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RNA interference of *β-N*-acetylglucosaminidase from the oriental armyworm moth, *Mythimna separata*

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Abstract

β-N-acetylglucosaminidase (NAG) is a key enzyme in chitin degradation, which makes it a possible target for biological pesticides. We identified a full-length cDNA sequence encoding NAG using RT-PCR and RACE technology from the oriental armyworm moth, *Mythimna separata* Walker (Lepidoptera Noctuidae). The NAG cDNA sequence (*MsNAG*) is 2,619 nucleotides long and contains a 1,785-nucleotide open reading frame, which encodes an amino acid sequence with 594 residues, and 3′ and 5′ untranslated regions. qRT-PCR was performed to examine the temporal and spatial expression pattern of *MsNAG*. The developmental stage-dependent expression pattern showed that *MsNAG* transcript level was significantly higher in the prepupal stage. Additionally, the transcript levels were higher on the last day compared with those on the first day from 4th to 6th instar larval stage. The transcript levels were also significantly different in different *M. separata* tissues, being highest in the salivary gland. The *MsNAG* transcript level was induced by the moulting hormone 20-hydroxyecdysone in first-day 5th instar larvae and examined using qRT-PCR method. *MsNAG* transcript abundance was highest 24 hours after injection with 10 μg/μL 20-hydroxyecdysone. RNA interference was used to induce *MsNAG* silencing. One-day-old 5th instar larvae were injected with *dsMsNAG* in the larval carcass and larval integuments. 48 hours after injection, the *MsNAG* transcript levels were inhibited by 80.6% and 81.1%, respectively. The RNA interference showed that *dsMsNAG* delayed the 5th instar larvae ecdysis time, reduced body weight, and even led to insect death. We concluded that *MsNAG* plays an important role in the moulting process of *M. separata*.

Key words: *Mythimna separata*, *β-N*-acetylglucosaminidase, RNA interference.

Introduction

Chitin is a linear polysaccharide made up of *β-N*-acetyl-D-glucosamine residues linked by *β*-1,4-glucosidic bonds and is the second most abundant biopolymer on earth (Wagner, 1994; Chen *et al*., 2015). During the insect moulting process, almost 90% of chitin is hydrolysed by two types of chitinolytic enzymes, chitinases (CHTs) and *β-N*-acetylglucosaminidases (NAGs) (Mommsen, 1980; Fukamizo and Kramer, 1985; Goo *et al*., 1999; Arakane *et al*., 2004). Chitin is degraded into various small chitin oligomers by CHTs, then the chitin oligomers are cleaved from the non-reducing end into monomeric *N*-acetylglucosamine by NAGs (Charpentier and Percheron, 1983). Chitin is a dominant constituent of the insect peritrophic matrix and cuticle. It deposits in the trachea as well as in the foregut and hindgut. NAGs gather in the moulting fluid between the epidermis and old procuticle (Merzendorfer and Zimoch, 2004). To complete their growth and development, insects must shed their old cuticles and synthesize new ones in a process that involves the synthesis and degradation of chitin (Zen *et al*., 1996; Kramer and Muthukrishnan, 1997). Chitin is the primary structural component of insects and it is not found in advanced animals and plants. Thus, CHTs and NAGs are potential targets as biological pesticides (Okada *et al*., 2007; Bao *et al*., 2016).

NAG (EC 3.2.1.52) belongings to the CAZy glycoside hydrolase family GH20 and is a key enzyme in the degradation of insect chitin (Cantarel *et al*., 2009). NAGs are present in microbes, arthropods, plants, and mammals, but show significant differences in their physiological functions. In insects, NAGs are involved in growth and development, moulting, and pupation (Tomiya *et al*., 2004; Intra *et al*., 2008).

NAGs have been identified from various insect species, including *Manduca sexta* (L.) (Zen *et al*., 1996), *Trichoplusia ni* (Hubner), *Choristoneura fumiferana* (Clemens) (Zheng *et al*., 2008), *Aedes aegypti* (L.) (Filho *et al*., 2002), *Tribolium castaneum* (Herbst) (Hogenkamp *et al*., 2008), *Bombyx mori* (L.) (Okada *et al*., 2014), *Xestia c-nigrum* (L.) (Fan *et al*., 2011), *Agrotis ipsilon* (Hufnagel) (Gao *et al*., 2012), *Locusta migratoria manilensis* (Meyen) (Rong *et al*., 2013), *Ostrinia furnacalis* (Guenee) (Liu *et al*., 2009), and *Mamestra brassicae* (L.) (Zhang *et al*., 2016). In *L. migratoria manilensis*, the NAG showed high transcript levels in the integument, foregut, and hindgut (Rong *et al*., 2013). In *T. castaneum*, four NAG-encoding genes had different stage- and tissue-specific expression patterns. Ecdysterone is converted to the moulting hormone 20-hydroxyecdysone (20E) in the gut of insects, and 20E is involved in regulating larval moulting, pupal metamorphosis, and adult differentiation. In *M. sexta* larvae, NAG expression level increased after 20E injections **(**Zen *et al*., 1996**)**, and in *M. brassicae*, NAG transcript levels changed after the injection of different concentrations of 20E (Zhang *et al*., 2016).

In *L. migratoria manilensis*, RNA interference (RNAi) targeting NAG resulted in 50% of the nymphs failing to moult and even died (Rong *et al*., 2013). RNAi targeting NAG of *Nilaparvata lugens* (Stal) effectively suppressed the gene transcript (Xi *et al*., 2015).

The oriental armyworm moth, *Mythimna separata* Walker, is a major pest of corn, wheat, and rice, and is a serious threat to food security. We cloned a novel NAG cDNA sequence (named *MsNAG*) from *M. separata* and investigated its developmental stage- and tissue-

dependent transcript expression patterns using quantitative real-time reverse transcription PCR (qRT-PCR). We injected 20E into *M. separata* larvae to explore its effect on *MsNAG* expression. We performed RNAi targeting *MsNAG* to determine whether the molting and growth processes were influenced by NAG.

Materials and methods

Experimental insect

Adults of *M. separata* were collected from a light trap at the agricultural station of the Northeast agricultural university, Harbin, China. The adults were reared in net cages and routinely fed with 5% honey water. After copulation, the females laid eggs in folded plastic ropes. Newly hatched larvae were transferred to plastic boxes and fed with fresh corn leaves at 25 ± 2 °C and 70% relative humidity, with a photoperiod of 14:10 (light: dark).

