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RNA interference to reveal roles of β-*N*-acetylglucosaminidase gene during molting process in *Locusta migratoria*

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Keywords: *Locusta migratoria*; chitin degradation; *β-N*-acetylglucosaminidase; RNAi

Running title: Roles of *β-N*-acetylglucosaminidase gene

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ABSTRACT: β-*N*-acetylglucosaminidases are crucial enzymes involved in chitin degradation in insects. We identified a β-*N*-acetylglucosaminidase gene (*LmNAG1*) from *Locusta migratoria*. The full-length cDNA of *LmNAG1* consists of 2667 nucleotides, including an open reading frame (ORF) of 1845 nucleotides encoding 614 amino acid residues, and 233- and 589-nucleotide non-coding regions at the 5'- and 3'-ends, respectively. Phylogenetic analysis grouped the cDNA-deduced LmNAG1 protein with the enzymatically characterized NAGs in group I. Analyses of stage- and tissue-dependent expression patterns of *LmNAG1* were carried out by real-time quantitative PCR. Our results showed that *LmNAG1*transcript level in the integument was significantly high in the last two days of the fourth and fifth-instar nymphs. *LmNAG1* was highly expressed in foregut and hindgut. RNA interference of *LmNAG1* resulted in an effective silence of the gene and a significantly reduced total LmNAG enzyme activity at 48 and 72 h after the injection of *LmNAG1* dsRNA. As compared with the control nymphs injected with *GFP* dsRNA, 50% of the ds*LmNAG1* injected nymphs were not able to molt successfully and eventually died. Our results suggest that *LmNAG1* plays an essential role in molting process of *L. migratoria*.

Key words: *Locusta migratoria*; chitin degradation; β-*N*-acetylglucosaminidase; RNA interference

Introduction

Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of a *N*-acetylglucosamine, a derivative of glucose, and is found in many places throughout the natural world (Arakane *et al*., 2004). It is the main component of the cell walls of fungi, the exoskeletons of arthropods, the radulas of mollusks, and the beaks of cephalopods. Chitin is widespread throughout insects and mainly functions as a scaffold material supporting the cuticle (Kramer and Koga, 1986) and peritrophic matrix that lines the midgut (Khajuria *et al*., 2010). In addition, chitin deposition occurs in the trachea and the lining of the foregut and hindgut (Merzendorfer and Zimoch, 2003; Zhang *et al*., 2010). Chitin is also found in the mouthpart and salivary glands of some insects (Kramer and Muthukrishnan, 2005).

The degradation of chitin in insects is accomplished by a binary enzyme system composed of chitinases and β-*N*-acetylglucosaminidases (NAGs, EC 3.2.1.30) which are also known as chitobiases. Chitin is first hydrolyzed by chitinases into various small oligomers, which later cleaved from the nonreducing end into the monomeric *N*-acetylglucosamine (GlcNAc) by NAGs. The binary enzyme system can synergize the catalytic activity in chitin degradation (Fukamizo and Kramer, 1985a, b). Several NAGs have been identified in various insect species including *Bombyx mori* (Nagamatsu *et al*., 1995), *Manduca sexta* (Zen *et al*., 1996), *Aedes aegypti* (Benedito *et al*., 2002), *Choristoneura fumiferana* (Zheng *et al*., 2008), *Tribolium castaneum* (Hogenkamp *et al*., 2008) and *Ostrinia furnacalis* (Liu *et al*., 2009). All these NAGs were proven to be involved in the process of insect chitin degradation. The NAGs in *B. mori* and *M. sexta* were present in the molting fluid and the timing of expression suggests a function in the cuticular chitin turnover during molting (Nagamatsu *et al*., 1995;

78 Zen *et al*., 1996). Furthermore, one *M. sexta* NAG was found to be under control of hormone
79 (Zen *et al*., 1996). Four putative NAG genes were identified from *T. castaneum*, and showed
80 different stage- and tissue-dependent expression patterns. However, knockdowns of the four
81 *TcNAG* genes resulted in similar phenotypes of developmental arrest and death of the insects
82 at the time of the next molt (Hogenkamp *et al*., 2008).

83 The migratory locust (*Locusta migratoria*), an orthopteran species, is one of the most
84 severe agricultural pests in prairies and croplands. The destructive outbreaks of *L. migratoria*
85 had been increasing in China (Xia, 2002). Because chitin is indispensable for insects as a
86 structural component and does not exist in higher animals (Cohen, 2001),
87 β-*N*-acetylglucosaminidase involved in chitin degradation could be a potential insect-specific
88 target for developing green pesticides. So far, the research concerning insect chitinolytic
89 β-*N*-acetylglucosaminidases mainly focuses on holometabolous insects including Lepidoptera
90 (*B. mori*, *M. sexta*, *C. fumiferana*, *O. furnacalis*), Diptera (*A. aegypti*) and Coleoptera (*T.*
91 *castaneum*). There is no information of *NAG*s from hemimetabolous insects. *L. migratoria*
92 has been long recognized as a model of hemimetabolous insects. Furthermore, the locust EST
93 (expressed sequence tag) database and amenability of *L. migratoria* (Zhang *et al*., 2010)
94 make it a practicable model species to study NAGs. In this paper, we report: 1) sequence
95 analysis of full-length cDNA of *LmNAG1* from *L. migratoria*; 2) phylogenetic analysis of
96 cDNA-deduced LmNAG1protein; 3) stage- and tissue-dependent expression patterns of
97 *LmNAG1*; and 4) biological function of *LmNAG1* as revealed by RNA interference (RNAi).

