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Research article

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Glycogen Phosphorylase and *Glycogen Synthase*: Gene Cloning and Expression Analysis Reveal Their Role in Trehalose Metabolism in the Brown Planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

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Abstract

RNA interference has been used to study insects' gene function and regulation. Glycogen synthase (GS) and glycogen phosphorylase (GP) are two key enzymes in carbohydrates' conversion in insects. Glycogen content and *GP* and *GS* gene expression in several tissues and developmental stages of the Brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) were analyzed in the present study, using quantitative reverse-transcription polymerase chain reaction to determine their response to double-stranded trehalases (dsTREs), trehalose-6-phosphate synthases (dsTPSs), and validamycin injection. The highest expression of both genes was detected in the wing bud, followed by leg and head tissues, and different expression patterns were shown across the developmental stages analyzed. Glycogen content significantly decreased 48 and 72 h after dsTPSs injection and 48 h after dsTREs injection. *GP* expression increased 48 h after dsTREs and dsTPSs injection and significantly decreased 72 h after dsTPSs, dsTRE1-1, and dsTRE1-2 injection. *GS* expression significantly decreased 48 h after dsTPS2 and dsTRE2 injection and 72 h after dsTRE1-1 and dsTRE1-2 injection. *GP* and *GS* expression and glycogen content significantly decreased 48 h after validamycin injection. The GP activity significantly decreased 48 h after validamycin injection, while GS activities of dsTPS1 and dsTRE2 injection groups were significantly higher than that of double-stranded GFP (dsGFP) 48 h after injection, respectively. Thus, glycogen is synthesized, released, and degraded across several insect tissues according to the need to maintain stable trehalose levels.

Key words: *Nilaparvata lugens*, RNA interference, glycogen, glycogen synthase, glycogen phosphorylase

Rice is one of the most important food crops worldwide, and it is mostly produced in China and other Asian countries. High and stable rice yields are important to guarantee food production but their safety is affected by 800 insect species, both in the field and during storage (Barrion and Litsinger 1994). In recent years, biological disasters, including pests and plant pathogens, have become a significant factor affecting rice output. Investigation showed that, from 2000 to 2010–2011, rice pests caused a loss of up to 48 million ha in China (Zhao et al. 2014). The hemimetabolous Brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), which causes huge yield losses directly, is one of the most destructive insect pests, as they can only feed and breed on rice or wild rice (Xi et al. 2014, 2015a,b). This pest damages rice plants by directly sucking the phloem sap and transmits plant viruses (Ghaffar et al. 2011, Yang et al. 2014). Originally, insecticide control was an important and very convenient way to control and decrease pest populations, but its improper use has led to pest resurgence and resistance, and to the accumulation of chemical residues (Xi et al. 2014). In *N. lugens*, gene-function studies have also shown that RNAi of target genes could be used a pest-control strategy (Wang et al. 2012, 2015; Xi et al. 2014, 2015a,b; Liu et al. 2015; Zhao et al. 2016; Yang et al. 2017).

Glycogen is an important metabolic and energy substance in insects (Tolmasky et al. 2001, Liu et al. 2009), and insects must accumulate glycogen before they enter diapause (Pullin 1996, Liu et al. 2009). For example, the ladybird *Coccinella septempunctata* Linnaeus accumulates a substantial amount of glycogen before entering diapauses

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(Ren et al. 2015) and *Zygaena trifolii* (Esper) (Lepidoptera: Zygaenidae) larvae that enter diapause store two times more glycogen than larvae that do not (Wipking et al. 1995). Glycogen is also one of the major carbohydrates found in insects, and it is mostly synthesized and stored in the fat body (Tang et al. 2012a). Here, it can be rapidly converted into dextrose or trehalose and then transported to other tissues when needed (Tang et al. 2012a). The synthesis and degradation of glycogen molecules require the concerted action of a set of enzymes, and are primarily regulated by glycogen synthase (GS) and glycogen phosphorylase (GP), respectively (Prats et al. 2005). When insects need energy for flying, trehalose is transported to the flight muscles leading to a decrease in the content of trehalose in the blood; the glycogen stored in the fat body is then converted to trehalose to maintain its concentration in the blood (Yu et al. 2008). Thus, in insects, glycogen usually acts in a supporting role rather than being the protagonist, as in mammals. However, under stress conditions, such as low temperature or diapause, glycogen is the essential sugar for some insects (Ren et al. 2015).

Trehalose, which is formed by two glucose units, is the "blood sugar" of insects (Tang et al. 2012b), while glucose is the "blood sugar" of mammals. Hemolymph sugars are composed of myo-inositol and trehalose (Moriwaki et al. 2003), and myo-inositol is the main sugar in the hemolymph of *N. lugens* (Kikuta et al. 2012); however, trehalose plays a key role in all developmental stages, including larvae, pupae, and adults (Becker et al. 1996, Elbein et al. 2003, Tang et al. 2014b, Zhao et al. 2016). In addition, trehalose acts as an energy reserve under low temperature, starvation, drought, and other environmental stresses (Tang et al. 2014a, Shi et al. 2016). This sugar is mainly synthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase and released in the hemolymph (Bell et al. 1992, Tang et al. 2016, Yang et al. 2017), reaching several tissues where it is degraded to glucose by trehalase (TRE or Treh). Insects have two kinds of trehalase (Shukla et al. 2015, Zhao et al. 2016, Tang et al. 2016), which participate and regulate the chitin biosynthesis pathway (Tang et al. 2012b). In addition, glucose-6-phosphate and uridine diphosphate (UDP)-glycogen are substrates for trehalose synthesis, and UDP-glycogen is the sole substrate for glycogen synthesis under the action of GS (Tang et al. 2012a). Glucose-1-phosphate, when degraded by GP, can be converted into glucose-6-phosphate and enter the trehalose synthesis pathway in the presence of phosphoglucomutase. Thus, trehalose and glycogen are two important and closely related sugars involved in insect physiological activities, which can be transformed into each other according to insects' physiological needs (Tang et al. 2012a).