Cloning of the full-length cDNA sequence of *MsNAG*

Total RNA was extracted from three *M. separata* first-day 5th instar larvae separately using Trizol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The extracts were treated with 1% diethylpyrocarbonate to prevent ribonuclease (RNase) contamination. We ground 100 mg insect material in 1 mL TRIzol® reagent, then centrifuged it at 4 °C for 10 minutes at 12,000 × g. The liquid supernatant was transferred into 1.5-mL Eppendorf tubes and 200 μL $CHCl_3$ was added. The mixture was oscillated for 15 seconds to blend, then left at room temperature for 2.5 minutes before being centrifuged at 4 °C for 10 minutes at 12,000 × g. The liquid supernatant was transferred into 1.5-mL Eppendorf tubes and the same volume of isopropyl alcohol was added. The mixture was slowly blown to blend, then left in an ice bath for 30 minutes before being centrifuged at 4 °C for 15 minutes at 12,000 × g. The precipitate was collected and washed with 75% ethanol, then centrifuged at 4 °C for 5 minutes at 12,000 × g. The above steps were repeated. The precipitate was dried for 15-25 minutes and then dissolved in 30-50 μL water. An Implen NanoPhotometer® P300 was used to determine the RNA concentration. First-strand cDNA was synthesized by reverse transcription in 20 μL reaction mixtures containing 1 μg of total RNA, 200 U PrimeScript™ reverse transcriptase (TaKaRa, Dalian, China), 20 U RNase inhibitor, 1 μL dNTP mixture (10 mM each), and 1 μL oligo $(dT)_{18}$ primer (50 μM), which were incubated at 42 °C for 1 hour. A *MsNAG* fragment was cloned by PCR using primers MsB-F and MsB-R (table 1), which were designed based on other NAG mRNA sequences (*Mamestra brassicae β-N*-acetylglucosaminidase mRNA, KP730442; *Agrotis ipsilon β-N*-acetylglucosaminidase mRNA, GU985280). We used the SMARTER RACE cDNA amplification kit (TaKaRa, Dalian, China) to synthesize 5′- and 3′-RACE Ready cDNA sequences with primers 3′-RACE-F, 3′-RACE-R, 5′-RACE-F, and 5′-RACE-R primers (table 1) following the manufacturer's instructions. The full-length cDNA sequence was assembled using ContigExpress software and confirmed by PCR with an initial denaturation for 1 minute at 94 °C, followed by 35 cycles of 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 120 seconds, and a final extension at 72 °C for 10 minutes. The PCR products were linked to a pEasy-T1 vector using a pEASY-T1 Cloning Kit (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α competent cells. Three positive recombinant bacteria clones were selected to confirm the full-length sequence by PCRs with primers Ms-ORF-F and Ms-ORF-R. The resulting sequences were verified by BLAST searches against NCBI nucleotide database.

Table 1. The primer sequences used in the study.

Primer name	Sequences of primers (5' to 3')	Application	Efficiency
MsB-F	TTGTCTAAGATCAACATCAACAAC	Fragment cloning	
MsB-R	ACCACAAAGCTGCTTCTCCT		
3'-RACE-F	CACCGTCTGCTTCAATGCGGAGCCCTG	3'-RACE	
3'-RACE-R	TCCTGAAGCTCTGGAACTACTTCCGG		
5'-RACE-F	GCTCCACGCAGTAGGTCGCCCAG	5'-RACE	
5'-RACE-R	CCACGCACCAGTCCGTACTGCACCAC		
Ms-ORF-F	ATGTGGCTTCAAAAATGTACT	Full-length cDNA identification	
Ms-ORF-R	TCTTTGGCAGTAGCCTTCGTT		
MsQ-F	TTCAGAACCGCTGGGACCT	qRT-PCR	0.95
MsQ-R	GCCTTGTAAGCCCTATCCTGT		
Ms-β-F	CCAACGGCATCCACGAGACCA		0.98
Ms-β-R	TCGGCGATACCAGGGTACAT		
T_7 *EGFP$_1$*-F	TAATACGACTCACTATAGGGGACGTAAACGGCCACAAGTT	RNAi	
T_7 *EGFP$_1$*-R	GGGGTGTTCTGCTGGTAGTG		
T_7 *EGFP$_2$*-F	GACGTAAACGGCCACAAGTT		
T_7 *EGFP$_2$*-R	TAATACGACTCACTATAGGGGGGGTGTTCTGCTGGTAGTG		
T_7 *MsNAG$_1$*-F	TAATACGACTCACTATAGGGAGACGAGGAACTTCTACACCATC		
T_7 *MsNAG$_1$*-R	CTATCCTGTGCCTTCATCTG		
T_7 *MsNAG$_2$*-F	CGAGGAACTTCTACACCATC		
T_7 *MsNAG$_2$*-R	TAATACGACTCACTATAGGGAGACTATCCTGTGCCTTCATCTG		

Construction of a phylogenetic tree

We used the deduced amino acid sequence of *MsNAG*, insect NAGs and related hexosaminidases searched by *MsNAG* in NABI database to construct a phylogenetic tree by using MEGA 6.0 software. The unweighted pair-group method with arithmetic averages (UPGMA) was used, and a bootstrap analysis of 5,000 replications was performed to evaluate the branch strengths of the phylogenetic tree.

Analysis of *MsNAG* expression patterns

We extracted RNA from *M. separata* first- and last-day of each instar larvae, prepupae, pupae, female and male adults as well as from seven different tissues of second-day 5th instar larvae using Trizol® reagent (Invitrogen). The seven tissues were foreguts, midguts, hindguts, fat bodies, salivary glands, Malpighian tubules, and integuments.

We used 2 μg of the RNAs to synthesize cDNAs using ReverTra Ace qPCR RT Kit (TOYOBO, Shanghai, China) according to the manufacturer's instructions. The expression patterns of *MsNAG* at different developmental stages and in different tissues were analysed by qRT-PCR using primers MsQ-F and MsQ-R (efficiency 0.95). β-actin was used as an internal reference gene with primers Ms-β-F and Ms-β-R (efficiency 0.98). The primers are listed in table 1. The qRT-PCR was performed as follows: initial denaturation of the cDNA at 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. A melting curve was constructed to confirm the amplification specificity of each qRT-PCR. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean expression ratio (± SE) of three biological replicates were calculated.

To confirm qRT-PCR data, an 18-cycle RT-PCR was used to analysis the *MsNAG* expression at different developmental stages and in different tissues. PCR was performed using the templates and primers used for the qRT-PCRs. The procedure was as follows: denaturation at 94 °C for 1 minutes, followed by 18 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 2 minutes, and a final extension at 72 °C for 10 minutes. PCR products were analysed by 1% agarose gel electrophoresis.

Induction of *MsNAG* by ecdysone

We obtained 20E solutions at concentrations of 2.5 μg/μL, 5 μg/μL, 10 μg/μL, and 20 μg/μL by dissolving 2.5 mg, 5 mg, 10 mg, and 20 mg 20E in 100 μL 1% dimethylsulfoxide (DMSO) then adding ddH_2O to 1 mL. Four experimental groups were used for every 20E concentration and a total of 225 one-day-old 5th instar larvae were dissected. Each experimental group and the control group included 45 larvae, which were divided into three replicates. The larvae in the experimental groups were injected with 2 μL of 20E at the different concentrations. The larvae in the control group were injected with 2 μL 0.1% DMSO. After 1, 3, 6, 12, 24, 48, and 96 hours, larvae were sampled and total RNA was extracted. The expression levels of *MsNAG* injected with different 20E concentrations were quantified using qRT-PCR. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean expression ratio (± SE) of three biological replicates were calculated. The RNA (2 μg) was used to synthesize cDNA by reverse transcription, then qRT-PCRs and 18-cycle PCRs were performed as described above.

