vascular bundles wherever they go. Heavy infestation in the fields causes death of the rice plants. The principal control methods of *N. lugens* are the use of chemical pesticides and the cultivation of resistant varieties of rice (Noda *et al*., 2008). However, strains of *N. lugens* that are resistant to chemical pesticides have expanded and appear to against the resistant rice (Wu *et al*., 2001; Noda *et al*., 2008). Therefore, more potent and powerful means are desperately needed for *N. lugens* control.

RNA interference (RNAi) is a mechanism for post-transcriptional gene silencing that has been developed as a powerful tool for studying gene function in a variety of organisms. Since RNAi was first discovered in the nematode *Caenorhabditis elegans* (Fire *et al*., 1998), scientists have explored a variety of efficient means to transport double-stranded RNA (dsRNA) into organisms, including microinjection, soaking, oral feeding and transgenic plant expression (Eaton *et al*., 2002; Baum *et al*., 2007; Mao *et al*., 2007; Chen *et al*., 2008; Tian *et al*., 2009). Two research groups, in particular, have made prodigious progress in exploiting transgenic plants that express insect dsRNAs for entomological research and insect pest control, suggesting that RNAi could be a

useful method of pest control. To achieve this goal, large scale screening methods of pest gene pools are required (Tian *et al*., 2009). For small insects, such as *N. lugens*, oral feeding is considered an excellent method because of its convenience and low rate of injury.

Trehalose is a disaccharide sugar that consists of two glucose molecules linked by an α, α-1, 1-glycosidic linkage. It is widely distributed amongst plants, microorganisms, fungi, nematodes and insects, but is absent in mammals (Elbein, 1974; Elbein *et al*., 2003; Tang *et al*., 2008). Trehalose always serves as a stabilizing agent of cellular structures under stress conditions and has a special capacity for the protection of cellular membranes and proteins from the undesirable effects of heat, cold or dehydration (Crowe *et al*., 1984; Garg *et al*., 2002; Elbein *et al*., 2003). In yeast and plants, the sugar can also be used as a signalling molecule to direct or regulate certain metabolic pathways, or to affect growth. It also serves as an energy and carbon source in many organisms (Muller *et al*., 1999; Arguelles, 2000; Bonini *et al*., 2000; Paul *et al*., 2001; Elbein *et al*., 2003). In insects, trehalose is the main blood sugar. It is synthesized in the fat body and released into the haemolymph (Wyatt, 1967; Becker *et al*., 1996; Thompson, 2003; Gu *et al*., 2009). The sugar is thought to be necessary for thermotolerance in the larvae and is involved in regulating pupal diapause (Wyatt, 1967; Thompson, 2003; Xu *et al*., 2009). Moreover, it is also considered as an important factor in the confrontation of environmental stress, especially at low temperature. Therefore, trehalose and its metabolism are pivotal for the growth, development and survival of insects (Xu *et al*., 2009). The biosynthesis of trehalose is known: trehalose-6-phosphate synthase (TPS: E.C.2.4.1.15), catalyses the reaction of uridine diphosphate glucose (UDPG) and glucose-6-phosphate to form trehalose-6-phosphate, which is converted into free trehalose by trehalose-6-phosphate phosphatase (TPP: E.C.3.1.3.12). As trehalose is the main reserve sugar in the haemolymph serving as a carbon and energy source for flying insects (Gu *et al*., 2009), it is meaningful to study the function of TPS, one of the main factors regulating trehalose levels in the insect haemolymph.

In this study, we report the existence and characterization of a *TPS* gene in *N. lugens* and show that feeding of *NlTPS* dsRNA solutions to *N. lugens* larvae resulted in a strong decline in the expression of the target gene and insect survival rates. Our results suggest that the use of feeding-based RNAi in *N. lugens* has great potential as a functional genomic tool. In addition, *NlTPS* may be a candidate gene for the construction of RNAi plants for use in insect pest control.

Results

Cloning and sequence analysis of NlTPS cDNA

A 1364 bp cDNA fragment was obtained using degenerate primers by PCR amplification. Sequence analysis showed that the deduced amino acid sequence of this cDNA fragment had 84% identity with the corresponding region of *Diabolocatantops pinguis* TPS (ACV32626). We used rapid amplification of cDNA ends (RACE) to clone the full-length cDNA encoding *N. lugens TPS*. The full-length *NlTPS* sequence is 3235 bp and has an open reading frame (ORF) of 2424 bp, a 5′,untranslated region (UTR) of 477 bp and a 3′,UTR of 334 bp (Fig. 1). The ORF of *NlTPS* encodes a polypeptide of 807 amino acids with a calculated molecular weight of 90.5 kDa. The *NlTPS* cDNA encodes a precursor polypeptide that contains both *TPS* and *TPP*, consistent with other *TPS* genes that have been characterized in other insects.

A phylogenetic tree was constructed based on the full-length sequences of known TPSs from insects and other organisms by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software (Fig. 2). Results using BLAST showed that the amino acid sequence of NlTPS has the greatest similarity to the TPSs from *D. pinguis* (Orthoptera) (79%) (ACV32626) and *Locusta migratoria manilensis* (Orthoptera) (79%) (ABV44614), followed by *Acyrthosiphon pisum* (Hemiptera) (76%) (XP_001944221), *Tribolium castaneum* (Coleoptera) (75%) (XP_975776), *Nasonia vitripennis* (Hymenoptera) (75%) (XP_001603693), *Helicoverpa armigera* (Lepidoptera) (74%) (ACH88521), *Aedes aegypti* (Diptera) (73%) (XP_001657813), *Spodoptera exigua* (Lepidoptera) (73%) (ABM66814), *Harmonia axyridis* (Coleoptera) (70%) (ACL50548) and *Drosophila melanogaster* (Diptera) (70%) (NP608827).

The tissue distribution and developmental expression of NlTPS mRNA

The expression of *NlTPS* mRNA was investigated in various tissues in one-day-old female *N. lugens* individuals. *TPS* was highly expressed in the fat body, and a slightly lower rate of expression was detected in the midgut and ovary, but not in the epidermis. The level of *β-ACTIN* was monitored in all tissues and used as a control to normalize for loading (Fig. 3). We used quantitative real-time PCR (qRT-PCR) to characterize the pattern of developmental expression of the *NlTPS* gene in all developmental stages (including larvae from the first to fifth instars and 1–15 days female adults). The resulting data showed that the *NlTPS* mRNA expression level was constant and low with a little change, and that it was only slightly higher at the late stage (days 9–15) in female adults (Fig. 4).