98 **Materials and methods**

Insects

The eggs of *L. migratoria* were provided by the Insect Protein Co., Ltd. of Cangzhou City in China. The eggs were incubated in a LED growth chamber (Shanghai, China) at 28±1°C with a 14:10-h (light: dark) photoperiod and 60% humidity. After hatch, insects were reared using wheat sprout and kept under the same temperature and light conditions. The developmental stages of the insect were synchronized at each molt by collecting newly molted nymphs. All the locust samples were freshly utilized or stored at -80 °C until use.

Identification of β-N-acetylglucosaminidase gene

Seventeen cDNA fragments putatively derived from NAG genes of *L. migratoria* were obtained by searching the locust EST database (http://locustdb.genomics. org.cn/). The fragments were analyzed and aligned into one unigene with GeneDoc software (http://www.nrbsc.org/gfx/genedoc/). The unigene cDNA putatively encoding NAG (LmNAG1) was further searched using BLASTX against the non-redundant database at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) to confirm its identity with other known insect *NAGs*.

Sequencing of LmNAG1 *cDNA*

To confirm the *LmNAG1* cDNA sequence, total RNA was extracted from fifth-instar nymphs using the RNAiso^{TM} Plus (TaKaRa, Japan) and 1.5 μg RNA was used to synthesize the first-strand cDNA using M-MLV Reverse Transcriptase (Promega, USA) and an oligo-$(dT)_{18}$ primer (TaKaRa, Japan). The PCR primers designed to obtain full-length cDNA sequence of *LmNAG1* are shown in Table 1. PCR was performed by initially denaturing the

cDNA template for 1 min at 94 °C followed by 39 cycles, each consisting of 30 s at 94°C, 30 s at 61°C and 90 s at 72°C, and final extension step of 10 min at 72°C. The PCR products were purified using E.Z.N.A.^{TM}Gel Extraction Kit (OMEGA, USA), and the purified product was subcloned into pGEM-T Easy Vector (Promega, USA) for sequencing from both directions.

Phylogenetic analysis of LmNAG1

ClustalW 2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to perform multiple sequence alignments prior to phylogenetic analysis. The MEGA 4.0 Beta program (Kumar *et al*., 2007) was used to construct the consensus phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA). To evaluate the branch strength of the phylogenetic tree, bootstrap analysis of 5000 replications was performed.

Stage-dependent expression patterns of LmNAG1

To determine whether *LmNAG1* gene was differentially expressed during developmental stages of *L. migratoria*, we dissected integument from each day of fourth and fifth-instar nymphs. Total RNA of the samples was isolated following the identical method as described above. Relative expression of *LmNAG1* was evaluated by real-time quantitative PCR (qPCR) using SYBR® Premix Ex Taq^{TM} II (TaKaRa, Japan) on Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, USA). The sequences of the primers used for qPCR analysis are shown in Table 1. The amplification efficiency of the gene was estimated by primer efficiency test. Three biological replications, each with three technical repeats, were performed for qPCR experiments. Because *L. migratoria* β-actin gene showed a stable expression in different tissues and at different developmental stages in our previous studies and therefore was used as an internal reference gene. Each reaction was carried out with 2 μl of 20-fold diluted first-strand cDNA, 0.4 mM of each primer in a total volume of 20 μl. The

optimized qPCR program was used for both *β-actin* and *LmNAG1*, which consisted of initial step at 95°C for 10 s followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Amplification specificity was verified using the dissociation curve. Relative expression of target gene in different stages was conducted according to threshold cycle (Ct) value based on $2^{-\Delta\Delta CT}$ method.

Tissue-dependent expression patterns of LmNAG1

Six selected tissues including the integument, foregut, midgut, gastric caeca, hindgut and wing pad were dissected from day 5, 6 and 7 of the fifth-instar nymphs of *L. migratoria*. The methods for RNA extraction and qPCR analysis were the same as described above.

Functional analysis of LmNAG1 *by RNAi*

To explore possible biological functions of *LmNAG1*, RNAi was performed by injecting sequence-specific double-stranded RNA (dsRNA) to locust nymphs. For dsRNA synthesis, cDNA template with T7 polymerase promoter sequence at both ends was prepared by PCR using the forward primer 5'-taatacgactcactatagggGTGGAGAGGGTGAAGG-3'and the reverse primer 5'-taatacgactcactatagggGGGCAGATTGTGTGGAC-3' for *GFP* and the primers for *LmNAG1* as shown in Table 1. The lower-case letters representing the promoter sequences were used for following *in vitro* transcription. The PCR products of *LmNAG1* and *GFP* were subcloned and sequenced to confirm their identities. The expected fragments were then separated on agarose gel and purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). After the concentrations of the purified fragments were determined by SpectraMax 190 microplate reader and SOFTmax software (Molecular Devices, USA), they were used for *in vitro* transcription by using T7 RiboMAX^{TM} Express RNAi System

(Promega, USA). The synthesized dsRNA were dissolved in nuclease-free water, and analyzed on 1.5% agarose gel to determine its quality. The final concentration of dsRNA was adjusted to 1.5 μg/μL.