Functional analysis of *MsNAG* by RNA interference

We designed primers using the E-RNAi webservice (http://www.dkfz.de/signaling/e-rnai3/) (shown in table 1) to synthesize templates by PCR. The templates were used to synthesize dsRNAs of *MsNAG* and enhanced green fluorescent protein (*EGFP*) using the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). We divided 180 one-day-old 5th instar larvae into two groups for *dsRNA* injection of *EGFP* for non-specific effects or *MsNAG* for RNAi. Each group contained 90 insects divided into three replicates. After injection with *dsMsNAG* or *dsEGFP*, total RNA was extracted from the larval carcass and integuments at 24, 48, and 72 hours. The RNA (2 μg) was used to synthesize cDNA for the qRT-PCRs. The expression levels of *MsNAG* treated with *dsMsNAG* or *dsEGFP* were quantified using qRT-PCR. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and mean expression ratio (± SE) of three biological replicates were calculated. The results were analysed to examine the suppressive effect of RNAi on *MsNAG* expression. Morphological data and mortality rate were also recorded.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 software (IBM Corp., Armonk, New York, USA). Differences among groups of means were examined using a one-way analysis of variance followed by Duncan's multiple range test ($P < 0.05$).

Results

Identification of *MsNAG*

RT-PCR and RACE were used to clone NAG from *M. separata*. We cloned and identified a novel NAG cDNA sequence from *M. separata*, referred to as *MsNAG* (GenBank accession number KY348777). The full-length cDNA sequence is 2,619 nucleotides long and contains a 1,785-nucleotide open reading frame and 3′, 5′ untranslated regions. The cDNA encodes an amino acid sequence with 594 residues that contain the conserved motif HMGGDEVSERCW (figure 1), which forms the catalytic site and is a characteristic of the GH20 family. A 21 amino acid putative signal peptide was detected using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) and three potential N-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The phylogenetic tree showed that the insect NAGs formed four groups: two groups of chitinolytic NAGs (Groups I and II), N-glycan-processing NAGs (Group III), and hexosaminidases (Group IV) (figure 2). *MsNAG* clustered with the Group I chitinolytic NAGs, many of which have been cloned from lepidopteran insect species,

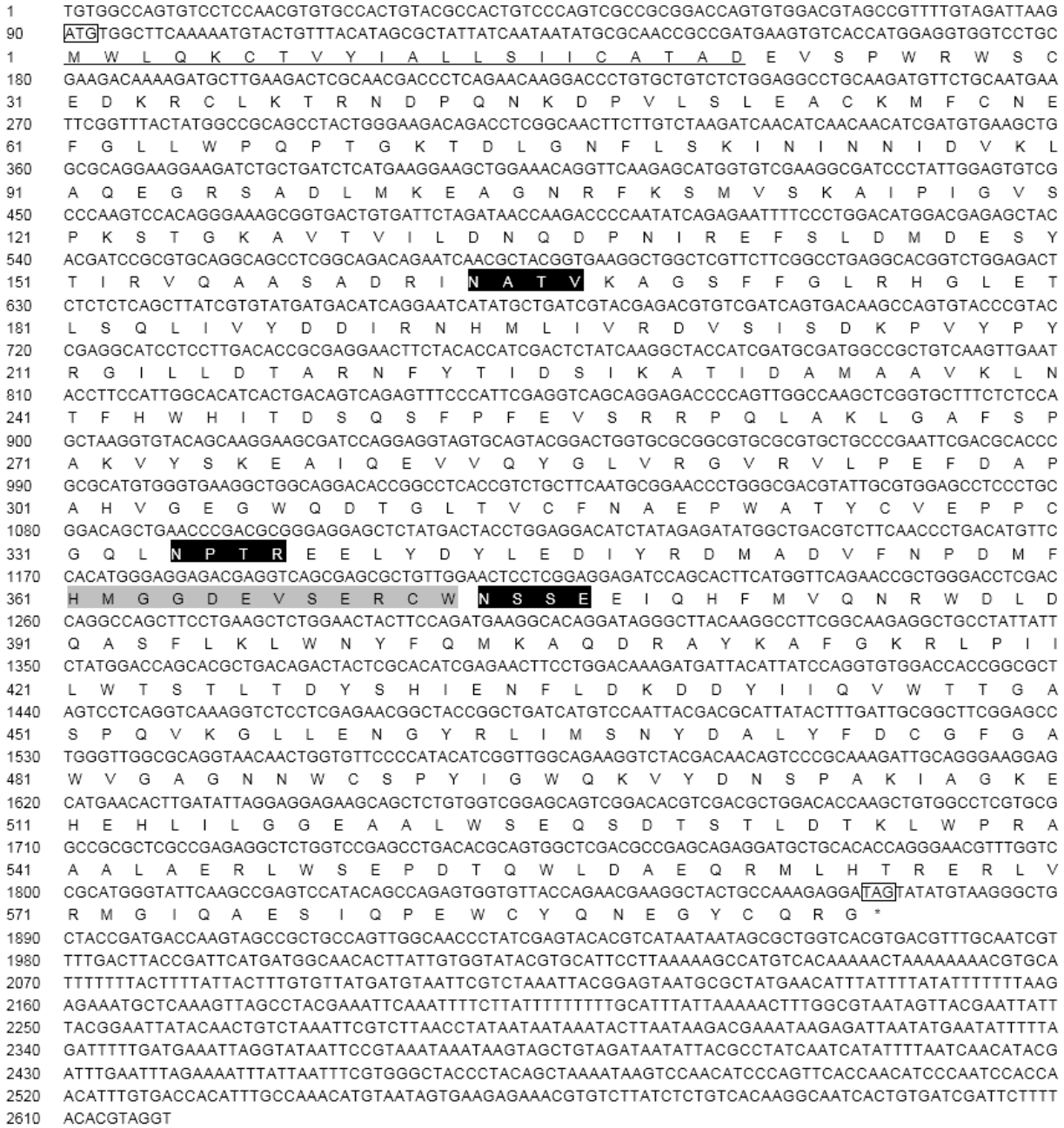

Figure 1. Nucleotide and deduced amino acid sequences of *MsNAG* cDNA from *M. separata*. The start (ATG) and stop (TAG) codons are boxed. The putative signal peptide is underlined. The three potential N-glycosylation sites are shaded in black. The conserved motif is highlighted in grey.

including *Agrotis ipsilon* (ADF56765), *Xestia c-nigrum* (ACR57832), *Mamestra brassicae* (AKR06190), *Trichoplusia ni* (AAL82580), and *Manduca sexta* (AAQ97603).

Stage- and tissue-specific expression of *MsNAG*

qRT-PCR and 18-cycle RT-PCR were used to examine spatiotemporal expression of *MsNAG*. *MsNAG* showed different expression patterns at different developmental stages. The *MsNAG* mRNA transcript was detected at all the developmental stages by qRT-PCR (figure 3A). The 18-cycle PCR results (figure 3B) were consistent with the qRT-PCR results, implying that *MsNAG* was expressed at all instar larval stages as well as at the prepupal, pupal, and adult stages.