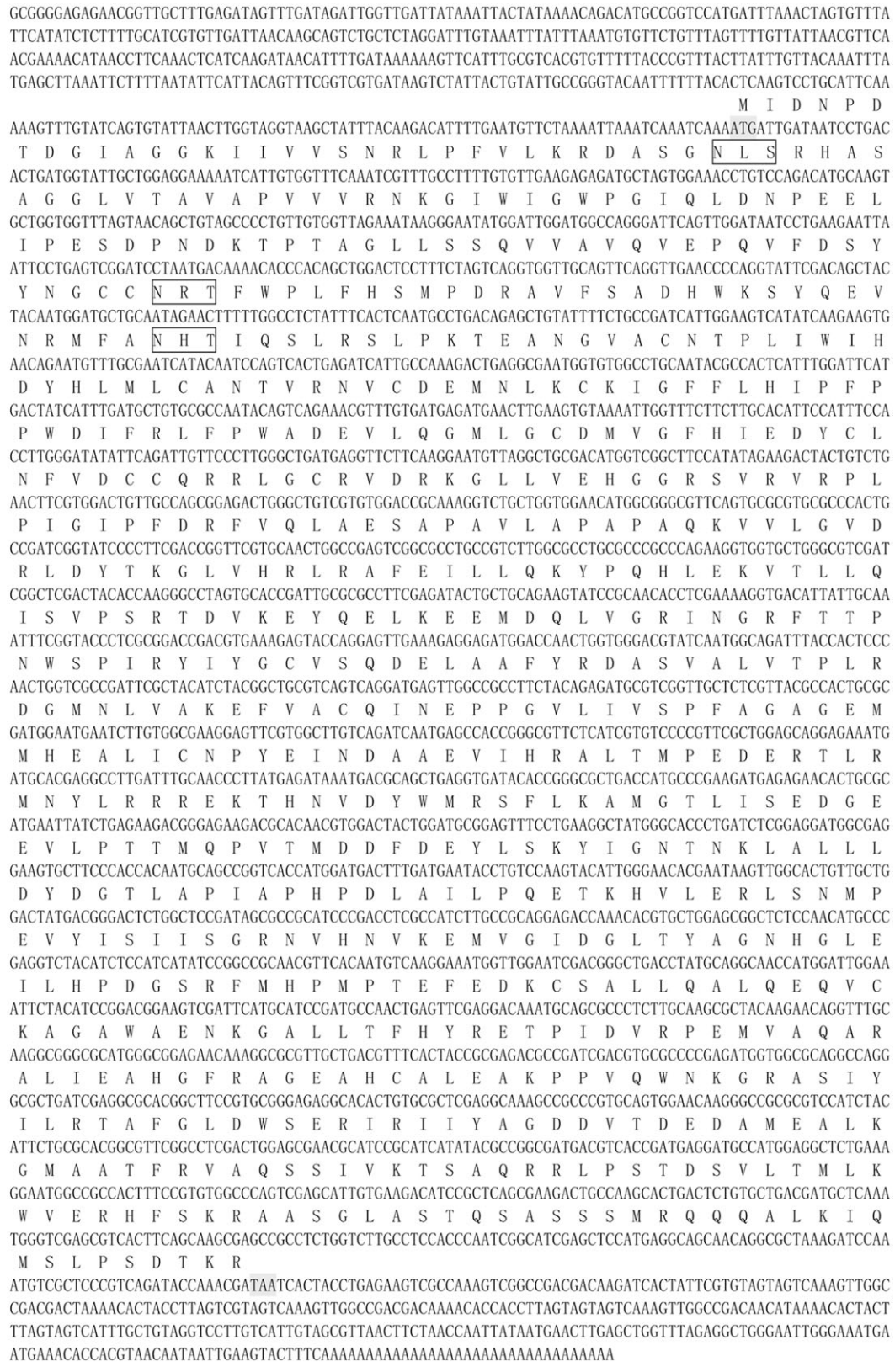

Figure 1. The nucleotide and deduced amino acid sequences of *Nilaparvata lugens* trehalose phosphate synthase. The deduced amino acid sequence is shown above the cDNA sequence. The start and stop codons are shaded in grey. Three potential N-glycosylation sites are boxed.

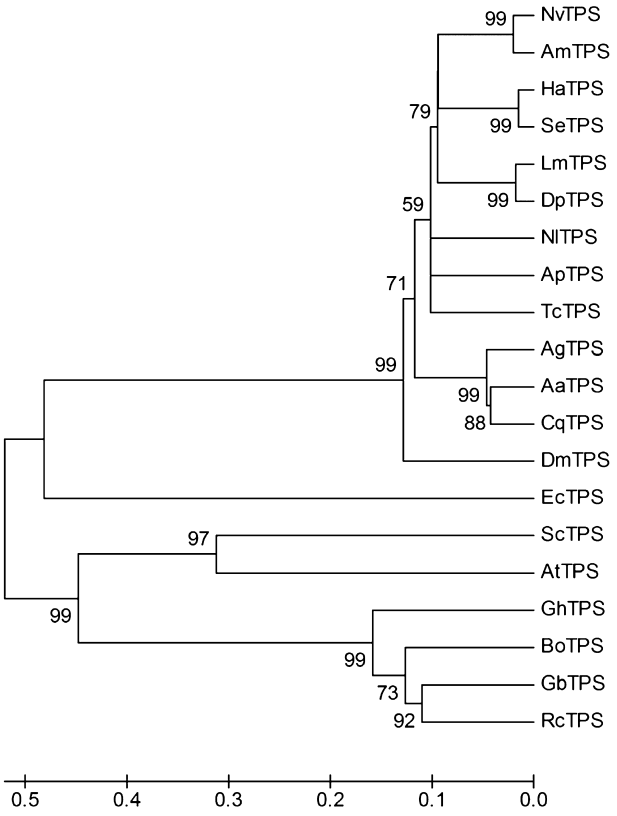

Figure 2. A phylogenetic tree of trehalose phosphate synthases (TPSs) in insects and other organisms. The tree was constructed by neighbor-joining method using MEGA software. The sequences were obtained from GenBank under the accession numbers: *DpTPS* (ACV32626), *LmTPS* (ABV44614), *ApTPS* (XP_001944221), *TcTPS* (XP_975776), *NvTPS* (XP_001603693), *HaTPS* (ACH88521), *AaTPS* (XP_001657813), *AmTPS* (XP_392397), *SeTPS* (ABM66814), *HaxTPS* (ACL50548), *AgTPS* (XP_317243), *CqTPS* (XP_001850998), *DmTPS* (NP608827), *ScTPS (*ABU44491*), EcTPS(*NP_416410*), AtTPS (*AAD30578*), GbTPS (*AAX16014*), RcTPS (*XP_002522255*), BoTPS (*ABD65165*)* and *GhTPS* (AAV65495).