Given its importance in insects as an energy source, many studies focused on glycogen function under stress pressures like starvation and cold hardness (Košťál et al. 2014; Heydari and Izadi 2014; Keshan et al. 2016; Wang et al. 2016a,b). The rate-limiting enzyme of glycogen degradation, GP, was first identified from rabbit skeletal muscle in 1936 (Cori and Cori 1936). In mammals, GP is classified into muscular, liver, and cerebral GP, and it degrades glycogen into glucose-1-phosphate. In 1977, the amino acid sequence of rabbit GP was reported (Titani et al. 1977), and the complete cDNA sequence of rabbit muscle GP was cloned in 1986 (Nakano and Fukui 1986). In insects, the complete cDNA sequence of GP was cloned and first reported from *Drosophila melanogaster* Meigen (Gabriella et al. 1999). In Lepidoptera, GP and GS genes have been reported and cloned from *Bombyx mori* Linnaeus, *Danaus plexippus* (Linnaeus), *Spodoptera exigua* (Hübner), and *Ostrinia furnacalis* (Guenée) (Tang et al. 2012a, Guo et al. 2016).

RNA interference (RNAi) has been widely used to investigate gene function in insects, especially for silencing important genes in *N. lugens* and other insects, by injecting double-stranded RNA (dsRNA) or single-stranded RNA to suppress gene expression (Belles 2010, Liu et al. 2010, Wang et al. 2012). Our previous studies showed that RNAi inhibited the expression of trehalase (*TRE* or *Treh*) or *TPS* in the chitin synthesis pathway, leading to molting deformities in most tested *N. lugens* and to their death (Zhao et al. 2016, Yang et al. 2017). These results suggested that inhibiting the expression of *TPS* and *TRE* might also affect the metabolism and the utilization of carbohydrates. Therefore, the present study aimed to further investigate the functions of *GS* and *GP* in *N. lugens*, by evaluating their expression patterns when trehalose synthesis and degradation pathways are inhibited.

Materials and Methods

N. lugens Rearing and Dissection

The *N. lugens* used in this study were collected from the rice fields located at China National Rice Research Institute, Hangzhou, Zhejiang, China, and were kindly provided by Professor Qiang Fu. Insects were fed fresh rice seedlings (*Oryza sativa* L. var.TN1), and kept in a stable environment at 25 ± 1 °C, 60–70% RH, and a photoperiod of 16/8 (L:D) h.

Brown planthopper individuals (three sets of 10 individuals) used in the gene expression analyses were obtained from several populations at the same developmental stage and growth rate. Tissues were sampled from the whole body of fifth instar nymphs in their first day (0 h) at this stage (5L-0), and every 12 h after that until adults were three days old (72 h). The head, leg, wing bud, cuticle, and fat body of fourth and fifth larval instars were dissected in a saline solution (0.75% NaCl) under an EZ4 microscope (Leica, Germany). Three biological replicates were used for each developmental stage and tissue/whole body sample. All samples were kept at −80 °C until RNA extraction.

Total RNA Extraction and cDNA Reverse Transcription

Total RNA was extracted from the tissues or whole bodies of *N. lugens* using the TRIzol reagent (Life Tech, Carlsbad, CA) as instructed by the manufacturer. RNA concentration was determined by measuring samples' absorbance at 260 nm in a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) (Yang et al. 2017), and purified RNA was stored at −80 °C before use. First-stand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's instructions.

Cloning GP and GS Genes

Primers were designed in Vector NTI Suite 7 software using cDNA as the template and according to the sequences of GP and GS obtained from transcriptome sequencing (Zhao et al. 2016, Yang et al. 2017). Full-length cDNAs of *NLGP* and *NLGS* genes were cloned using the primer pairs presented in Table 1. Amplification reactions were performed in a 25 μl final volume, containing polymerase chain reaction (PCR) buffer, 0.1 mM dNTPs, 0.2 μM each primer, and 0.5 U of HiFi-Taq DNA polymerase (Transgene, Beijing, China). The cycling conditions were as follows: 10 min of initial denaturation at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 180 s, and 10 min for a final extension at 72 °C. The PCR products were then analyzed by agarose gel electrophoresis, and those with the expected size (approximately 2,500 and 2,200 bp) were excised from the gel and purified using a DNA gel-extraction kit. The purified DNA was ligated into the pMD18-T vector (TaKaRa, Dalian, China) and Sanger sequenced.

Table 1. Primers used for the cloning and qRT-PCR of the *GS* and *GP* genes in *N. lugens* (NL)

Gene	Forward (5′–3′)	Reverse (5′–3′)	Function
NLGS	ATGTCTCGAGAACGTGCCAATA	TTATGTTACCTCTTTTTCATCATCC	Gene cloning
NLGP	ATGGCTACGCCACAATCAGATG	TTAAGCTTCACGAGGCTCATGTG	
NLGS	GCTCCAAAGCCTATGTTTCTACTG	TGGTAACCCCTGTCCCTCA	qRT-PCR
NLGP	GCTGCCTATGGCTATGGTATTC	TCTGAGTGTTGACCCACTTCTTG	
NL18S	CGCTACTACCGATTGAA	GGAAACCTTGTTACGACTT	

Sequence Analysis

The amino acid sequences of *NLGP* and *NLGS* were translated from cDNA sequences using the tool available at the ExPASy Proteomics website (http://expasy.org/tools/dna.html). The NLGP and NLGS putative sequences obtained were compared to protein sequences deposited in GenBank, using the BLAST-N or BLAST-X tools available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

NLGP and *NLGS* Expression in Several Tissues and Developmental Stages Using Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Complementary DNA synthesis and qRT-PCR were performed to analyze the distribution of *NLGP* and *NLGS* using gene-specific primers (Table 1). Based on the previously cloned *NLGP* and *NLGS* cDNAs, two pairs of specific primers were designed in Vector NTI Suite 7 software to amplify the unique regions found in the cDNA alignment. Using 1 μg total RNA as template, and a specifically designed *NL18S* primer pair (Table 1) the stability of 18S RNA was demonstrated in a PCR performed under the following conditions: 95 °C for 5 min, 28 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min.