MsNAG expression level was higher on the last day than on the first day at 4th instar, 5th instar and 6th instar larval stage. *MsNAG* expression level was highest on the last day at 6th instar larval stage, which was also the prepupal stage, being 95.2-fold higher than its expression at the first-day 1st instar larval stage, then its expression level decreased at the pupal stage, but the expression level at this stage still showed significant difference with its expression at the first-day 1st instar larval stage.

MsNAG showed different expression patterns in different tissues by both qRT-PCR (figure 4A) and 18-cycle PCR (figure 4B). In salivary glands, *MsNAG*

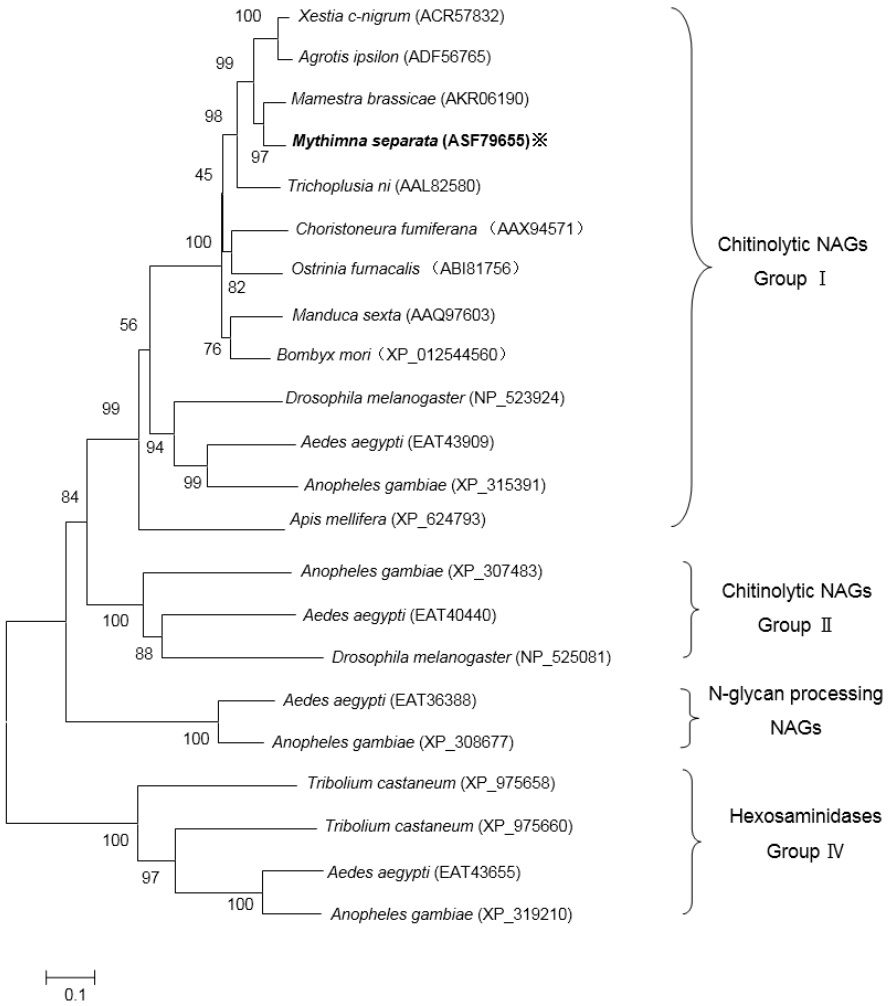

Figure 2. Phylogenetic tree of insect NAGs and related hexosaminidases from the NCBI databases. The consensus phylogenetic tree was constructed according to the unweighted pair group method with arithmetic means (UPGMA) using MEGA 6.0 and a bootstrap analysis of 5,000 replications was performed to evaluate the branch strengths of the phylogenetic tree.

expression level was significantly higher than in the other tested tissues and was 53.3-fold higher than in hindgut. In integuments, *MsNAG* expression level was 29.3-fold higher than in hindgut. In midgut, Malpighian tubules, foregut and fat bodies, *MsNAG* expression levels were 14.3-, 6.8-, 2.9- and 2.7-fold higher than in hindgut.

Effect of ecdysone on *MsNAG*

20E at different concentrations were used to induce the expression of *MsNAG*. We injected one-day-old 5th instar larvae with four different concentrations of 20E (the experimental groups) or DMSO (the control group). The qRT-PCR results showed that 20E influenced the expression of *MsNAG* at the mRNA level (figure 5A). The 18-cycle PCR produced the same result (figure 5B). *MsNAG* expression level was highest at 24 hours after injection with 10 μg/μL 20E, being 2.5-fold higher than that injection with DMSO. At every time point, the *MsNAG* expression levels were higher in the four experimental groups than in the control, and the expression levels increased as the 20E concentrations increased from 2.5 to 10 μg/μL, but decreased at 20 μg/μL.

We found that the trend in *MsNAG* expression variations was consistent among four different concentrations of 20E treatment groups at the different time points. *MsNAG* expression increased from 1 to 24 hours reaching its highest level at 24 hours, decreased to its lowest point at 48 hours, then increased at 96 hours.

Developmental duration of the four experimental groups after injection with 2.5 μg/μL, 5 μg/μL, 10 μg/μL, or 20 μg/μL 20E showed that the time points when larvae finished the 5th instar larvae ecdysis were earlier by an average of 0.8, 3.0, 4.9, and 0.6 hours, respectively, than that of the larvae in the control group.

Influence of RNA interference on *MsNAG*

RNAi was used to examine the suppressive effect on *MsNAG* expression and insects' morphological data and mortality rate. At 24 hours point of injection of *dsMsNAG*, the *MsNAG* expression levels in larval carcass and integuments were repressed by 76.5% and 67.9% and, at 48 hours point, the expression levels were repressed by 80.6% and 81.1% compared with the expression levels injection of *dsEGFP* (figure 6).