Survival rates after feeding-based RNAi

RNAi was achieved in *N. lugens* by feeding synchronous groups of third instar larvae. We were interested in whether inhibition of the expression of *NlTPS* mRNA leads

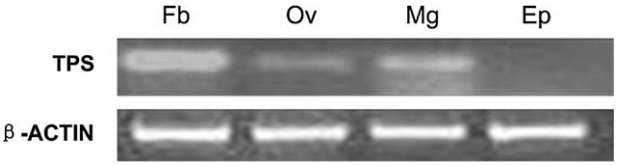

Figure 3. The tissue distribution of *Nilaparvata lugens trehalose phosphate synthase* (*NlTPS*) mRNA. An analysis of *NlTPS* mRNA in tissues using reverse transcription PCR (RT-PCR). Total RNAs (1 μg each) were reversed transcribed to cDNA using an oligo dT_{18} primer from the fat body (Fb), midgut (Mg), epidermis (Ep) or ovary (Ov) of about 100 one-day-old female *N. lugens* adults. The cDNA was used as a template for PCR reactions using *NlTPS*-specific primers (35 cycles) or *β-ACTIN* specific primers (30 cycles). The PCR products were analysed using an agarose gel and stained with ethidium bromide.

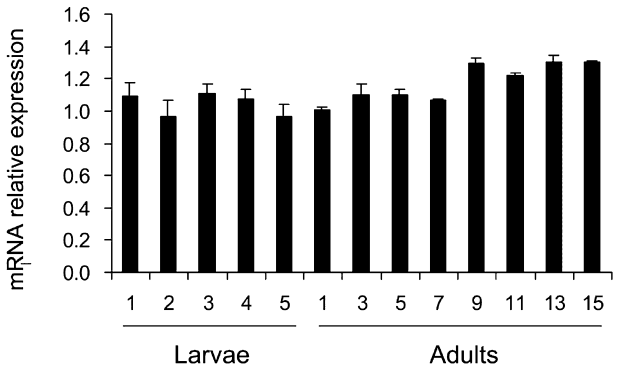

Figure 4. The developmental expression pattern of *Nilaparvata lugens trehalose phosphate synthase* (*NlTPS*) mRNA. The levels of *NlTPS* mRNA were detected using quantitative real-time PCR. Total RNAs (1 μg each) were prepared from individuals of *N. lugens* collected at all stages of development from the first to fifth instar larvae and female adults, and transcribed to cDNA using an oligo dT_{18} primer. The mRNA level was normalized relative to the *β-ACTIN* transcript levels. The data represent the mean values ± SE of three replicates.

to a lethal phenotype in *N. lugens* larvae. Larvae were continuously fed a diet containing dsRNA until the first day of the adult stage, using three different dsRNA concentrations (0.02, 0.1, 05 μg/μl). Prior to 1 day post-ingestion, no obvious abnormal phenotypes were observed compared to the double-stranded RNA of green fluorescent protein (dsGFP) group. However, after continuous feeding with the dsTPS-containing (high concentration: 0.5 μg/μl) diet, the average survival rates decreased to 75.56, 64.44, 55.56 and 40.00% on the second, fourth, seventh and 10th day, respectively. These were significantly lower than those of the insects in the dsGFP groups (Fig. 5). In the larvae fed on medium (0.1 μg/μl) and low doses

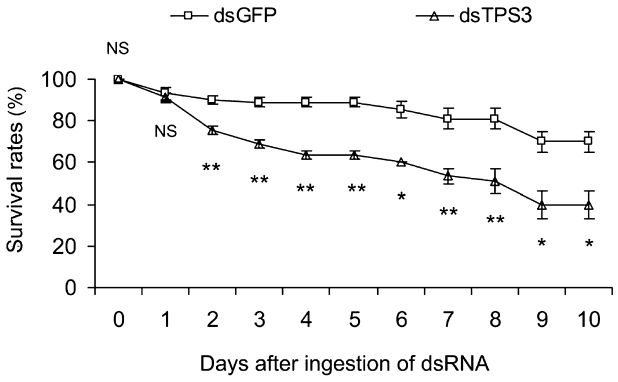

Figure 5. The cumulative survival rates of *Nilaparvata lugens* after ingestion of high dose double-stranded RNA (dsRNA; 0.5 μg/μl, dsTPS3). An asterisk indicates significant differences of the survival rates between the treated and control groups measured on the same day, as determined using a *t*-test ($P < 0.05$). Double asterisks indicate differences at the $P < 0.01$ level. All error bars represent the SE of the mean, as determined from three independent replicates. TPS, trehalose phosphate synthase.

(0.02 μg/μl) of dsTPS or control larvae, no obvious differences were found in the survival rate (data not shown).

Quantitative analyses of the mRNA and enzyme activity after feeding-based RNAi

To better obtain a quantitative measure of the efficiency of feeding for RNAi, we detected the transcript level of *NlTPS* using qRT-PCR and measured the level of enzyme activity using a colorimetric method. Larvae that were used in the qRT-PCR analysis were continuously fed a diet containing dsRNA using three different dsRNA concentrations. qRT-PCR analysis revealed that the mRNA abundance of *NlTPS* substantially decreased after feeding 0.5 μg/μl *NlTPS* dsRNA for 2, 4, 7 and 10 days($P < 0.01$, *t*-test). In particular, the abundance of *NlTPS* mRNA dropped significantly after 7 days to approximately 70% of its original level. *NlTPS* mRNA knockdown was also found in medium dose (0.1 μg/μl) dsTPS feeding larvae for 4, 7 and 10 days. However, ingestion of 0.02 μg/μl *NlTPS* dsRNA did not result in an obvious decrease in *NlTPS* mRNA levels (Fig. 6A). The level of TPS enzyme activity also declined on the second, fourth, seventh and 10th day after ingestion of 0.5 μg/μl dsTPS, as compared to the negative dsGFP control (Fig. 6B). In particular, on the seventh day, the enzyme activity had decreased by 50% when compared to the control group. However, on the 10th day, the level of enzyme activity had a slight increase to 60%. This result confirms that the *TPS* gene is susceptible to silencing by RNAi using the feeding method.