Two-day-old second and fifth-instar nymphs were used for dsRNA injection. Three micrograms of each dsRNA for second-instar nymphs and 6 μg for fifth-instar nymphs were injected into the abdomen between the second and third abdominal segments using a manual microinjector (Ningbo, China). Control groups were injected with equivalent volumes of ds*GFP* alone. The nymphs were incubated in a chamber at 30±1°C for day and 28±1°C for night with L14:D10 photoperiod and 50% humidity. The injected nymphs were checked frequently and carefully for possible phenotypic changes. The nymph samples used to monitor gene expression were collected at different time points after the injection by using qPCR.

Total enzyme activity of LmNAG after RNAi

To assess the contribution of *LmNAG1* to the total NAG chitinolytic activity, LmNAG total activities were determined at different time points in fifth-instar nymphs after the ds*LmNAG1* or ds*GFP* injection. The assay for LmNAG total activity was based on the method of Espie and Roff (1995) with modifications as follows. After the samples were collected, they were frozen in liquid nitrogen and immediately homogenized in 0.15 M citrate phosphate butter (pH 5.5) containing 0.04% (v/v) proteinase inhibitor cocktail (Sigma, St. Louis, USA) at 0°C and centrifuged at 10,000×g for 3 min at 4°C. A 25-μL appropriately diluted supernatant was incubated with 10 μL 1.0 mM fluorogenic substrate

[4-methylumbelliferyl-N-acetyl-β-glucosaminide (4-MU-β-GlcNAc)] in a 96-well plate at 25 ^{o}C for 10 min. The reaction was stopped by adding 200 μL 0.5 mol/L glycine-NaOH (pH 10.3). The liberated 4-methylumbelliferone (4-MU) was fluorometrically measured at 365 nm excitation and 445 nm emission wave lengths with SpectraMax M5 (Molecular Devices, USA). Two control wells were used: one contained 25 μL supernatant diluent and 10 μL citrate phosphate buffer (pH 5.5), and the other contained 25 μL citrate phosphate buffer (pH 5.5) and 10 μL 1.0 mM 4-MU-β-GlcNAc. Conversion of fluorescence to the concentration of the liberated 4-methylumbelliferone was based on a standard curve made by using 4-methylumbelliferone (Sigma). The protein concentration in the supernatant was determined using Enhanced BCA Protein Assay Kit (Sangon Biotech, Shanghai, China). The enzymatic activity is presented as nmol methylumbelliferone liberated per mg protein per min.

Statistical analysis

The data were analyzed using two-way analysis of variance (ANOVA) for significant differences between the treated group and the control group. For the relative expression of *LmNAG1*, data were first transformed using arcsine square root transformation before the ANOVA. Fisher's least significant difference (LSD) multiple comparisons were then used to separate the means among the samples. A level of $P < 0.05$ was accepted as statistically significant. All the statistical analyses were performed using IBM® SPSS 16.0 (SPSS Inc., Chicago, IL). All the results were expressed as mean ± standard errors,

Results

207 *Analysis of* LmNAG1 *cDNA and deduced amino acid sequences*

208 A full-length cDNA sequence of *LmNAG1* was obtained from PCR amplification using
209 the cDNA template prepared from the insect whole body. The cDNA consists of 2667
210 nucleotides, including an open reading frame (ORF) of 1845 nucleotides that encode 614
211 amino acid residues and 233- and 589-nucleotide non-coding regions at the 5'- and 3'-ends,
212 respectively (Fig. 1). The calculated molecular mass (MM) and isoelectric point (pI) of the
213 predicted protein are about 69.2 kDa and 5.37, respectively, by using the ExPASy Proteomics
214 website.

215 By using TMHMM Server v. 2.0 (Krogh *et al*., 2001), no transmembrane helix was
216 predicted. One signal peptide motif was found by SignalP 4.0 Server (http://www.cbs.dtu.dk/
217 services/SignalP/) and the cleavage site is between A18 and C19. The active sites R211,
218 D354, E355, W405, W424, Y450, D452, E491 and W489 of human β-hexosaminidase B
219 (Maier et al., 2003) were also recognized in LmNAG1.

220 *Phylogenetic analysis of LmNAG1*

221 Phylogenetic analysis of the NAGs from *L. migratoria* and several other insect species,
222 and the related hexosaminidases was carried out by using UPGMA (Fig. 2). To evaluate the
223 branch strength of the tree, a bootstrap analysis of 5000 replications was performed. In
224 addition to UPGMA, another distance-based method, neighbor joining, was employed for
225 comparative purposes as well as for maximum parsimony and minimum evolution analyses.
226 All of the trees generated by the four methods were consistent, grouping the proteins into five
227 major groups: NAG group I, NAG group II, *N*-glycan processing NAGs (group III),

hexosaminidases (group IV) and ENGs (group V) (Leonard *et al*., 2006). LmNAG1 was grouped with the enzymatically characterized NAGs in group I.

Developmental stage and tissue-dependent expression patterns of LmNAG1

The developmental expression of *LmNAG1* in the integument of *L. migratoria* was examined by qPCR (Fig. 3). The results indicated that the expression level of *LmNAG1* was significantly high in the last two days of fourth and fifth-instar nymphs. qPCR was carried out to analyze the expression patterns of *LmNAG1* in selected tissues from day 5 (N5D5), 6 (N5D6) and 7 (N5D7) of the fifth-instar nymphs. The results showed that the expression of *LmNAG1* in foregut and hindgut were 3- to 4- fold, respectively, higher than those in integument, midgut and wing pad on day 5 (Fig. 4). However, the expression of *LmNAG1* in foregut was highest on both days 6 and 7 among all the tissues examined. The expression of *LmNAG1* in hindgut was also relatively high on both days 6 and 7.