Larvae in the RNAi experimental groups showed

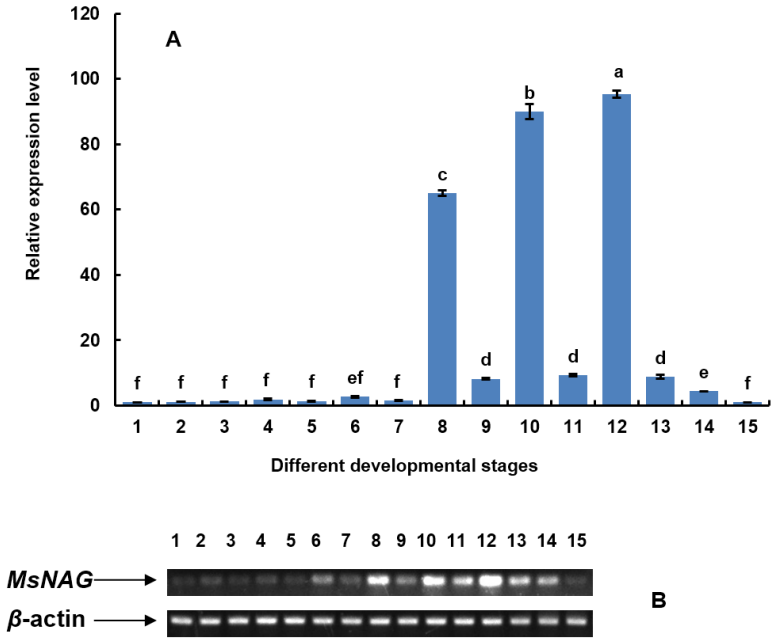

Figure 3. Developmental stage-dependent expression pattern of *MsNAG*. **(A)** Relative expression levels at different developmental stages. The numbers on the *x* axis indicate the different stages as follows: 1: first-day 1st instar larvae; 2: last-day 1st instar larvae; 3: first- day 2nd instar larvae; 4: last-day 2nd instar larvae; 5: first-day 3rd instar larvae; 6: last-day 3rd instar larvae; 7: first-day 4th instar larvae; 8: last-day 4th instar larvae; 9: first-day 5th instar larvae; 10: last-day 5th instar larvae; 11: first-day 6th instar larvae; 12: last-day 6th instar larvae (prepupae); 13: pupae; 14: female adults; 15: male adults. Three biological replicates were conducted. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and means ± SEs were calculated. The relative expression levels were calculated as the ratios of relative copy numbers in individuals at different developmental stages. Statistical differences at $P < 0.05$ are indicated by different lowercase letters. **(B)** Result of the 18-cycle PCR agarose gel electrophoresis. The numbers above the *MsNAG* and β-actin bands correspond to the order of the expression level bars in (A).

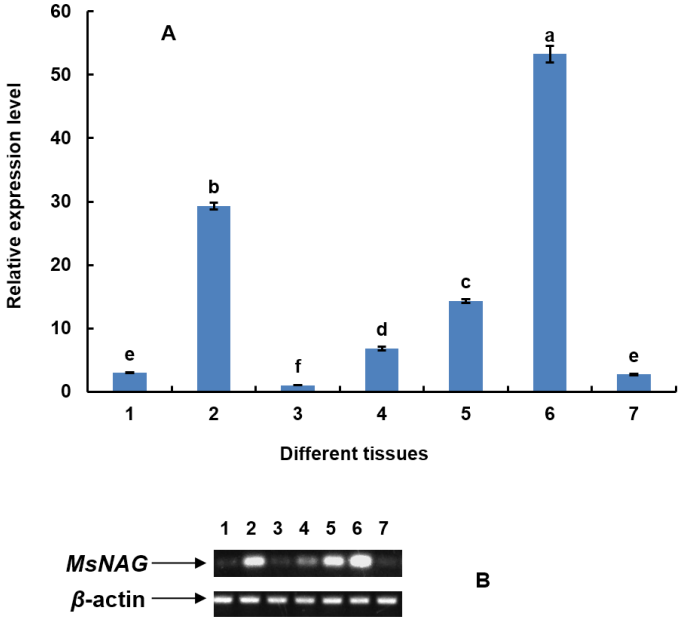

Figure 4. Tissue-dependent expression pattern of *MsNAG*. **(A)** Relative expression levels in different tissues. The numbers on the *x* axis indicate the different tissues as follows: 1: foreguts; 2: integuments; 3: hindguts; 4: Malpighian tubules; 5: midguts; 6: salivary glands; 7: fat bodies. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and means ± SEs were calculated. Statistical differences at $P < 0.05$ are indicated by different lowercase letters. The RNA was extracted from the tissues of 5th instar larvae. **(B)** Result of the 18-cycle PCR agarose gel electrophoresis. The numbers above the *MsNAG* and β-actin bands correspond to the order of the expression level bars in (A).

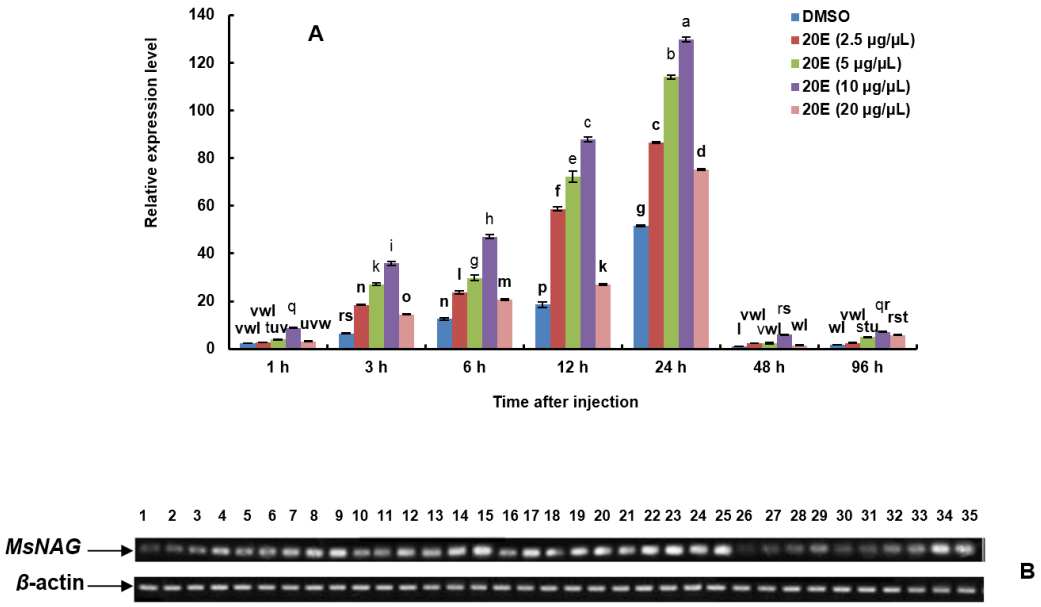

Figure 5. Effect of 20-hydroxyecdysone (20E) on the expression pattern of *MsNAG*. **(A)** Relative expression levels in different doses of 20E. Total RNA was extracted from second-day 5th instar larvae injected with 20E concentrations of 2.5, 5, 10, and 20 μg/μL. The time points on the *x* axis indicate hours after the insects injected with 20 E. DMSO is the control group. The relative transcript levels were measured using the $2^{-\Delta\Delta CT}$ method and means ± SEs were calculated from three individuals with three technical replicates each. Statistical differences at $P < 0.05$ are indicated by different lowercase letters. **(B)** Result of the 18-cycle PCR agarose gel electrophoresis. The numbers above the *MsNAG* and β-actin bands correspond to the order of the expression level bars in (A).