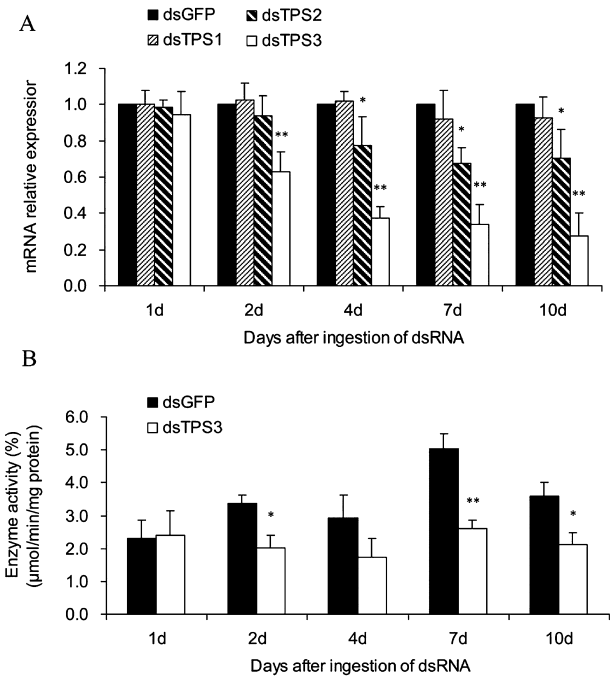

Figure 6. Detection of the mRNA level and enzyme activity level after feeding-based RNA interference (RNAi). (A) The levels of *Nilaparvata lugens trehalose phosphate synthase* (*NlTPS*) mRNA after feeding double-stranded TPS (dsTPS) or double-stranded RNA of green fluorescent protein (dsGFP). dsTPS1, dsTPS2 and dsTPS3 represent the larvae fed on 0.02, 0.1, 0.5 μg/μl dsRNA concentrations, respectively. The mRNA expression level in the dsGFP group was designated as one. The results of the quantitative real-time PCR detection were normalized to the housekeeping gene, *β-ACTIN*. (B) Changes in TPS enzyme activity. dsTPS3 represents the larvae fed on 0.5 μg/μl dsRNA concentrations. An asterisk indicates significant differences in the survival rates between the treated and control groups measured on the same day, as determined using a *t*-test ($P < 0.05$). Double asterisks indicate differences at the $P < 0.01$ level. All error bars represent the SE of the mean, as determined from three or four independent replicates.

Discussion

Previous studies have demonstrated that feeding-based RNAi can specifically lead to gene silencing in several insects. For example, the ingestion of dsRNA (13 μg per larva) of a salivary *nitrophorin 2* gene in the hemipteran species *Rhodnius prolixus* reduced the expression of the target gene and the saliva of normal insects prolonged the coagulation of plasma for approximately four times longer than in knockdown insects (Araujoa *et al*., 2006). Moreover, the oral delivery of an artificial diet containing bacterially expressed dsRNA of chitin synthase A to larvae of the lepidopteran species *S. exigua* not only suppressed its transcription levels, but also led to lethal phenotypes (Tian *et al*., 2009). Thus, until now, it was not clear whether the hypothesis that RNAi can be used as a functional genomic tool in the Hemiptera species *N. lugens* is tenable. Here, we have demonstrated the feasibility of RNAi via feeding and shown that it can specifically inhibit the transcription level of a target gene in *N. lugens*. Knockdown of the target gene *NlTPS* not only suppresses its transcription and enzyme activity levels, but also affects larval growth and development, leading to an increasing mortality rate over time.

In recent years, outbreaks of *N. lugens* have occurred more frequently in China (Wang *et al*., 2008). It is a major migratory insect pest (Cheng *et al*., 1979) that can migrate from Vietnam, which is considered to be the major source for the north-bound migration to China every year (Huo *et al*., 2002). Only a few *N. lugens* individuals can live through the winter in China. Therefore, the migration nature of *N. lugens* is the most probable reason for its outbreaks in China. Previous research has demonstrated that a high concentration of trehalose in the haemolymph in the flight muscle is vital for flight in many insects (van der Horsta *et al*., 1978; Becker *et al*., 1996). For example, the concentration of trehalose in the blood of *Phormia regina* was found to determine the rate of energy expenditure during flight (Clegg & Evans, 1961). It has also been found that trehalose contributes to flight muscle energy metabolism substantially during durative tethered flight

(van der Horsta *et al*., 1978). These findings led us to presume that the expression level of *TPS*, as the key factor in trehalose synthesis, would affect the level of trehalose in the haemolymph, possibly affecting flight ability or growth and development. Based on this, *TPS* could be a feasible target for RNAi-based pest control measures. In this study, we first cloned and characterized a cDNA sequence from *N. lugens* encoding *TPS*, which shares a high identity (79%) with the *D. pinguis TPS* gene that codes for both *TPS* and *TPP*. Pattern analysis of the developmental expression of the *TPS* gene suggested that the expression level of *TPS* mRNA in *N. lugens* was almost constant throughout the life cycle, a result consistent with that of *HaTPS* in a nondiapause type from *Helicoverpa armigera* (Xu *et al*., 2009). In addition, *NlTPS* was expressed mainly in the fat body, the major tissue for trehalose synthesis in *Bombyx mori* (Murphy & Wyatt, 1965; Becker *et al*., 1996). However, slightly lower expression levels were also detected in the midgut and ovary, indicating that these tissues could also produce trehalose. The result is surprising in comparison with some previous studies in other insects (Murphy & Wyatt, 1965; Becker *et al*., 1996). However, similar results have been obtained with the Lepidoptera species *H. armigera* and the blue crab *Callinectes sapidus*, in which the expression of *TPS* was ubiquitous in many tissues (Chung, 2008; Xu *et al*., 2009).