The expression of *NLGP* and *NLGS* in the several tissues and developmental stages was estimated by qRT-PCR using a Bio-Rad CFX96 system and the SsoFast EvaGreen Supermix (both from Bio-Rad Laboratories, Hercules, CA). Each reaction was performed in a 20 μl final volume, containing 1 μl cDNA (or standard), 1 μl (10 μM) each primer, 7 μl RNAase- and DNAase-free water, and 10 μl SsoFast EvaGreen Supermix. The reactions were performed the following conditions: 3 min at 95 °C, followed by 40 cycles of 5 sat 95 °C and annealing at 55–62.5 °C for 20 s, with a melting curve at 65–95 °C, as instructed by the manufacturer.

dsRNA Synthesis and Injections

Using the *N. lugen*s cDNA template and specific primers containing the T7 promoter sequence at their 5' ends (Table 2), regions of three *NLTRE* and two *NLTPS* genes were amplified by qRT-PCR. The profile used in the reactions included 40 cycles at 95 °C for 30 s, 58 °C for 30s, 72 °C for 45 s, and a last extension at 72 °C for 10 min. Purified *TRE* and *TPS* amplicons were transcribed in vitro to synthesize dsRNA using T7 RiboMax Express RNAi System (Promega Corporation, Madison, WI) (Zhao et al. 2016). A green fluorescence protein (*GFP*) amplicon was used as a control. Sense and antisense strands were first produced in two separate transcriptive procedures and then mixed for annealing. Reactions were incubated for 10 min at 70 °C and then placed on an ice bath for 20 min. Finally, dsRNAs were precipitated with 95% ethanol and 3 M sodium acetate (pH 5.2), washed with 70% ethanol, air dried, and resuspended. The integrity and quantity of dsRNAs were evaluated by spectroscopy analysis with Nanodrop 2000 (Thermo Fisher Scientific) and by agarose gel electrophoresis.

Using an IM-31 microinjector (NARISHIGE, Tokyo, Japan), dsTRE1-1, dsTRE1-2, dsTRE2, dsTPS1, and dsTPS2 (200 ng of each) were injected into the abdomen of *N. lugens* nymphs. Control groups were injected with dsGFP or with 0.1 to 10 μg/μl of validamycin, which is a specific trehalase inhibitor. The efficiency of gene knockdown resulting from RNAi was calculated as the ratio of gene expression between insects injected with target dsRNAs and GFP dsRNA, determined at 48 and 72 h after injection. Data for the validamycin-inhibited groups were collected 48 h after injection.

Measurement of Glycogen Content

Glycogen content (mg glucose/g total protein) was measured as described by Santos et al. (2008), using 100 μl of the supernatant . These were incubated for 4 h at 37 °C in the presence of 20 μl (1 U) amyloglucosidase (EC 3.2.1.3, Sigma, Darmstadt, Germany) diluted in 100 mM sodium acetate (pH 5.5) to hydrolyze glycogen. The amount of glucose generated from glycogen was determined using a Glucose Assay Kit (GAGO20-1KT, Sigma Jurong Town, Singapore), following the manufacturer's instructions. Controls were prepared in the absence of the enzyme, and their amount of glycogen was calculated by excluding endogenous glucose.

Quantification of *GP* and *GS* mRNA Expression Levels

The effects of RNAi on the transcript expression of *GP* and *GS* genes were analyzed by qRT-PCR. Total RNA (1 μg) from each sample was reverse-transcribed to generate first-strand cDNA using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). Through qRT-PCR, relative gene expressions were detected using the Bio-Rad CFX96 system and the SsoFast EvaGreen Supermix (both from Bio-Rad Laboratories), as described in section before. Ten-fold serial dilutions of pooled total RNA were used in standard curves.

GP and GS Enzyme Activities Assays

Protein content in the enzyme source was measured using BCA Protein Assay Kit (Sangon Biotech, Shanghai, China), according to Chutipongtanate (2012), BSA was using as standard protein. The kits used to measure GS and GP activities were purchased from Genmed Scientifics, Inc. (USA), and the manufacturer's instructions were followed. In the continuous circulatory system consisting of GS, pyruvate kinase, and lactate dehydrogenase (LDH), GS activity was quantified by measuring the change in absorbance values after reduced nicotinamide adenine dinucleotide (NADH) was oxidized at 340 nm. One unit of GS activity was defined as causing the oxidation of 1 μmol NADH/min per gram of protein at 30°C pH 8.2. In the continuous reaction system consisting of GP, phosphoglucomutase, and glucose-6 phosphate dehydrogenase, GP activity was assayed by measuring the reduction of oxidized nicotinamide adenine dinucleotide phosphate (NADP) during the process of glycogen breakdown at 340 nm and was expressed in micromole NADP/min per gram of protein. One unit of GP activity was defined by the