Determinations of transcript level of LmNAG1 and total enzyme activity of LmNAGs after RNAi

qPCR was carried out to determine the silencing efficiency of *LmNAG1* at different time points (24, 48 and 72 h) after dsRNA injections in fifth-instar nymphs (Fig. 5A). Our results showed that the *LmNAG1* transcript level was repressed by 90 to 95% at the three time points. Meanwhile, the total enzyme activity of LmNAG was significantly lower in *LmNAG1* dsRNA-injected nymphs than in ds*GFP*-injected nymphs at 48 and 72 h (Fig. 5B).

Functional analysis of LmNAG1 *by RNAi*

248 To assess the effect of RNAi of *LmNAG1* on the development of *L. migratoria*,
249 sequence-specific dsRNA for *LmNAG1* and *GFP* were synthesized *in vitro* and injected into
250 the second and fifth-instar nymphs. In the control nymphs injected with ds*GFP*, their molting
251 time span was 15- 20 min. In the nymphs injected with ds*LmNAG1*, however, their molting
252 time was >1 h in the abnormal individuals. These abnormal individuals typically showed
253 half-ecdysis and eventually died. In a total of 30 second-instar nymphs injected with
254 *LmNAG1* dsRNA, 9 died before third instar and only 21 survived to third instar. Among the
255 21 survivors, 6 died before fourth instar and only 15 survived to fourth instar (Fig. 6A). Thus,
256 the accumulative mortality of the nymphs from the second to fifth instars was 50.0%. In
257 contrast, there was no mortality observed in the control during the same period of time. In the
258 fifth-instar nymph group, only 19 out of 40 individuals survived to adults which led to a
259 mortality of 52.5% (Fig. 6B). During the nymph-nymph molting, the affected nymphs were
260 unable to completely shed their old cuticle (Fig. 6C). In the case of nymph-adult molting, the
261 affected individual was severely embedded by incompletely digested nymphal cuticle. As
262 such, a strongly twisted body was observed as a terminal phenotype in the RNAi-affected
263 individual (Fig. 6D).

264 **Discussion**

265 β-*N*-acetylglucosaminidases belong to the CAZy glycoside hydrolase family 20 (GH20).
266 NAGs have been implicated in various cellular processes, including chitin catabolism,
267 *N*-glycan processing (Léonard *et al*., 2006) and in the catabolism of free oligosaccharides in
268 the cytosol (Suzuki *et al*., 2002).

The NAGs, as the immediate downstream enzymes of chitinases, are key enzymes involved in chitin degradation in all chitin-containing organisms. Bacterial NAGs have been intensively studied due to their important physiological role in cell wall recycling. In marine chitinolytic bacteria such as *Vibrio furnissii* and *Alteromonas* sp., NAGs participate in chitin degradation and chitinase inducer formation (Keyhani and Roseman, 1996; Tsujibo *et al*., 1999). Fungal NAGs are well known to play a crucial physiological role in the complex chitinolytic system in the cell wall of growing fungi. The so-called "tandem binary system", produced under a severe regime of catabolite repression, consists of chitinases and NAGs and is responsible for the control of cell wall chitin lysis (Rast *et al*., 1991, 2003). So far, *NAG* genes that function in chitin metabolism have been characterized in at least six insect species, including four lepidopterans including *B. mori* (Nagamatsu *et al*., 1995), *M. sexta* (Zen *et al*., 1996), *C. fumiferana* (Zheng *et al*., 2008), and *O. furnacalis* (Liu *et al*., 2009); one dipteran, *A. aegypti* (Benedito *et al*., 2002) and one coleopteran, *T. castaneum* (Hogenkamp *et al*., 2008). All these insect species are holometabolous, which means they go through a complete metamorphosis with all four life stages (egg, larva, pupa and adult). However, there has been no report on NAGs in hemimetabolous insects to date.

As a typical hemimetabolous insect, *L. migratoria* has three life cycle stages: egg, nymph and adult, bypassing the pupal stage. The locust has also been served as a model in physiology, neuroscience, developmental and evolutionary biology (He *et al*., 2004; He *et al*., 2006; Wei *et al*., 2007; Simpson and Sword, 2008). Studies on *NAGs* in diverse orders of insects are important for in-depth understanding of insect chitin degradation. In this paper, we report, for the first time, a full-length cDNA encoding chitinolytic NAG from *L. migratoria*.

291 After we identified several putative NAG ESTs from the locust EST database, we
292 sequenced the full-length cDNA of *LmNAG1* putatively encoding a
293 β-*N*-acetylglucosaminidase in *L. migratoria* (Fig. 1). The amino acid sequence was deduced
294 and subjected to a battery of analyses including the prediction of signal peptides, molecular
295 mass and isoelectric point. The cDNA-deduced LmNAG1 was predicted to contain a signal
296 peptide and is, therefore, probably a secreted protein. The amino acid residues R211, D354,
297 E355, W405, W424, Y450, D452, E491 and W489, which are catalytically important active
298 sites of human β-hexosaminidase B (Maier *et al*., 2003), are also highly conserved in
299 LmNAG1.