A variety of efficient methods for the delivery of dsRNA into insects has been explored in recent years to knock down specific gene expression. Owing to its effectiveness, microinjection remains the most direct and popular method of knocking down the expression of target genes. However, it is very difficult to achieve RNAi via microinjection for small insects, such as *N. lugens* nymphae, where the injection procedure may cause a high mortality rate. In our experiment, feeding-based RNAi was used because of its convenience and low rate of injury. Successful knockdown of the *TPS* gene at the mRNA expression levels and the enzyme activity levels was confirmed in our RNAi experimental system. However, some gene and enzyme activity remained, indicating that the silencing was incomplete. This effect was also observed after ingestion of *CELL-1* dsRNA by the termite *Reticulitermes flavipes* (Zhou *et al*., 2008) or ingestion of *Nitrophorin 2* dsRNA by the insect *Rhodnius prolixus* (Araujoa *et al*., 2006). In contrast, the midgut *aminopeptidase N* gene in *Spodoptera litura* (Rajagopal *et al*., 2002) and the *vitellogenin* gene in adult honeybees (Amdam *et al*., 2003) were almost totally silenced by abdominal injections of dsRNA. We also analysed the efficiency and the time at which the effect of dsRNA on gene expression began. An obvious decrease in the level of *NlTPS* gene expression was observed after dsRNA ingestion for 2 days, and this is similar to the reduction in the expression of the *HEX-2* or *CELL-1* mRNA in *Re. flavipes* that is observed on the second day after dsRNA ingestion (Zhou *et al*., 2008). However, this observation differs from the results found in *S. exigua,* as the silencing of *chitin synthase A* reaches a maximum effect on days 7 and 9 (Tian *et al*., 2009). These results suggest that the efficiency of RNAi in different insect orders varies greatly. Furthermore, continuous ingestion of 0.5 μg/μl *NlTPS* dsRNA led to higher larvae mortality rates after 2 days. It is similar to what occurs in the loss-of-function *TPS-1* mutant in *Drosophila melanogaster* (Chen *et al*., 2002). However, in *C. elegans*, RNAi knockdown of *TPS* gene expression does not produce any obvious loss-of-function phenotypes, despite a decrease in the trehalose content to less than 10% of the normal level in treated worms (Pelleronea *et al*., 2003). One possible reason is that the *TPS* gene in insect species has been reported to exist as a single copy in a haploid genome (Xu *et al*., 2009), whereas the presence of two *TPS* genes in *C. elegans* presents a more complex situation that is, as yet, not understood. Another possible explanation is that because trehalose is the main blood sugar in insects, its metabolism is pivotal for insect growth, development and survival. Nevertheless, whether trehalose synthesis is also essential in *C. elegans* remains unknown (Pelleronea *et al*., 2003). Even though *NlTPS* mRNA knockdown was also found in medium dose dsTPS (0.1 μg/μl) feeding larvae, we did not find any significant difference in survival rates compared with that of controls. These results suggest that only a sufficient decrease in *NlTPS* transcriptional level has an effect on survival of *N. lugens*.

Although the purpose of our experiments was to determine whether feeding-based RNAi can be used as a functional-genomic tool in *N. lugens* and as a potential pesticide mode of action, our research team is also interested in the molecular mechanism of RNAi in the Hemiptera insect *N. lugens.* Recent discoveries from other plants and nematode models provide some insight into this issue. Systemic RNAi defective protein-1 (SID-1) is a key protein involved in the amplification of the silencing signal and intercellular transportation (Buchon & Vaury, 2006; Jose & Hunter, 2007), and it appears to be necessary for systemic RNAi (Winston *et al*., 2002). SID-1 has been found in *Schistocerca americana* (Dong & Friedrich, 2005), *Apis mellifera*, *Bombyx mori*, *S. exigua* (Tian *et al*., 2009), *Tribolium castaneum* (George & Gene, 2006) and *Aphis gossypii* (Xu & Han, 2006), but is absent in *Drosophila melanogaster* (Roignant *et al*., 2003) and *Anopheles gambiae* (Landin *et al*., 2002; Voinnet, 2005), which show cell-autonomous RNAi but not systemic RNAi. Therefore, SID-based passive transport is probably the main molecular mechanism of RNAi signalling in insects (Zhou *et al*., 2008). Recently, our research team has cloned a cDNA fragment corresponding to the SID-1 like protein

(unpublished data) in *N. lugens*, which may account for the systemic RNAi effect in this insect after ingestion of dsRNA. Further study on this item will be required to develop a deep understanding of the molecular basis of RNAi in Hemiptera.

In summary, the results presented here not only provide the first demonstration of gene silencing via RNAi in *N. lugens* and verify that the *TPS* gene is a feasible candidate for *N. lugens* RNAi targeting, but also provide a feasible approach to developing a bioassay to screen target genes for the construction of RNAi-based transgenic plants for pest control.

Experimental procedures

Insect rearing

The colony of *N. lugens* used was a laboratory strain obtained from Guangdong Academy of Agricultural Sciences in September 2007 and has been reared in continuous laboratory culture since then. The insects were maintained in wooden cages (50 × 50 × 70 cm) with rice seedlings at 26–28 °C with a 16:8 h light : dark photoperiod.

RNA isolation and cDNA synthesis

Total RNA was isolated from five individuals of fifth instar *N. lugens* larvae using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Synthesis of the first-strand cDNA was carried out according to the reverse transcriptase XL (AMV) (TaKaRa, Kyoto, Japan) protocol using an oligo dT_{18} primer. The first-strand cDNA (1 μl) was used as a template for PCR in a reaction containing 0.1 mM deoxynucleotide triphosphate, 5 mM of each primer and 1.0 U HiFi-Taq DNA polymerase (TransGen Biotech, Beijing, China) in a total volume of 25 μl. Two pairs of degenerate primers (Table 1), TPS-F1/TPS-R1 and TPS-F2/TPS-R2, were designed from the conserved *TPS* cDNA sequences of other insects. The first PCR reaction was performed using the primers TPS-F1 and TPS-R1 and the following conditions: three cycles of 30 s at 95 °C, 30 s at 45 °C and 60 s at 72 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 48 °C and 60 s at 72 °C, with a final extension at 72 °C for 10 min. A second PCR was carried out using the nested primers TPS-F2 and TPS-R2 using the same conditions. The amplified product was separated in an agarose gel and purified using a gel extraction kit (Omega, Bio-tec, Inc, GA, USA). Purified DNA was ligated into the pMD18-T vector (TaKaRa) and sequenced completely from both directions.