Table 2. Primers used in dsRNA synthesis

Gene	Application	Primer name	Primer sequence (5′–3′)
NlTPS1	RNAi	DSNLTPS1-F	ACCAGGAGTTGAAGGAGGAG
		DSNLTPS1-R	GATCAGGGTGCCCATAGC
		DSNLTPS1-FT	T7-ACCAGGAGTTGAAGGAGGAG
		DSNLTPS1-RT	T7-GATCAGGGTGCCCATAGC
NlTPS2	RNAi	DSNLTPS2-F	CACCAAAGGTCTAAGGCACA
		DSNLTPS2-R	CATCGTTGATCTCGTAGGGA
		DSNLTPS2-FT	T7-CACCAAAGGTCTAAGGCACA
		DSNLTPS2-RT	T7-CATCGTTGATCTCGTAGGGA
NlTRE1-1	RNAi	DSNLTRE1-1-F	GATGCAATCAAGGAGGTGTTATGGC
		DSNLTRE1-1-R	CGTATTCACCTCCACCTCCGT
		DSNLTRE1-1-FT	T7-GATGCAATCAAGGAGGTGTTATGGC
		DSNLTRE1-1-RT	T7-CGTATTCACCTCCACCTCCGT
NlTRE1-2	RNAi	DSNLTRE1-2-F	AGATGAAGGCATGTGGTTCG
		DSNLTRE1-2-R	CATCGATTCGCCAACTGGTAAGC
		DSNLTRE1-2-FT	T7-AGATGAAGGCATGTGGTTCG
		DSNLTRE1-2-RT	T7-CATCGATTCGCCAACTGGTAAGC
NlTRE2	RNAi	DSNLTRE2-F	CCAACTGCTATGACACCGACAAG
		DSNLTRE2-R	GGGTTCAGATCCTGCCGTCGCT
		DSNLTRE2-FT	T7-CCAACTGCTATGACACCGACAAG
		DSNLTRE2-RT	T7-GGGTTCAGATCCTGCCGTCGCT
NlGFP	RNAi	DSNLGFP-F	AAGGGCGAGGAGCTGTTCACCG
		DSNLGFP-R	CAGCAGGACCATGTGATCGCGC
		DSNLGFP-FT	T7- AAGGGCGAGGAGCTGTTCACCG
		DSNLGFP-RT	T7- CAGCAGGACCATGTGATCGCGC

T7 sequence: GGATCCTAATACGACTCACTATAGG.

catalysis of 1 μmol glycogen and orthophosphate into glucose-1 phosphate per minute at 30°C and pH 6.8 (Hao et al. 2013).

Statistical Analyses

The mRNA expression levels in the noninjected and dsGFP-injected groups were the designated controls. All data obtained in the present study were analyzed through one-way analysis of variance and presented as means ± SEs of three to six biological replicates. In Duncan's new multiple range tests, a $P < 0.01$ or 0.05 was considered as extremely significant or significant, respectively.

Results

GP and GS cDNA Sequence Analyses

The molecular weight and isoelectric point of complete cDNA and amino acid sequences of GP and GS in *N. lugens* found using ExPaSy were 97.28 kDa and 6.10 for GP (Fig. 1A) and 84.14 kDa and 6.20 for GS (Fig. 1B), respectively.

Tissue Expression of GP and GS

According to the qRT-PCR results, *GP* and *GS* had similar trends in mRNA expression but different levels of gene expression among the five tissues: head, leg, wing bud, cuticle, and fat body. As evidenced in Fig. 2, the highest expression of both genes was registered in wing bud tissues, followed by leg and head tissues, and the lowest in cuticle and fat body tissues. The quantitative analysis of *GP* and *GS* expression in the different tissues used the expression obtained in head tissues as the control levels for both genes. The expression of *GP* in the wing bud was significantly different ($P < 0.05$) from that in other tissues, and its expression in leg tissues was significantly higher than in head, cuticle, and fat body tissues (Fig. 2A). An identical pattern was found for *GS* expression, with levels in the wing bud being extremely different ($P < 0.01$) from that in other tissues (Fig. 2B). Overall, results showed that *GP* and *GS* were differentially expressed across the several tissues analyzed, and that their expression was significantly higher in the wing bud of the Brown planthopper, followed by leg and head tissues.

Expression of GP and GS in Several Developmental Stages

We analyzed the expression of *GP* and *GS* in different developmental stages, from fifth-instar nymph to adult. The expression of *GP* was highest in 12- to 48-h fifth-instar nymphs and lowest in 60-h fifth-instar nymphs to 72-h adults (Fig. 3). A different pattern was obtained for *GS*: its expression was highest in 12-, 36-, 48-, and 60-h fifth-instar nymphs and in 24-, 60-, and 72-h adults and lowest in 0-, 72-, and 84-h fifth-instar nymphs and 0- and 36-h adults.

Changes in Glycogen During *TPS* and *TRE* Genes Knockdown

After the successful RNAi targeting of *NLTPS*, a significant decrease in the expression of the two *TPS* genes was observed at 48 and 72 h after dsTPSs injections (Yang et al. 2017); RNAi targeting of *NLTRE* produced similar results (Zhao et al. 2016). In the present study, glycogen contents were detected at 48 and 72 h after dsTPSs, dsTREs, and dsGFP RNA injections, as well as at 48 h after validamycin injection at different concentrations. A highly significant decrease in glycogen content was detected at 48 and 72 h after injection in the dsTPS RNAi treatment, compared to the control treatment (Fig. 4A). In the dsTRE-injected groups, glycogen content decreased significantly at 48 h after dsTRE1-2 and dsTRE2 injection and increased significantly at 72 h after dsTRE1-1 and dsTRE2 injection, compared to the control treatment (Fig. 4B).

Effects of NLTPS and NLTRE RNAi on the Expression of *GP* and *GS* Genes

Results evidenced an increase in *GP* gene expression 48 h after dsTPS and dsTRE injections although this increase differed

Fig. 1. Complementary DNA and putative amino acid sequences of *NLGP* and *NLGS*, as submitted to GenBank. (A) *NLGP*, accession number FJ809918; (B) *NLGS*, accession number FJ754277. Initiation and termination codons, as well as the termination codon before the first Methionine are indicated in bold-italics.

between treatments (Fig. 5A and B). An extreme decrease in *GP* expression was registered 72 h after dsTPS2 and dsTRE1-1 injections ($P < 0.01$), and it significantly decreased 72 h after dsTRE1-2 injection ($P < 0.05$). The expression of *GS* increased 48 h after dsTPS1, dsTRE1-1, and dsTRE1-2 injections and significantly decreased 48 h after dsTPS2 and dsTRE2 injections ($P < 0.01$)