300 To date, a total of five major groups were identified in the phylogenetic tree of NAGs
301 using the UPGMA method, which include chitinolytic NAGs group I, chitinolytic NAGs
302 Group II, *N*-glycan processing NAGs (Group Ⅲ), hexosaminidases (Group Ⅳ), and ENGs
303 (Group Ⅴ) (Fig. 2). The obtained LmNAG1 falls within the chitinolytic NAG group I,
304 containing the well-characterized NAG1, which has been shown for its catalytic activity in
305 degradation of chitin oligosaccharides. Taken together, LmNAG1 is likely a principal
306 chitinolytic NAG in *L. migratoria*.

307 The developmental expression of *LmNAG1* was detected in each day during the fourth
308 and fifth-instar nymphal stages (Fig. 3). The expression level was significantly high in the
309 last two days of each instar, which is consistent with the ecdysone (20-hydroxyecdysone) titer
310 during the same developmental stages of *L. migratoria* (Feyereisen and Durst, 1980), and
311 with the results showing an up-regulation of a *M. sexta NAG* gene by 20-E (Zen *et al*., 1996).
312 However, in the last three days of the fifth-instar nymphs, *LmNAG1* expression was

313 remarkably high in foregut and hindgut (Fig. 4). This result implies that *LmNAG1* may also
314 play an important role in chitin metabolism in foregut and hindgut of *L. migratoria*
315 (Merzendorfer and Zimoch, 2003).

316 Our most compelling evidence suggesting that *LmNAG1* plays an important role in
317 chitin degradation during the molt was provided by RNAi. Specifically, injection of dsRNA
318 corresponding to a unique region of the *LmNAG1* transcript resulted in a dramatic
319 suppression of its transcript level in the second and fifth-instar nymphs of *L. migratoria* (Fig.
320 5A). Such a gene silencing significantly reduced total LmNAG enzyme activity at 48 and 72
321 h after *LmNAG1* dsRNA injections (Fig. 5B), suggesting that LmNAG1 contributed to a
322 significant amount of total LmNAG enzyme activity. Furthermore, the injection of *LmNAG1*
323 dsRNA at developmental stages prior to the nymph-nymph (second-instar) and nymph-adult
324 (fifth-instar) molts resulted in the arrest of development and subsequently over 50% of
325 mortality during their molting processes (Fig. 6A and 6B). The terminal phenotypes resulting
326 from *LmNAG1* knockdown is consistent with its role in chitin turnover during molting (Fig.
327 6C and 6D). Therefore, *LmNAG1* is essential for insect molts and knockdown of its transcript
328 alone can lead to a disrupted ecdysis and insect mortality.

329 In summary, our study revealed that *LmNAG1* was essential for the growth and
330 development of *L. migratoria*, which sheds new lights on chitin metabolism in a
331 hemimetabolous insect. Because *LmNAG1* plays an important role in locust molting, our
332 findings suggest that *LmNAG1* could potentially serve as a practicable target for developing
333 novel strategies for insect pest control. Pesticide screening by inhibiting LmNAG1 enzyme
334 activity and developing RNAi-based methods by silencing *LmNAG1* gene could be possible

strategies of using *LmNAG1* for insect control.

Disclosure：

All authors have declared no conflicts of interest, including specific financial interests and relationships and affiliations (other than those affiliations listed in the title page of the manuscript) relevant to the subject of this manuscript.

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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, 291-304.

Benedito, P.D. F., Francisco, J.A. L., Na´gila, F.C. S., Vale´ria, P., Sheila, T. P., Paulo, F.P. P. (2002) Presence of chitinase and *beta-N*-acetylgluco- saminidase in the *Aedes aegypti*: a chitinolytic system involving peritrophic matrix formation and degradation. *Insect Biochemistry and Molecular Biology*, 32, 1723-1729.

Cohen, E. (2001) Chitin synthesis and inhibition: a revisit. *Pest Management Science*, 57, 946-950.

Feyereisen, R., Durst, F. (1980) Development of microsomal cytochrome P450 monooxygenases during the last larval instar of the locust, *locusta migratoria*: Correlation with the hemolymph 20-hydroxyecdysone titer. *Molecular and Cellular Endocrinology,* 20:157-169.

Fukamizo, T. Kramer, K. J. (1985a) Mechanism of chitin oligosaccharides hydrolysis by the binary chitinase system in insect molting fluid. *Insect Biochemistry*, 15, 1-7.

Fukamizo, T. Kramer, K. J. (1985b) Mechanism of chitin hydrolysis by the binary chitinase system in insect molting fluid. *Insect Biochemistry*, 15, 141-145.

He, Y.P., Ma, E.B., Zhu, K.Y. (2004) Characterizations of general esterases in relation to malathion susceptibility in two field populations of the oriental migratory locust, *Locusta migratoria manilensis* (Meyen). *Pesticide Biochemistry and Physiology*, 78, 103-113.

He, Z.B., Cao, Y.Q., Yin, Y.P., Wang, Z.K., Chen, B., Peng, G.X., Xia, Y.X.(2006) Role of hunchback in segment patterning of *Locusta migratoria manilensis* revealed by

parental RNAi. *Development, Growth and Differentiation*, 48, 439-445.