Amplification of the full-length cDNA

A BD SMART RACE cDNA amplification kit (BD Bioscience Clontech, Mountain View, CA, USA) was used to obtain the full-length *NlTPS* cDNA. The specific primers 5-GSP-1 and 5-GSP-2 were used for 5′,RACE and 3-GSP-1 and 3-GSP-2 were used for 3′,RACE (Table 1). These were synthesized based on the cDNA sequence obtained from the cDNA fragment identified. PCR was performed using the GSP-1 primer and Universal Primer Mix (UPM, Clontech) by denaturing at 95 °C for 30 s and using 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. Nested PCR was carried out with the first round PCR product as a template and the Nested Universal Primer A (NUP, Clontech) and GSP-2 primers. The RACE products were purified and sequenced as described above. Potential N-glycosylation sites were predicted using the NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), and evolutionary analyses were conducted using MEGA software version 4.1.

Table 1. PCR primers used in this study

Primers	Primer sequence
Degenerate primers	
TPS-F1	5′-TTCTGGCCWYTRTTCCAYTC-3′
TPS-F2	5′-TTCTTCYTKCAYATYCCRTTCC-3′
TPS-R1	5′-GCCATACCYTTWAGRGCC-3′
TPS-R2	5′-CGGTARTGGAAHGTHAGCA-3′
For cDNA cloning	
5-GSP-1	5′-ACTCCTGGTACTCTTTCACGTCG-3′
5-GSP-2	5′-CAACAGTCCACGAAGTTCAGAC-3′
3-GSP-1	5′-GTTGGCACTGTTGCTGGACTA-3′
3-GSP-2	5′-GCTGACCTATGCAGGCAACCATG-3′
For reverse transcription PCR and real-time PCR	
QTPS-F	5′-TTGCCAAAGACTGAGGCGAATG-3′
QTPS-R	5′-CCTCATCAGCCCAAGGGAACAA-3′
ACTIN-F	5′-TGCGTGACATCAAGGAGAAGC-3′
ACTIN-R	5′- CCATACCCAAGAAGGAAGGCT-3′
For dsRNA synthesis	
TPSDS-F	5′-GCAAAGGTCTGCTGGTGGAAC-3′
TPSDS-R	5′-CAGATAATTCATGCGCAGTGTTCT-3′
GFP-F	5′-AAGGGCGAGGAGCTGTTCACCG-3′
GFP-R	5′-CAGCAGGACCATGTGATCGCGC-3′

GFP, green fluorescent protein; GSP, gene specific primer; TPS, trehalose phosphate synthase.

Developmental expression analysis

The expression of *TPS* in *N. lugens* was estimated by qRT-PCR using a LightCycler480 system (Roche Diagnostics Indianapolis, IN, USA) and SYBR Premix Ex Taq (TaKaRa). Total RNA was isolated from the first to fifth instar larvae and 1–15-day-old female adults as described above. The primer pair QTPS-F and QTPS-R (Table 1) was designed to determine the expression of *TPS*. Cycling for each reaction was carried out in a final volume of 10 μl containing 1 μl of the cDNA sample (or standard), 0.2 μl (10 picomoles/ul) of each primer and 5 μl SYBR premix Ex Taq. After 10 s of initial denaturation at 95 °C, the cycling protocol consisted of 40 cycles of denaturation at 95 °C for 5 s, annealing at 58 °C for 15 s and elongation at 72 °C for 20 s. A *β-ACTIN* (EU179846) cDNA fragment was amplified with ACTIN-F and ACTIN-R primers (Table 1) as an internal control. Standard curves were obtained using a 10-fold serial dilution of cDNA pooled from 30 individuals. All the data are presented as the relative mRNA expression and were the means of four individual measurements ± SE.

Tissue distribution analysis

To investigate the expression of *NlTPS* in different tissues, we extracted the total RNA from the fat body, ovary, epidermis and midgut. We carefully collected one to two organs/individual, rinsed them in 1 × phosphate-buffered saline buffer several times and then mixed the organs from 15–30 individuals. Samples from

these organs were used for reverse transcription. PCR amplification was performed using the primers QTPS-F/QTPS-R and ACTIN-F/ACTIN-R (Table 1) as described above by denaturing at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Each PCR product (5 μl) was electrophoresed and detected by ethidium bromide staining.

Synthesis of dsRNA

To synthesize dsRNA, a 659 bp fragment (*NlTPS*) was amplified by PCR using the *NlTPS* cDNA described above as a template. The forward primer was TPSDS-F and the reverse primer was TPSDS-R (Table 1). The amplification reactions comprised 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, with a final extension step of 72 °C for 10 min. The PCR products were verified by nucleotide sequencing. Only sequences in which products from the forward and reverse sequences aligned well (98%) were used as templates for dsRNA synthesis. The *GFP* gene (ACY56286) was used as control dsRNA. The PCR primers GFP-F and GFP-R were used to amplify the *GFP* fragment (688 bp) (Table 1). dsRNAs were prepared using the T7 RiboMax Express RNAi System (Promega, Madison, WI, USA). Sense and antisense dsRNAs generated in separate reactions were annealed by mixing both transcription reactions, incubating at 70 °C for 10 min and slowly cooled to room temperature over 20 min, yielding dsRNA. The dsRNA was then precipitated by adding 2.5 volume of 95% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2), washing with 70% ethanol, drying and resuspension in an appropriate amount of nuclease-free water. The purified dsRNAs were quantified by spectroscopy and examined by agarose gel electrophoresis to ensure their integrity.