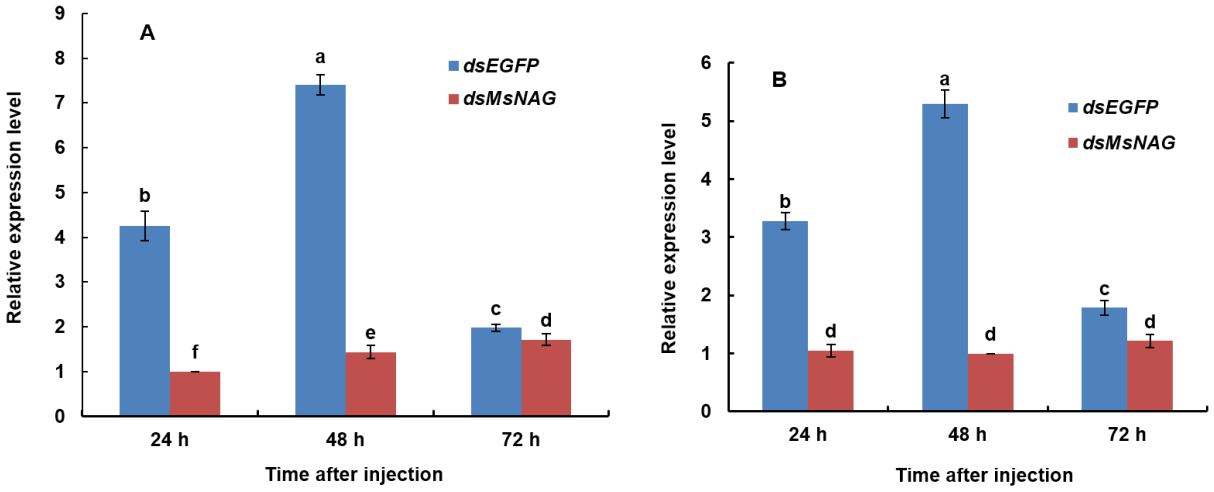

Figure 6. Influence of RNAi on *MsNAG* expression levels. Relative *MsNAG* expression in **(A)** insect bodies and **(B)** integuments at different times after the larvae were injected with *dsEGFP* or *dsMsNAG*. Total RNA was extracted at 24, 48, and 72 hours after injection with *dsRNA*. Means ± SEs were calculated to measure *MsNAG* expression ratios using $2^{-\Delta\Delta CT}$ values from three individuals with three technical replicates each. Significant differences are indicated by different lowercase letters ($P < 0.05$).

stunted growth. The developmental duration of 5th to 6th instar larvae with no RNAi treatment (sterilized water injection) or after *dsEGFP* injection were an average of 83.50 hours and 83.80 hours, respectively, and an average of 95.57 hours after *dsMsNAG* injection (figure 7). The *dsMsNAG* treatment group showed different morphological differences. After injection of *dsRNA* 24 and 48 hours, the body weight of larvae in the control group were significantly higher than those in the treatment group. After 72 hours of treatment, the body weight of larvae did not show significant difference between *dsMsNAG* and *dsEGFP* treatment (figure 8A). But at the pupal stage, we could examine the significant difference between *dsMsNAG* and *dsEGFP* treatment (figure 8B). After injection of *dsRNA*, the average body length of larvae and pupae in the control group did not show

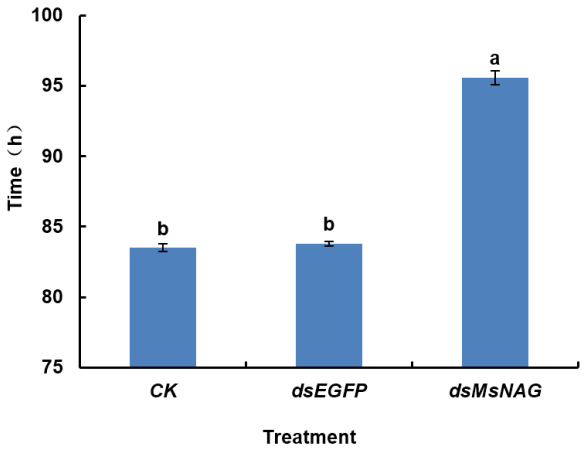

Figure 7. Developmental duration of larvae from 5th to 6th larva-larva ecdysis after different RNAi treatments. No RNAi treatment or sterilized water injection was acted as control (CK). Means ± SEs were calculated to measure *MsNAG* expression ratios using $2^{-\Delta\Delta CT}$ values from three individuals with three technical replicates each. Significant differences are indicated by different lowercase letters ($P < 0.05$).

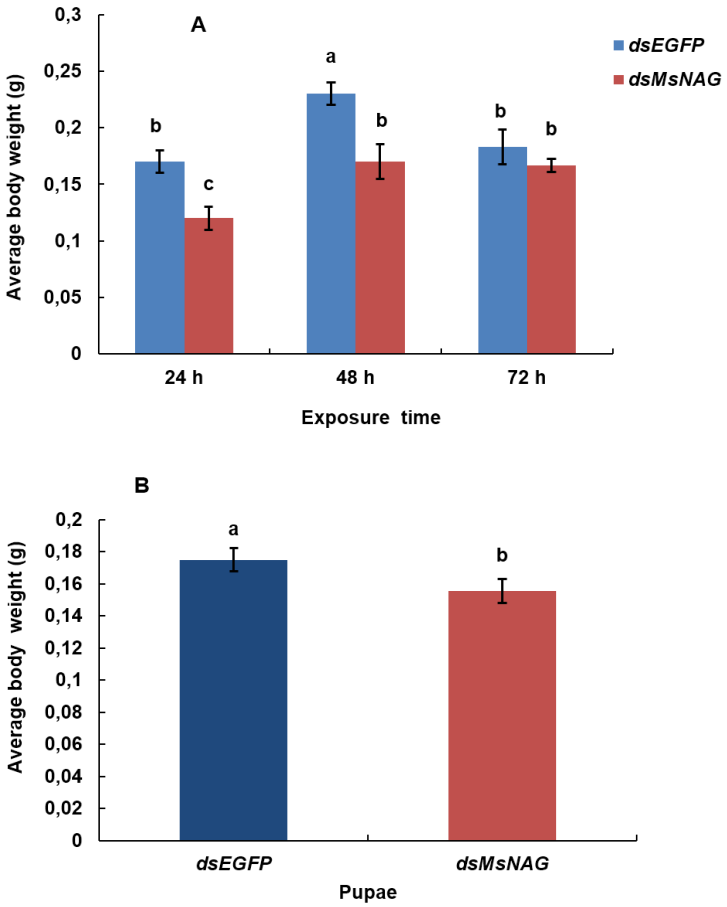

Figure 8. Body weights changes after RNAi. **(A)** Average body weights of larvae at 24, 48, 72 hours after injecting with *dsEGFP* or *dsMsNAG*. **(B)** Average body weights of pupae after injecting with *dsEGFP* or *dsMsNAG*. Significant differences are indicated by different lowercase letters ($P < 0.05$).

significant difference with those in the treatment group (figure 9).

After pupation, the pupal morphology of the control group was normal and the pupal integuments were smooth and sound, whereas the pupal integuments in the treatment group were ceasmic (figure 10). In the *dsMsNAG* treatment group, deaths occurred from 5th instar larvae to adults. The mortality rate reached 52.0% in the last stage (figure 11).