Hogenkamp, D. G., Arakane, Y., Kramer, K.J., Muthukrishnan, S., Beeman, R. W. (2008). Characterization and expression of the *β-N*-acetylhexosaminidase gene family of *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, 38, 478-489.

Keyhani, N.O, Roseman, S. (1996) Chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Molecular cloning, isolation and characterization of a periplasmic β-*N*-acetylglucosaminidase. *Journal of Biological Chemistry*, 271, 33425-32.

Khajuria, C., Buschman, L.L., Chen, M. S., Muthukrishnan, S., Zhu, K.Y. (2010) A gut-specific chitinase gene essential for regulation of chitin content of peritrophic matrix and growth of *Ostrinia nubilalis* larvae. *Insect Biochemistry and Molecular Biology*, 40, 621-629.

Kramer, K.J., Koga, D. (1986) Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochemistry*, 16, 851-877.

Kramer, K. J., Muthukrishnan, S. (2005) Chitin metabolism in insects: a revisit. In: Gilbert, L., I., Iatrou, K., and Gill, S. eds., Comprehensive Molecular Insect Science. Vol. 4, Biochemistry and Molecular Biology, Chapter 11. Elsevier Press, Oxford, UK.

Léonard, R., Rendić, D., Rabouille, C., Wilson, I. B., Preat, T., Altmann, F. (2006) The *Drosophila fused lobes* gene encodes an *N*-acetylglucosaminidase involved in N-glycan processing. *Journal of Biological Chemistry*, 281, 4867-4875.

Liu, T., Liu F.Y., Yang, Q., Yang, J. (2009) Expression, purification and characterization of the chitinolytic *β-N*-acetyl-*D*-hexosaminidase from the insect *Ostrinia furnacalis*. *Protein Expression and Purification*, 68, 99-103.

Maier, T., Strater, N., Schuette, C. G., Klingenstein, R., Sandhoff, K., Saenger, W. (2003) The X-ray

crystal structure of human β-hexosaminidase B provides new insights into Sandhoff Disease. *Journal of Molecular Biology*, 328, 669-681.

Merzendorfer, H., Zimoch, L. (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *Journal of Experimental Biology*, 206, 4393-4412.

Nagamatsu, Y., Yanagisawa, I., Kimoto, M., Okamoto, E., Koga, D. (1995) Purification of a chitooligosaccharidolytic beta-N-acetylglucosaminidase from *Bombyx mori* larvae during metamorphosis and the nucleotide sequence of its cDNA. *Bioscience, Biotechnology and Biochemistry*, 59, 219-225.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O. (2003) Cell wall-associated enzymes in fungi. *Phytochemistry*, 64, 339-66.

Rast, D.M., Horsch, M., Furter, R., Gooday, G.W. (1991) A complex chitinolytic system in exponentially growing mycelium of Mucor rouxii: properties and function. *Journal of General Microbiology*, 137, 2797-810.

Simpson, S.J., Sword, G.A. (2008) Locusts. *Current Biology*, 18, 364-366.

Suzuki, T., Yano, K., Sugimoto, S., Kitajima, K., Lennarz, W. J., Inoue, S., Inoue, Y., Emori, Y. (2002) Endo-*beta*-*N*-acetylglucosaminidase, an enzyme involved in processing of free oligosaccharides in the cytosol. *Proceedings of the National Academy of Sciences* (USA), 99, 9691-9696.

Tsujibo, H., Kondo, N., Tanaka K., Miyamoto, K., Baba, N., Inamori, Y. (1999) Molecular analysis of the gene encoding a novel transglycosylative enzyme from Alteromonas sp. strain O-7 and its physiological role in the chitinolytic system. *Journal of Bacteriology*, 181, 5461-6.

Wei, Z., Yin, Y.P., Zhang, B.S.,Wang, Z.K., Peng, G.X., Cao, Y.Q., Xia, Y.X. (2007) Cloning of a novel protease required for the molting of *Locusta migratoria manilensis*. *Development, Growth*

and Differentiation, 49, 611-621.

Xia, J.Y., Huang, H. (2002) Analysis on the outbreak of *Locusta migratoria manilensis* and its control strategies. *Plant Protection Technology and Extension*, 22, 7-10.

Zen, K. C., Choi, H. K., Krishnamachary, N., Muthukrishnan, S., Kramer, K. J. (1996) Cloning, expression, and hormonal regulation of an insect *beta-N*-acetylglucosaminidase gene. *Insect Biochemistry and Molecular Biology*, 26, 435-444.

Zhang, J.Z., Liu, X.J., Zhang, J.Q., Li, D.Q., Sun, Y., Guo, Y.P., Ma, E.B., Zhu K.Y. (2010) Silencing of two alternative splicing-derived mRNA variants of chitin synthase 1 gene by RNAi is lethal to the oriental migratory locust, *Locusta migratoria manilensis* (Meyen). *Insect Biochemistry and Molecular Biology*, 40, 824-833.

Zheng, Y.P., Krell, P.J., Doucet, D., Arif, B.M., Feng, Q. L. (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, 49-59.

Table 1 Primers used for full-length cDNA cloning, qPCR analysis and dsRNA synthesis of putative *LmNAG1*.