Insect bioassays

In the dsRNA ingestion experiment, we used the rearing procedure of Fu *et al.* (2001) with some modifications. For rearing on artificial diets, we used glass cylinders, 9.0 cm in length and 2.0 cm in diameter, as feeding chambers. The dsRNA was added as a supplement to the *N. lugens* artificial diet and held between two layers of stretched Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL, USA) that were located at the two open ends of the chamber. This was renewed every day. The dsRNA concentrations were designated as high dose (0.5 μg/μl), medium dose (0.1 μg/μl) and low dose (0.02 μg/μl). The chambers were covered with a piece of black cotton cloth, but the two ends where the artificial diet was placed were exposed to light. Insects could feed on the diets by puncturing the inner Parafilm M membrane of the diet pouch. Individuals of third instar larvae were pre-reared on artificial diets for 1 day before initiation of the assays. Then, 15 individuals were transferred into each chamber, every 30 individuals were used in each set of repetition and three replicates were used. Mortality data were recorded every day (1–10 days) until the first adult emerged. The rearing experiments were carried out in a growth cabinet with a humidifier at 27 °C, using 90% relative humidity, with a 16:8 h light: dark photoperiod.

Gene silencing analysis

For experiments employing different concentrations of dsRNA (0.02, 0.1, 0.5 μg/μl), the feeding protocols were the same as described above. Three synchronous larvae were selected randomly at 1, 2, 4, 7 and 10 days (adult) after ingestion for subsequent RNA extraction and four replicates were used. The mRNA expression level in the dsGFP group was designated as one. The qRT-PCR methods, primers and reaction conditions were as described above. All the data are presented as the relative mRNA expression rates (as the means of four individual measurements ± SE).

TPS activity measurement assay

The activity of TPS was determined as described previously (Xu *et al.*, 2009) with some modifications. Five individuals were homogenized with120 μl of 500 mM phosphate buffer (pH 7.0), and the homogenate was centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant (60 μl) was transferred to another Eppendorf tube and mixed with 10 μl 0.05 M uridine-5,diphosphoglucose (Sigma, St Louis, MO, USA), 10 μl 0.1 M glucose-6-phosphate (Roche Diagnostics), 10 μl 0.25 M $MgSO_4$ and 10 μl 0.01 M ethylene diamine tetraacetic acid. The blanks contained all of the same components except the glucose-6-phosphate. The mixtures were then incubated at 37 °C for 20 min, heated for 3 min at l00 °C and cooled. After the addition of 50 μl 500 mM phosphate buffer containing 0.5 μmol phosphopyruvate (Sigma) and 15 units of pyruvate kinase (Roche Diagnostics), the tubes were incubated again for 20 min at 37 °C. Cold 10% trichloroacetic acid (1 ml) was then added to each tube. After centrifuging at 12 000 *g* for 10 min at 4 °C, 600 μl of the supernatant was added to 200 μl 2 M HCl containing 0.1% 2,4-dinitrophenylhydrazine and the sample was incubated at 25 °C for 5 min before being extracted with 600 μl benzene and 1.2 ml 10% sodium carbonate sequentially. The optical density of 500 μl of the extract mixed with 500 μl 1.5 M NaOH was measured at 520 nm, as reported by Friedemann & Haugen (1943). The TPS activity was determined by reference to a calibration curve constructed using standard UDP (Sigma). The concentration of total protein was determined using the BCA Protein Assay Kit (Paragon Biotech, Guangzhou, China).

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References

Akira, O., Tomonari, W., Yoshito, S., Masaya, M., Akiko, F. and Masamichi, C. (2005) A migration analysis of the rice planthopper *Nilaparvata lugens* from the Philippines to East Asia with three-dimensional computer simulations. *Popul Ecol* **47**: 143–150.

Amdam, G.V., Simoes, Z.L.P., Guidugli, K.R., Norberg, K. and Omholt, S.W. (2003) Disruption of *vitellogenin* gene function in adult honeybees by intraabdominal injection of double-stranded RNA. *BMC Biotechnol* **3**: 1–8.

Araujoa, R.N., Santosa, A., Pintoa, F.S., Gontijoa, N.F., Lehaneb, M.J. and Pereiraa, M.H. (2006) RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. *Insect Biochem Mol Biol* **36**: 683–693.

Arguelles, J. (2000) Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Arch Microbiol* **174**: 217–224.

Baum, J., Bogaert, T., Clinton, W., Heck, G. and Feldmann, P. (2007) Control of coleopteran insect pests through RNA interference. *Nat Biotech* **25**: 1322–1326.

Becker, A., Schloer, P., Steel, J.E. and Wegener, G. (1996) The regulation of trehalose metabolism in insects. *Cell Mol Life Sci* **52**: 433–439.

Bonini, B.M., Van-vaeck, C., Larsson, C., Gustafsson, L., Pingsheng, M.A., Winderickx, J. *et al*. (2000) Expression of *Escherichia coli* otsA in a *Saccharomyces cerevisiae tps1* mutant restores trehalose 6-phosphate levels and partly restores growth and fermentation with glucose and control of glucose influx into glycolysis. *Biochem J* **350**: 261–268.

Buchon, N. and Vaury, C. (2006) RNAi: a defensive RNA-silencing against viruses and transposable elements. *Heredity* **96**: 195–202.

Chen, Q., Ma, E., Behar, K.L., Xu, T. and Haddad, G.G. (2002) Role of trehalose phosphate synthase in anoxia tolerance and development in *Drosophila melanogaster*. *J Biol Chem* **277**: 3274–3279.

Chen, X.F., Tian, H.G., Zou, L., Tang, B., Hu, J. and Zhang, W.Q. (2008) Disruption of *Spodoptera exigua* larval development by silencing *chitin synthase gene A* with RNA interference. *Bull Entomol Res* **98**: 613–619.

Cheng, X., Chen, R. and Xi, X. (1979) Study on migration patterns of *Nilaparvata lugens* (Stal). *Acta Entomol Sin* **22**: 1–21.

Chung, J.S. (2008) A *trehalose 6-phosphate synthase* gene of the hemocytes of the blue crab, *Callinectes sapidus*: cloning, the expression, its enzyme activity and relationship to hemolymph trehalose levels. *Saline Systems* **4**: 1–8.

Clegg, J.S. and Evans, D.R. (1961) Blood trehalose and flight metabolism in the blowfly. *Science* **7**: 54–55.

Crowe, J., Crowe, L. and Chapman, D. (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**: 701–703.

Dong, Y. and Friedrich, M. (2005) Nymphal RNAi: systemic RNAi mediated gene knockdown in juvenile grasshoppers. *BMC Biotechnol* **5**: 1–7.