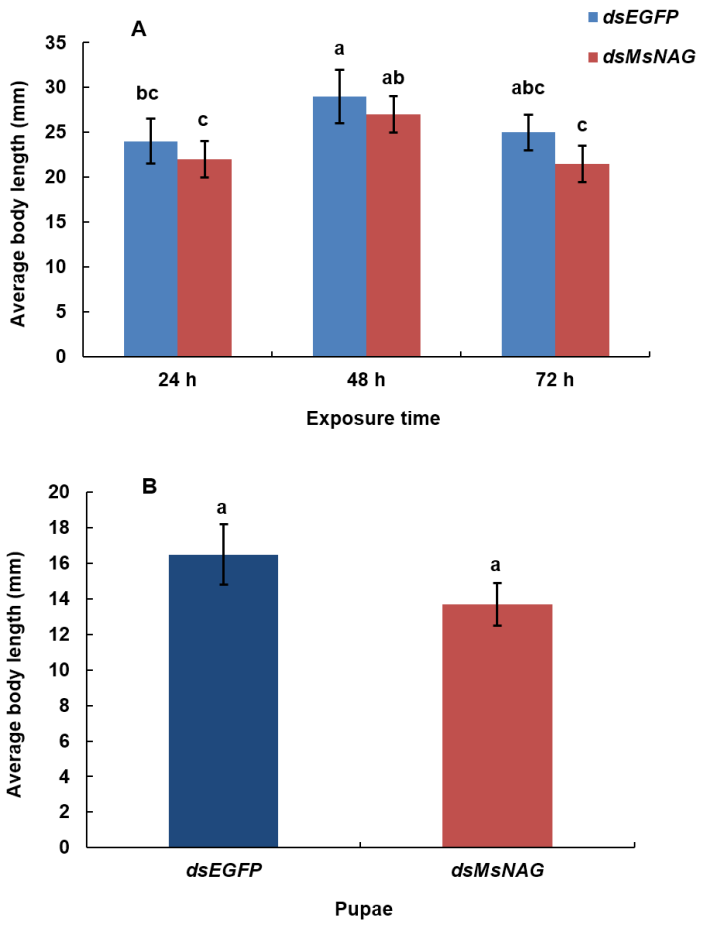

Figure 9. Body length changes after RNAi. **(A)** Average body length of larvae at 24, 48, 72 hours after injecting with *dsEGFP* or *dsMsNAG*. **(B)** Average body length of pupae after injecting with *dsEGFP* or *dsMsNAG*. Significant differences are indicated by different lowercase letters ($P < 0.05$).

Figure 10. The pupal shape changes after larval treatment with *dsRNA*. **(A)** pupa from *dsEGFP*-treated larva. **(B)** pupa from *dsMsNAG*-treated larva.

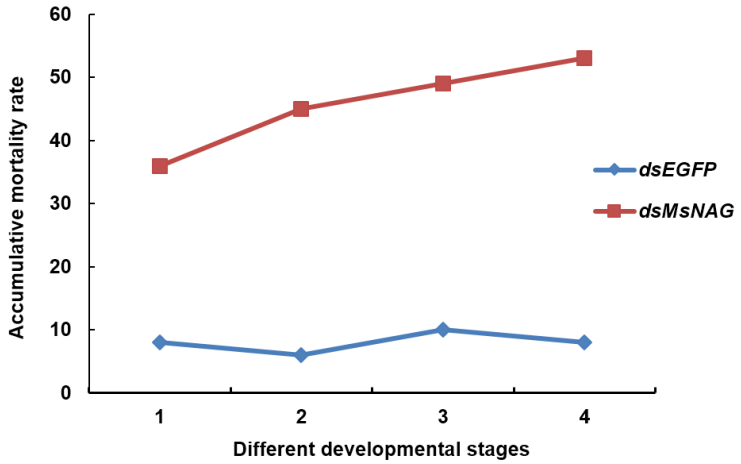

Figure 11. Accumulative mortality rate of *M. separata* at different developmental stages after *dsRNA* injection. The numbers on the *x* axis indicate the different stages as follows: 1: 5th instar larvae; 2: 6th instar larvae; 3: pupae; 4: adults.

Discussion

MsNAG is a key glycosidase enzyme involved in the bio-degradation of chitin. To further understand the NAG system and its role in *M. separata*, a novel NAG, *MsNAG*, was cloned and characterized. The deduced amino acid sequence of *MsNAG* included a conserved motif HMGGDEVSERCW of the 20 glycosyl hydrolase family, as well as several other highly conserved motifs. The phylogenetic tree showed that NAGs were divided into four groups and *MsNAG* belonged to the chitinolytic NAGs of Group I.

In our study, *MsNAG* expression levels at developmental stages were highest at prepupal stage, which expressed high level of *MsNAG* to complete the larval-pupal transformation. A study on NAG in *M. brassicae* found that the mRNA expression level increased gradually from the 1st to 6th instar larval stages and peaked in the prepupal stage (Zhang *et al*., 2016). In this study, we found that *MsNAG* expression levels were higher on the last day than those on the first day from 4th to 6th instar larval stage. Consistent with this result, Zheng and colleagues found that the gene expression levels were high on the last days in 5th and 6th instar larvae, which were ready to moult (Zheng *et al*., 2010).

MsNAG expression levels varied in tissues and was highest in the salivary glands. This may be related to the lumen of the salivary duct being lined with a cuticle. *MsNAG* was expressed highly in integuments and midguts, and was also expressed in Malpighian tubules, foreguts, fat bodies and hindguts. Chitin is one of the chemical components deposited in trachea in insects. Trachea is spread in almost all tissues. Though in some tissues, they maybe lack of cuticle, they contain chitin that need NAG to metabolize.

NAG expression level was promoted after injection of 20E into larvae of *M. sexta* and *M. brassicae* (Zen *et al*., 1996; Zhang *et al*., 2016). *MsNAG* expression was also successfully induced by ecdysone. *MsNAG* expression levels could be induced by after injections of four different 20E.

When the 20E concentrations increased from 2.5 to 10 μg/μL, *MsNAG* expression levels reached highest point. When 20 μg/μL of 20E was used, induction level decreased. That means high level of 20E could not induce high level of *MsNAG* mRNA. The larval stage transformations were controlled by a balance of ecdysing and juvenile hormones. When the level of ecdysone increased, moulting resulted. However, high doses of hormones can destroy the normal physiological activity of some organs, affecting growth (Ryerse and Stephen, 1978).

MsNAG expression levels increased from 1 to 12 hours, peaking at 12 hours, but decreased at 24 hours after injection with 20E. The expression levels were lowest at 48 hours and then increased at 96 hours. We inferred that *MsNAG* of larvae showed high expression level to moult at 24 hours, but descended at 48 hours with the accomplishment of moulting, and ascended at 96 hours because insects gradually entered the next moulting process. Based on observation, larvae shed their cuticles from 24 to 48 hours after injection with 20E, but the shedding process occurred earlier in the four concentrations of 20E treatment groups. Thus, ecdysone accelerated the moulting process.

RNAi has been successfully conducted in some insects. RNAi targeting NAG1 in *L. migratoria manilensis* induced gene silencing, 50% of the *dsLmNAG1*-injected nymphs were not able to moult successfully and eventually died (Rong *et al*., 2013). RNAi reduced the *MbNAG* mRNA expression level and induced both abnormal molting phenotypes and a high mortality rate in *M. brassicae* larvae (Zhang *et al*., 2016). *MsNAG* transcript levels in larval carcass and integuments were repressed significantly after *dsMsNAG* injection. The developmental duration of larvae injected with *dsMsNAG* was 11.8 hours longer than *dsEGFP* injection from fourth to the fifth larva-larva ecdysis on average. Some morphologi-

cal differences, such as reduced body weight, short body length were observed after injection of *dsMsNAG*. About half of the total insects died at last. The result indicated that the gene of *MsNAG* could be successfully silenced by RNAi, and *MsNAG* is associated with insect moulting and affect the process of moulting.