Application of primers	Primer sequence (5'-3')	PCR Product Size (bp)
Full-length verification	F1: GAGTGTTTTCCTTCAAAGTCGTCAT R1: TGCTCCTCAAACTAATCTGCACACT	2667
qPCR analysis	F2: CCCTCGCAGAACGTTTGTG R2: TGTAGACACCACTCAGGCTCGAT	133
dsRNA synthesis	F3: taatacgactcactatagggGGTGATAACGGATTGGATGG R3: taatacgactcactatagggCAGACTCGTCAGCCTGTTCA	494

Figure Legends:

Fig. 1. Nucleotide and deduced amino acid sequences of *LmNAG1* cDNA. The start codon (ATG), stop codon (TAA) and putative polyadenylation signal (AATAAA) are highlighted in black. The Signal peptide cleavage site, which is between A18 and C19, is highlighted and underlined in blue. The catalytically active sites are shaded in gray and underlined in black.

Fig. 2. A dendrogram showing the phylogenetic relationships of other metazoan β-*N*-acetylglucosaminidases. MEGA 4 was used to construct the consensus phylogenetic tree using UPGMA. Bootstrap analyses of 5000 replications are shown. Protein sequences extracted from GenBank include: MsNAG, *Manduca sexta* (AY368703); BmNAG, *Bombyx mori* (AF326597); TnNAG, *Trichoplusia ni* (AY078172); DmNAG1(DmHEXO1), *Drosophila melanogaster* (NM_079200); AgNAG1, *Anopheles gambiae* (XP_315391); AaNAG1, *Aedes aegypti* (EAT43909); AmNAG1, *Apis mellifera* (XM_624790); DmNAG2(DmHEXO2), *Drosophila melanogaster* (NM_080342); AgNAG2, *Anopheles gambiae* (XM_307483); AaNAG2, *Aedes aegypti*(EAT40440); AmFDL, *Apis mellifera* (XP_394963); DmFDL, *Drosophila melanogaster* (NP_725178); AgFDL, *Anopheles gambiae* (XP_308677); AaFDL, *Aedes aegypti* (EAT36388); SfHEX1, *Spodoptera frugiperda* (DQ183187); SfHEX2, *Spodoptera frugiperda* (DQ249307); BmHEX, *Bombyx mori* (AY601817); HsHEXA, *Homo sapiens* (NM_000520); HsHEXB, *Homo sapiens* (NM_000521); MmHEXA, *Mus musculus* (NM_010421); MmHEXB, *Mus musculus* (NM_010422); AmHEX, *Apis mellifera* (XM_001122538); TcHEX1, *Tribolium castaneum* (XM_970563); TcHEX2, *Tribolium castaneum* (XM_970567); TcHEX3, *Tribolium castaneum* (XM_970565); AaHEX, *Aedes aegypti* (EAT43655); AgHEX, *Anopheles gambiae* (XM_319210).

518

519 **Fig. 3.** Relative expression levels of β-*N*- acetylglucosaminidase gene (*LmNAG1*) in integument

520 as determined by qPCR in each day of fourth- (N4) and fifth- (N5) instar nymphs. β-actin

521 was used as an internal reference gene. The relative expression (fold) was calculated based on the

522 value of the lowest expression which was ascribed an arbitrary value of 1. Different letters on the

523 bars indicate significant difference of the expression among different developmental stages based

524 on three biological replications ($P < 0.05$, Fisher's LSD; n=3).

525

526 **Fig. 4.** Relative expression levels of β-*N*-acetylglucosaminidase gene (*LmNAG1*) as determined by qPCR

527 in integument (IN), foregut (FG), midgut (MG), gastric caeca (GC), hindgut (HG), and wing pad (WP) on

528 day 5,6 and 7 of fifth-instar nymphs of *L. migratoria*. β-actin was used as an internal reference gene. The

529 relative expression (fold) was calculated based on the value of the lowest expression which was ascribed

530 an arbitrary value of 1. Different letters on the bars of the histogram indicate significant difference in gene

531 expression among different samples based on three biological replications ($P < 0.05$, Fisher's LSD test;

532 n=3).

533

534 **Fig. 5.** (A) Analysis of relative transcript levels of *LmNAG1* at three time points (24, 48, 72h) after

535 ds*NAG1* injection. β-actin was used as an internal reference gene. (B) Total NAG activity in fifth-instar

536 nymphs at 24, 48 and 72 h after ds*LmNAG1* and ds*GFP* injections. Asterisk on the bars indicate that the

537 means are significantly different among the control and treatments ($*P < 0.05$, $**P < 0.01$, Fisher's LSD

538 test; n=3).

539

Fig. 6. Effect of *LmNAG1* dsRNA injected into second and fifth-instar nymphs on development of *L. migratoria.* Survivorships of the nymphs at different developmental stages following the ds*NAG1* or ds*GFP* injection at the second-instar (A) or fifth-instar (B). Representative phenotypes of the nymphs after the ds*NAG1* injection at the second-instar (C) or fifth-instar (D). These phenotypes include stunting development; disable to detach the old cuticle during the molting, twisted body and abnormal body parts. Red arrows indicate the abnormalities. The relative expression levels of *NAG1* after RNAi in the fifth-instar nymphs were shown in Fig. 5A. Because similar suppression levels were achieved by RNAi in the second and fifth-instar nymphs, the relative expression levels of *NAG1* in the second-instar nymphs were not shown.