Eaton, B.A., Fetter, R.D. and Davis, G.W. (2002) Dynactin is necessary for synapse stabilization. *Neuron* **34**: 729–741.

Elbein, A.D. (1974) The metabolism of alpha, alpha-trehalose. *Adv Carbohydr Chem Biochem* **30**: 227–256.

Elbein, A.D., Pan, Y.T., Pastuszak, I. and Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology* **13**: R17–R27.

Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver, S. and Mello, C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**: 806–811.

Friedemann, T.E. and Haugen, G.E. (1943) Pyruvic acid: II. The determination of keto acid in blood and urine. *J Biol Chem* **147**: 415–442.

Fu, Q., Zhang, Z.Y., Hu, C., Lai, F.X. and Sun, Z. (2001) A chemically defined diet enables continuous rearing of the brown planthopper, *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae). *Appl Entomol Zool* **36**: 111–116.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Do Choi, Y., Kochian, L.V. *et al*. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc Natl Acad Sci USA* **99**: 15898–15903.

George, M. and Gene, E. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**: 931–949.

Gu, J., Shao, Y., Zhang, C., Liu, Z. and Zhang, Y. (2009) Characterization of putative soluble and membrane-bound trehalase in a hemipteran insect, *Nilaparvata lugens*. *J Insect Physiol* **55**: 997–1002.

van der Horsta, D.J., van Doorna, J.M. and Beenakkersa, A.M.T. (1978) Dynamics in the haemolymph trehalose pool during flight of the locust, *Locusta migratoria*. *Insect Biochem* **8**: 413–416.

Huo, Z., Chen, L., Ye, C. and Liu, L. (2002) Effect of climate on outbreak of China rice planthopper. *J Nat Disasters* **11**: 97–102.

Jose, A.M. and Hunter, C.P. (2007) Transport of sequence-specific RNA interference information between cells. *Annu Rev Genet* **41**: 305–330.

Landin, S., Moita, L. and Kocher, T. (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene. *EMBO Rep* **3**: 852–856.

Mao, Y., Cai, W., Wang, J., Hong, G., Tao, X., Wang, L. *et al*. (2007) Silencing a cotton bollworm *P450 monooxygenase* gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat Biotech* **25**: 1307–1313.

Muller, J., Wiemken, A. and Aeschbacher, R. (1999) Trehaolse metabolism in sugar sensing and plant development. *Plant Sci* **147**: 37–47.

Murphy, T. and Wyatt, G. (1965) The enzyme of glycogen and trehalose synthesis in silk moth fat body. *J Biol Chem* **240**: 1500–1508.

Noda, H., Kawai, S., Koizumi, Y., Matsui, K., Zhang, Q., Furukawa, S. *et al*. (2008) Annotated ESTs from various tissues of the brown planthopper *Nilaparvata lugens*: a genomic resource for studying agricultural pests. *BMC Genomics* **9**: 1–18.

Paul, M., Pellny, T. and Goddijn, O. (2001) Enhancing photosynthesis with sugar signals. *Trends Plant Sci* **6**: 197–200.

Pelleronea, F.I., Archera, S.K., Behma, C.A., Grantc, W.N., Laceyb, M.J. and Somerville, A.C. (2003) Trehalose metabolism genes in *Caenorhabditis elegans* and filarial nematodes. *Int J Parasitol* **33**: 1195–1206.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P. and Bhatnagar, R.K. (2002) Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as Bacillus thuringiensis toxin receptor. *J Biol Chem* **277**: 46849–46851.

Riley, J.R., Cheng, X., Zhang, X., Reynolds, D.R., Xu, G., Smith, A.D. *et al*. (2008) The long-distance migration of *Nilaparvata lugens* (Stål) (Delphacidae) in China: radar observations of mass return flight in the autumn. *Ecol Eng* **16**: 471–489.

Roignant, J., Carre, C. and Mugat, B. (2003) Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA* **9**: 299–308.

Rosenberg, L.J. and Magor, J.I. (1983) Flight duration of the brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae). *Ecol Entomol* **8**: 341–350.

Tang, B., Chen, X., Liu, Y., Tian, H., Liu, J., Hu, J. *et al*. (2008) Characterization and expression patterns of a membrane-bound trehalase from *Spodoptera exigua*. *BMC Mol Biol* **9**: 1–12.

Thompson, S.N. (2003) Trehalose-the insect 'blood' sugar. *Adv Insect Physiol* **31**: 203–285.

Tian, H., Peng, H., Yao, Q., Chen, H., Xie, Q., Tang, B. *et al*. (2009) Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS One* **4**: 1–13.

Voinnet, O. (2005) Non-cell autonomous RNA silencing. *FEBS Lett* **579**: 5858–5871.

Wang, M. (2007) Outbreak status, current prevention and sustainable management technique of *Nilaparvata lugens*. *J Anhui Agric Sci* **35**: 2944–2945.

Wang, Y., Chen, J., Zhu, Y., Ma, C., Huang, Y. and Shen, J. (2008) Susceptibility to neonicotinoids and risk of resistance development in the brown planthopper, *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae). *Pest Manag Sci* **64**: 1278–1284.

Winston, W., Molodowitch, C. and Hunter, C. (2002) Systemic RNAi in *C.elegans* requires the putative transmembrane protein SID-1. *Science* **295**: 2456–2459.

Wu, J., Xu, J., Yuan, S., Liu, J., Jian, Y. and Xu, J. (2001) Pesticide-induced susceptibility of rice to brown planthopper *Nilaparvata lugens*. *Entomol Exp Appl* **100**: 119–126.

Wyatt, G.R. (1967) The biochemistry of sugars and polysaccharides in insects. *Adv Insect Physiol* **4**: 287–360.

Xu, J., Bao, B., Zhang, Z., Yi, Y. and Xu, W. (2009) Identification of a novel gene encoding the trehalose phosphate synthase in the cotton bollworm, *Helicoverpa armigera*. *Glycobiology* **19**: 250–257.

Xu, W. and Han, Z. (2006) Cloning and phylogenetic analysis of *sid-1-like* genes from aphids. *J Insect Sci* **8**: 1–6.

Zhou, X., Wheeler, M.M., Oi, F.M. and Scharf, M.E. (2008) RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochem Mol Biol* **38**: 805–815.