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References

ARAKANE Y., HOGENKAMP D. G., ZHU Y. C., KRAMER K. J., SPECHT C. A., BEEMAN R. W., KANOST M. R., MUTHUKRISHNAN S., 2004.- Characterization of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage in one of the genes during development.- *Insect Biochemistry and Molecular Biology*, 34 (3): 291-304.

BAO W., CAO B., ZHANG Y., WURIYANGHAN H., 2016.- Silencing of *Mythimna separata* chitinase genes via oral delivery of in planta-expressed RNAi effectors from a recombinant plant virus.- *Biotechnology Letters*, 38 (11): 1961-1966.

CANTAREL B. L., COUTINHO P. M., RANCUREL C., BERNARD T., LOMBARD V., HENRISSAT B., 2009.- The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics.- *Nucleic Acids Research*, 37: D233-D238.

CHARPENTIER M., PERCHERON F., 1983.- The chitin-degrading enzyme system of a *Streptomyces* species.- *The International Journal of Biochemistry*, 15 (3): 289-292.

CHEN F., CHEN X. Z., QIN L. N., TAO Y., DONG Z. Y., 2015.- Characterization and homologous overexpression of an *N*-acetylglucosaminidase Nag1 from *Trichoderma reesei*.- *Biochemical and Biophysical Research Communications*, 459 (2): 184-188.

DORDAL M. S., WANG F. F., GOLDWASSER E., 1985- The role of carbohydrate in erythropoietin action.- *Endocrinology*, 116 (6): 2293-2299.

FAN D., GAO Y. L., GUO B. Z., HAN L. L., ZHOU C. M., WANG X. Y., LIU J., 2011.- cDNA cloning and prokaryotic expression of *β-N*-acetylglucosaminidase from *Xestia c-nigrum*.- *Plant Protection*, 37 (5): 50-55.

FILHO B. P., LEMOS F. J., SECUNDINO N. F., PASCOA V., PEREIRA S. T., PIMENTA P. F., 2002.- Presence of chitinase and *β-N*-acetylglucosaminidase in the *Aedes aegypti* a chitinolytic system involving peritrophic matrix formation and degradation.- *Insect Biochemistry and Molecular Biology*, 32 (12): 1723-1729.

FUKAMIZO T., KRAMER K. J., 1985.- Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid.- *Insect Biochemistry*, 15 (2): 141-145.

GAO Y., WU L., GUO B. Z., FAN D., 2012- cDNA cloning and mRNA expression of *β-N*-acetylglucosaminidase in different tissues of *Agrotis ipsilon* Hufngel.- *Chinese Journal of Applied Entomology*, 49 (6): 1496-1502.

GEISLER C., AUMILLER J. D., 2008.- A fused lobes gene encodes the processing *β-N*-acetylglucosaminidase in Sf9 cells.- *Journal of Biology Chemistry*, 283 (17): 11330-11339.

GOO T. W., HWANG J. S., 1999.- Molecular cloning and characterization of a gene encoding a beta-N-acetylglucosaminidase homologue from *Bombyx mandarina*.- *Korean Journal of Sericultural Science*, 41: 147-153.

HOGENKAMP D. G., ARAKANE Y., KRAMER K. J., 2008- Characterization and expression of the *β-N*-acetylhexosaminidase gene family of *Tribolium castaneum*.- *Insect Biochemistry and Molecular Biology*, 38 (4): 478-489.

INTRA J., PAVESI G., HORNER D. S., 2008.- Phylogenetic analyses suggest multiple changes of substrate specificity within the glycosyl hydrolase 20 family.- *Bmc Evolutional Biology*, 8 (1): 214-220.

KRAMER K. J., MUTHUKRISHNAN S., 1997.- Insect chitinases: molecular biology and potential use as biopesticides.- *Insect Biochemistry and Molecular Biology*, 27 (11): 887-900.

LIU T., LIU F., YANG Q., 2009.- Expression, purification and characterization of the chitinolytic *beta-N*-acetyl-*D*-hexosaminidase from the insect *Ostrinia furnacalis*.- *Protein Expression and Purification*, 68 (1): 99-103.

MERZENDORFER H., ZIMOCH L., 2004.- Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases.- *Journal Experimental Botany*, 206 (24): 4393-4412.

MOMMSEN T. P., 1980.- Chitinase and *β-N*-acetylglucosaminidase from the digestive fluid of the spider, *Cupiennius salei*.- *Biochimica et Biophysica Acta Biomembranes*, 612 (2): 361-372.

OKADA T., ISHIYAMA S., SEZUTSU H., USAMI A., TAMURA T., MITA T., FUJIYAMA K., SEKI T., 2007.- Molecular cloning and expression of two novel *β-N*-acetylglucosaminidases from silkworm *Bombyx mori*.- *Bioscience Biotechnology and Biochemistry*, 71 (7): 1626-1635.

RONG S., LI D. Q., ZHANG X. Y., LI S., ZHU K. Y., GUO Y. P., MA E. B., ZHANG J. Z., 2013.- RNA interference to reveal roles of *β-N*-acetylglucosaminidase gene during molting process in *Locusta migratoria*.- *Insect Science*, 20 (1): 109-119.

RYERSE J. S., 1978.- The hormonal control of malpighian tubule structure and function during larval-pupal-adult development of the skipper butterfly *Calpodes ethlius*, 274 pp., *PhD Thesis*, Western University, London, Ontario, Canada.

TOMIYA N., NARANG S., LEE Y. C., BETENBAUGH M. J., 2004.- Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines.- *Glycoconjugate Journal*, 21 (6): 343-360.

WAGNER G. P., 1994- Evolution and multi-functionality of the chitin system.- *EXS*, 69 (69): 559-577.

XI Y., PAN P. L., ZHANG C. X., 2015.- The *β-N*-acetylhexosaminidase gene family in the brown planthopper, *Nilaparvata lugens*.- *Insect Molecular Biology*, 24 (6): 601-610.

ZEN K. C., CHOI H. K., KRISHNAMACHARY N., MUTHUKRISHNAN S., KRAMER K. J., 1996.- Cloning, expression, and hormonal regulation of an insect *β-N*-acetylglucosaminidase gene.- *Insect Biochemistry and Molecular Biology*, 26 (5): 435-444.

ZHANG H., ZHAO K., FAN D., 2016.- Molecular cloning and RNA interference analysis of *β-N*-acetylglucosaminidase in *Mamestra brassicae* L.- *Journal Asia-Pacific Entomology*, 19 (3): 721-728.

ZHENG Y. P., KRELL P. J., DOUCET D., 2008.- Cloning, expression, and localization of a molt-related *β-N*-acetylglucosaminidase in the spruce budworm, *Choristoneura fumiferana*.- *Archives of Insect Biochemistry and Physiology*, 68 (1): 49-59.

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