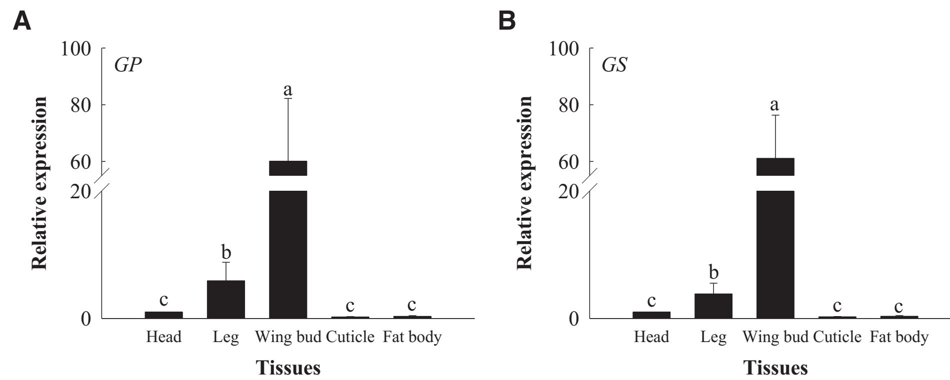

Fig. 2. Expression of *GP* (A) and *GS* (B) in the five different tissues of *Nilaparvata lugens* analyzed. Total RNA was extracted from head, leg, wing bud, cuticle, and fat body tissues and the expression of both genes was obtained by quantitative real-time PCR, using 18S RNA as the internal control. Values are means ± SEs from three independent measurements. The relative expression of each gene was determined in relation to that obtained in the head of *N. lugens* adults. Different letters indicate significant differences according to Duncan's test ($P < 0.05$).

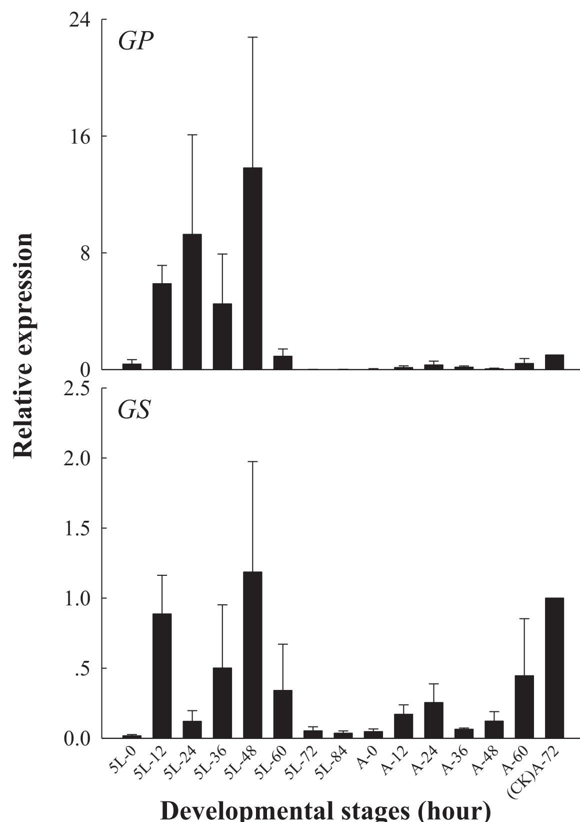

Fig. 3. Relative expression of *GP* and *GS* in the several developmental stages of *Nilaparvata lugens*, as measured by quantitative real-time PCR and using 18S RNA as the internal control. Values are means± SEs from three independent measurements. The age of Brown planthopperwas defined as follows: 5L-0, 0-h fifth-instar nymph; 5L-12, 12-h fifth-instar nymph; 5L-24, 24-h fifth-instar nymph; 5L-36, 36-h fifth-instar nymph; 5L-48, 48-h fifth-instar nymph; 5L-60, 60-h fifth-instar nymph; 5L-72, 72-h fifth-instar nymph; 5L-84, 84-h fifth-instar nymph; A-0, 0-h adults; A-12, 12-h adults; A-24, 24-h adults; A-36, 36-h adults; A-48, 48-h adults; A-60, 60-h adults; A-72, 72-h adults.

(Fig. 5C and D). The expression of *GS* decreased 72 h after the two TPS genes were knocked down and decreased significantly 72 h after the two dsTRE1s were injected (Fig. 5D).

Effects of Validamycin on the Expression of *GP* and *GS* Genes and on Glycogen Content

Results showed that *GP* expression decreased significantly ($P < 0.05$ or $P < 0.01$) 48 h after the injection of validamycin at several concentrations. Whereas *GP* expression decreased with increasing levels of validamycin (Fig. 6A), *GS* expression increased significantly 48 h after 0.1 μg/μl validamycin injection, but presented an extremely significant decrease 48 h after 0.5 to 10 μg/μl validamycin injections, showing a weak increasing trend with increasing concentrations of validamycin (Fig. 6B). Glycogen content significantly decreased ($P < 0.01$) 48 h after validamycin was injected, irrespective of its concentration (Fig. 6C).

Enzyme Activity of GP and GS After RNAi and Validamycin Injection

There was no significant difference in GP activity among the first six groups. The lowest GP activity was the group of validamycin injected and it significantly ($P < 0.05$) decreased compared with dsGFP injection group (Fig. 7A). GS activities of dsTPS1 and dsTRE2 groups were significantly higher than that of dsGFP group after 48-h injection. Furthermore, GS activities were increased compared with the dsGFP injection group, but there was no significant ($P < 0.05$) difference among dsTPS2, two dsTRE1 and validamycin injection groups (Fig. 7B).

Discussion

GS cDNA has been cloned from many organisms, including the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Farkas et al. 1990, 1991), rat liver (Bai et al. 1990), the amoeba *Dictyostelium discoideum* Raper (Williamson et al. 1996), the mold *Neurospora crassa* Shear & B. O. Dodge (de Paula et al. 2002) and *S. exigua* (Tang et al. 2012a). GP cDNA was first cloned from human brain (Newgard et al. 1988), *D. discoideum* (Rutherford et al. 1988), *Escherichia coli* (Migula) Castellani & Chalmers (Choi et al. 1989), *S. cerevisiae* (Hwang et al. 1989), *Dictyostelium* (Rutherford et al. 1992), *D. melanogaster* (Gabriella et al. 1999), *S. exigua* (Tang et al. 2012a), and *O. furnacalis* (Guo et al. 2016); it was first cloned from human muscle in 1989 and in insects, *GP* was cloned for the fruit fly *D. melanogaster* (Tick et al. 1999). The analysis of *N. lugens* GS and GP protein sequences and of the catalytic

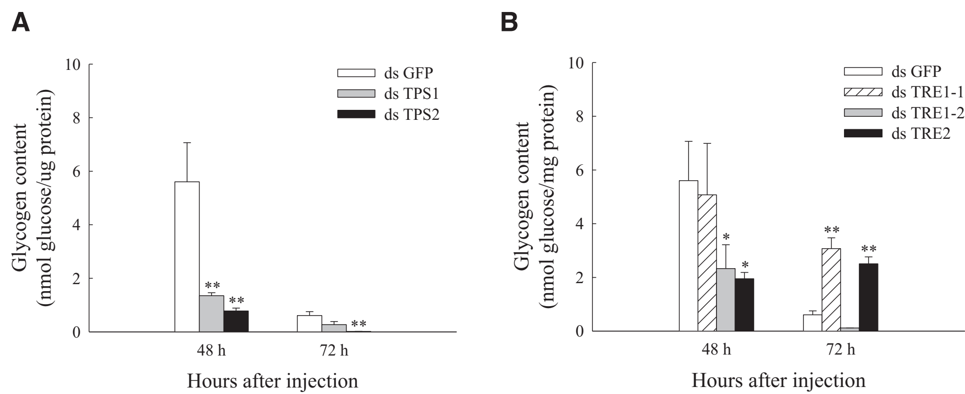

Fig. 4. Glycogen content after RNAi targeting of two dsTPS and three dsTRE. *Nilaparvata lugens* nymphs were divided into six groups and each was injected with dsGFP, dsNlTPS1, dsNlTPS2, dsTRE1-1, dsTRE1-2, or dsTRE2. Insects were collected 48 (A) and 72 h (B) after dsRNA injection and their glycogen content was determined, in triplicate. Values are means ± SEs from three independent measurements. *Indicates significant differences at $P<0.05$ and ** indicates significant differences at $P<0.01$. GFP was used as control.

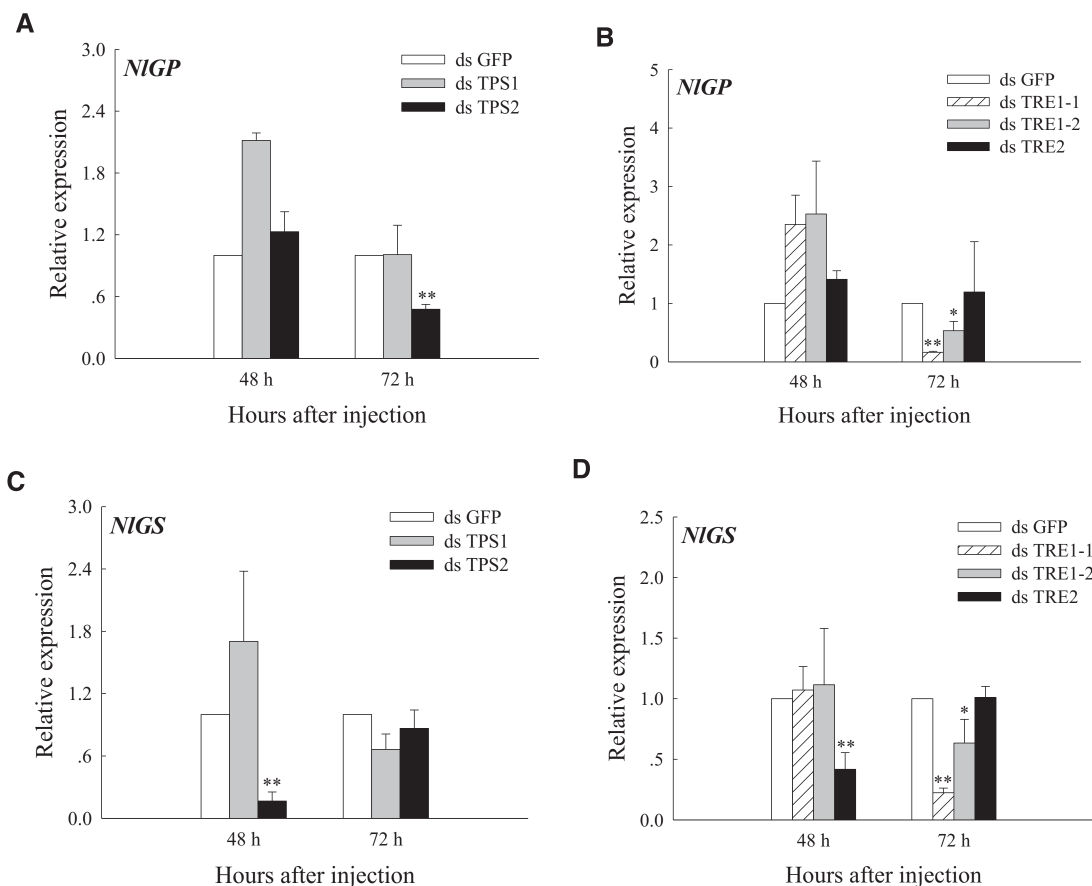

Fig. 5. Relative expression of *GP* and *GS* after RNAi targeting of two dsTPS and three dsTRE. The expressions of *GP* (**A,B**) and *GS* (**C,D**) were measured 48 and 72 h after dsTPS1, dsTPS2, dsTRE1-1, dsTRE1-1, dsTRE2, and dsGFP injection, quantitative real-time PCR and using 18S RNA as the internal control. Values are means ± SEs from three independent measurements. *Significant differences at $P<0.05$, and **indicates significant differences at $P<0.01$. Green fluorescence protein (GFP) was used as control.

mechanisms of GS and GP enzymes revealed these were conserved (Fig. 1). Amino acid sequence alignment revealed a high degree of conservation between invertebrate and vertebrate orthologs (Bacca et al. 2005, Tang et al. 2012a). Two GS isoforms, GS1 and GS2, have been found in *S. cerevisiae* (Farkas et al. 1990, 1991), but not in insects, where only one GS and one GP have been cloned or reported from genomic analyses.

In insects, glycogen synthesized by the fat body (or by other tissues) is released in the hemolymph and then transported to several tissues where it is used as a major energy source (Tang et al. 2012a). Insect tissues contain GS, which catalyzes glycogen synthesis from UDP-glucose, and GP, which catalyzes the hydrolysis of glycogen to glucose-1-phosphate. Thus, in insects, GP is an essential enzyme for the uptake or utilization of glycogen (Tick et al. 1999). Unlike

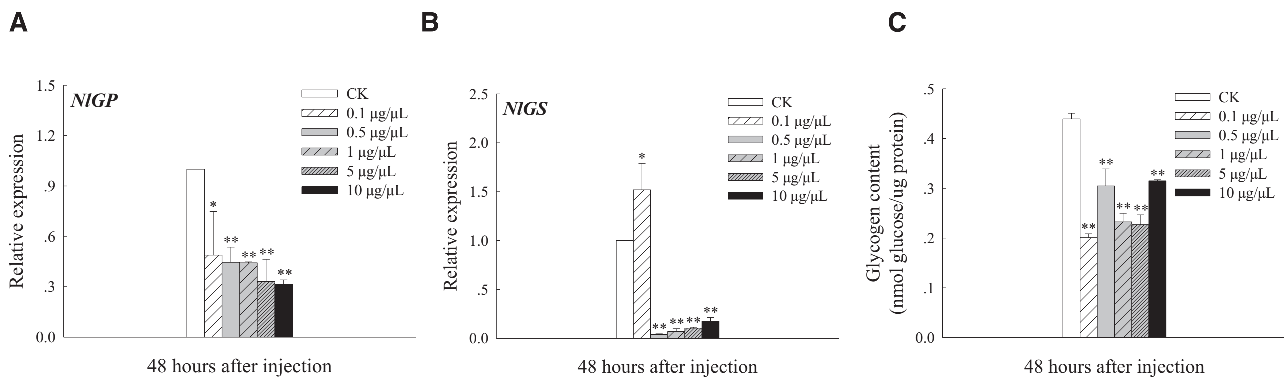

Fig.6. Contents of *GP*, *GS*, and glycogen after the injection of validamycin at several concentrations. The six groups of *Nilaparvata lugens* nymphs were injected with a solution of validamycin or deionized water. Insects were collected 48 h after injection of validamycin and the expression of *GP* (A) and *GS* (B) in relation to that of 18S mRNA was determined by quantitative real-time PCR. Glycogen content (C) was also measured 48 h after validamycin injections. Every measurement was performed in triplicate. Values are means ± SEs from three independent measurements. *Indicates significant differences at $P<0.05$ and **indicates significant differences at $P<0.01$. Deionized water was injected in the control group (CK).

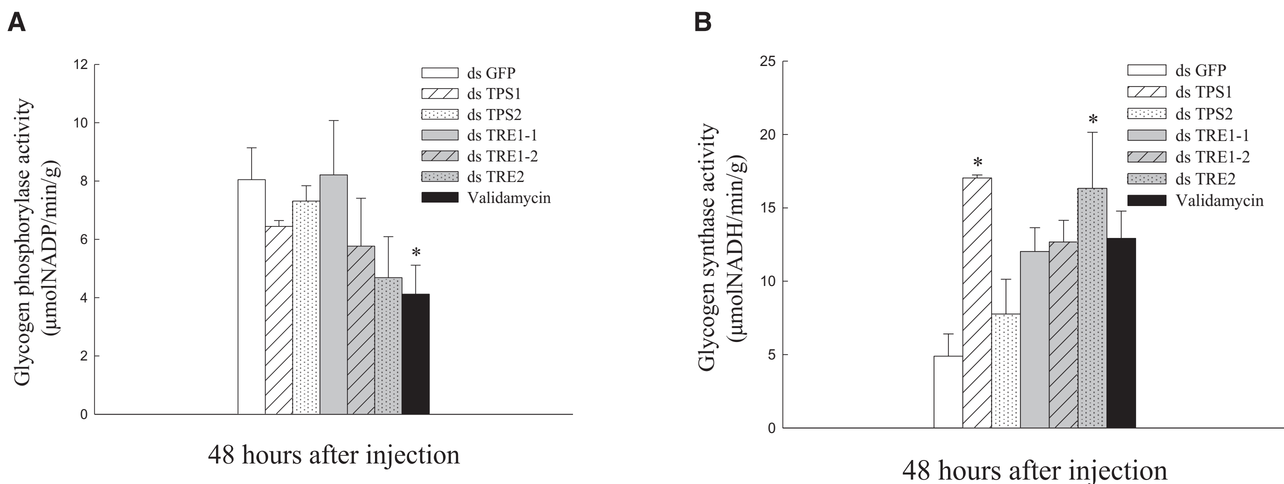

Fig. 7. Enzyme activity of GP and GS after RNAi targeting of two dsTPS, three dsTRE and the injection of validamycin. Enzyme activity of GP (A) and GS (B) were measured 48 h after dsTPS1, dsTPS2, dsTRE1-1, dsTRE1-1, dsTRE2, dsGFP and validamycin injection. Values are means ± SEs from three independent measurements. *Significant differences at $P<0.05$. GFP was used as control.

trehalose and *TPS* genes that were only expressed in the fat body and in trehalose-synthesis tissues (Tang et al. 2010), *GS* and *GP* transcripts were detected in the brain, fat body, mid-gut, Malpighian tubules, spermary, and tracheae of *S. exigua* larvae, suggesting they were expressed in different tissues and organs (Tang et al. 2012a). Similarly, in our study, *GS* and *GP* transcripts were also expressed in the head, leg, wing bud, cuticle, and fat body of *N. lugens* (Fig. 2). In addition, *GP* was not expressed in *S. exigua* epidermis (Tang et al. 2014b) and it presented the lowest levels in the cuticle of *N. lugens*, whereas the highest expression of this gene was found in wing bud tissues (Fig. 2). Overall, the widespread expression of *GS* and *GP* in insect tissues suggested they synthesize and degrade glycogen, and that flying insects such as *N. lugens* might need to store more glycogen than non-flying insects. In the present study, the expression of *GS* increased from 0- to 72-h adults, whereas *GP* was expressed at relatively lower levels (Fig. 3). Expression levels of *GS* also increased with pupa development in *S. exigua*, whereas GP was expressed at a lower level (Tang et al. 2014b). Thus, glycogen metabolism differs among insect species and more studies are needed to examine it in several kinds of insects, or among populations of the same insect species such as the long- and short-winged populations of *N. lugens*.

Glycogen is hydrolyzed under carbon starvation and accumulated during the diauxic growth phase or in response to carbon, nitrogen, sulfur, or phosphorus limitations (Johnston et al. 1992). When the ladybug *C. septempunctata* enters diapause during winter, lipids, total sugars, and glycogen contents increase significantly, while trehalose content is similar to that of insects that are not in diapauses (Ren et al. 2015). In the beetle *Pityogenes chalcographus* Linnaeus, trehalose and glycogen contents were higher in November than in other months (except March in the case of glycogen) while the content of glycerol was high all months (Košťál et al. 2014). Trehalose can be accumulated during late autumn and early winter (Kostál et al. 2007), and sugars can be inter-converted when insects are under cold hardness, starvation, or other stress conditions: molecular trehalose can be hydrolyzed to two glucose molecules by TRE and glycogen can be transformed into trehalose by the trehalose and glycogen metabolism pathway (Tang et al. 2012a). In the present study, glycogen content changed after *TPS* or *TRE* knockdown, following trehalose changes similar to those observed under stress conditions. Our results showed that glycogen decreased 48 h

after dsTPS, dsTRE, and validamycin injection (Figs. 4 and 6C), whereas trehalose content increased 48 and 72 h after dsTPSs, dsTRE1-1, and validamycin injection (Zhao et al. 2016, Tang et al. 2016, Yang et al. 2017). Similarly, glycogen content increased 72 h after dsTRE1-1 and dsTRE2 were injected (Fig. 4B), whereas trehalose decreased significantly. Thus, as insect trehalose content seems to require some balance, glycogen content decreased due to its conversion into trehalose when trehalose synthesis or degradation pathways were inhibited. Overall, although trehalose is the "blood sugar" of insects, other sugars, including glycogen, also play an important role in insect physiological activity, especially when TPS or TRE RNAi inhibit trehalose pathways or when validamycin inhibits trehalase.

Many previous studies reported that the trehalose metabolism regulating insect's chitin synthesis and degradation was seriously affected when TPS or TRE were inhibited, preventing insects from completing their developmental and molting process (Chen et al. 2010a,b; Zhao et al. 2016; Yang et al. 2017). Our results showed that the chitin synthesis pathway and chitinase genes expression were regulated by trehalose metabolism. Although *GP* and *GS* expressions were correlated with TPS and TRE, *GP* expression increased 48 h after dsTPSs injection whereas *GS* expression decreased after dsTPS2 injection (Fig. 5A and C). The expression of *GP* increased 48 h after dsTREs injection, whereas *GS* was maintained at a similar level or decreased (Fig. 5B and D). Glycogen was also affected by dsTPSs and dsTREs, generally decreasing significantly 48 h after their injection (Fig. 4). Glycogen contents changed differently 72 h post dsRNAs injection, following the changes observed in *GP* and *GS* expression. Glycogen content and *GP* and *GS* expression also decreased significantly when validamycin was injected, except for *GS* when 0.1 μg/μl of validamycin was injected (Fig. 6).

Synthesis and degradation of glycogen molecules involves the concerted action of a set of enzymes, with GS and GP activity primarily controlling this process, respectively (Prats et al. 2005). When GS activity is high and GP activity is low, glycogenesis exceeds glycogenolysis, resulting in glycogen accumulation. However, when GP activity is high and GS activity is low, glycogenolysis exceeds glycogenesis, resulting in glycogen reduction (Hao et al. 2013). The activity of GP decreased significantly at 48 h when validamycin were injected. The activity of the two kinds of trehalase also decreased significantly at 48 h and 72 h when validamycin were injected (Tang et al. 2016), and it decreased at 48 h after the three dsTREs were injected, while TRE expression increased (Zhao et al. 2016). In comparison, there was no significant difference in GP activity among the dsTRE and dsTPS injection groups (Fig. 7A). However, in the process of glycogen metabolism, the degradation of glycogen requires the action of 3 enzymes, and it can be regulated by strict and complex structure and hormone (Buschiazzo et al. 2004). In this atudy, there was a significant difference in GS activity for dsTPS1 or dsTRE2 compared with dsGFP, respectively. And there were no significant difference in GS activity among dsTPS2, two dsTRE1 and Validamycin injection groups. (Fig. 7B) Glycogen content has been detected under different stress conditions, including starvation, cold hardness, over-winter or seasonal acclimation, and diapauses in previous studies (Koštál et al. 2014; Heydari and Izadi 2014; Ren et al. 2015; Keshan et al. 2016; Wang et al. 2016a,b), but few studies reported *GP* and *GS* expression levels and enzyme activity. Thus, more studies are needed to understand their function in insect physiology, including *GP* and *GS* RNAi to study the specific function of these two genes in glycogen and trehalose metabolism regulation, as well as in energy and chitin metabolism regulation in insects.

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