Fig. 1

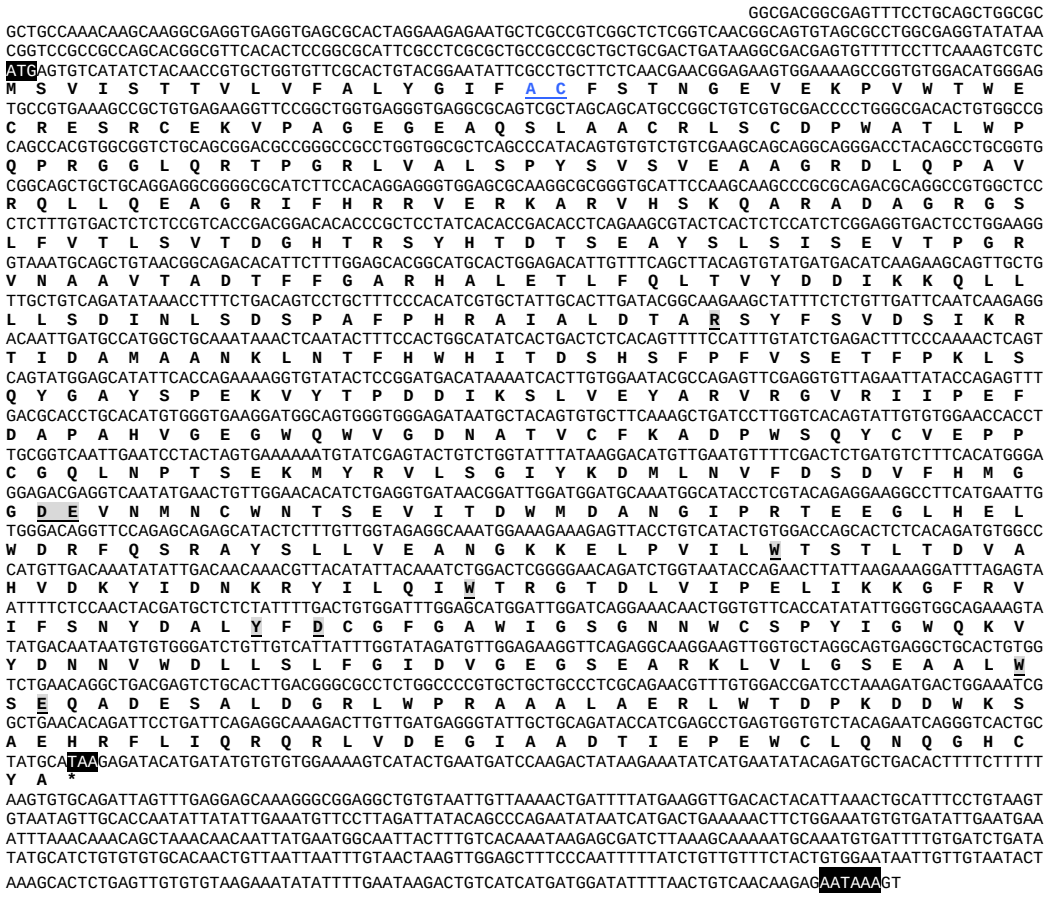
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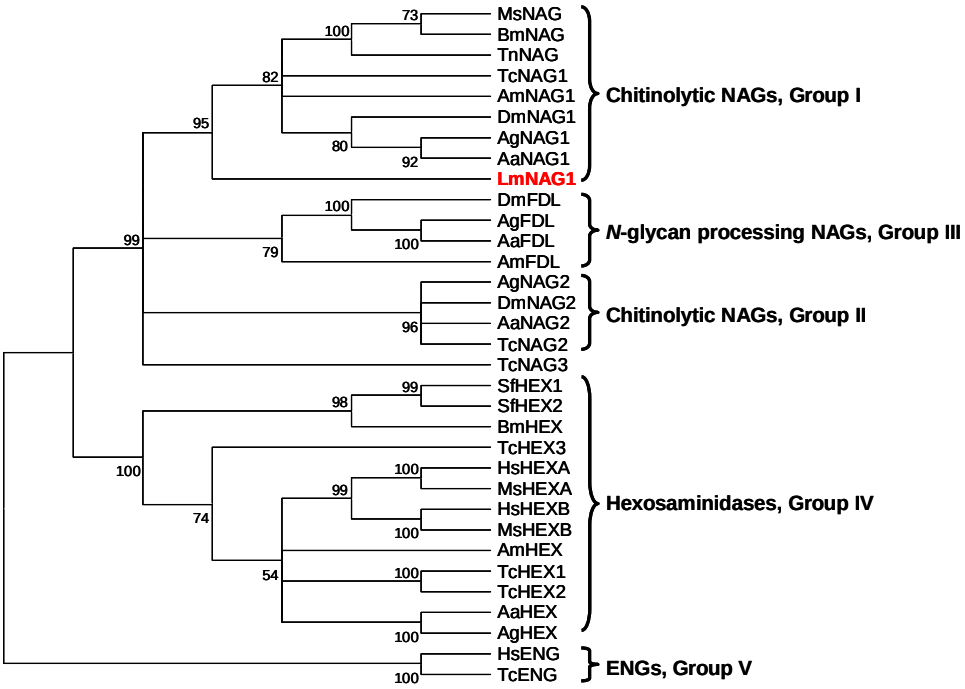
Fig.2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HsENG
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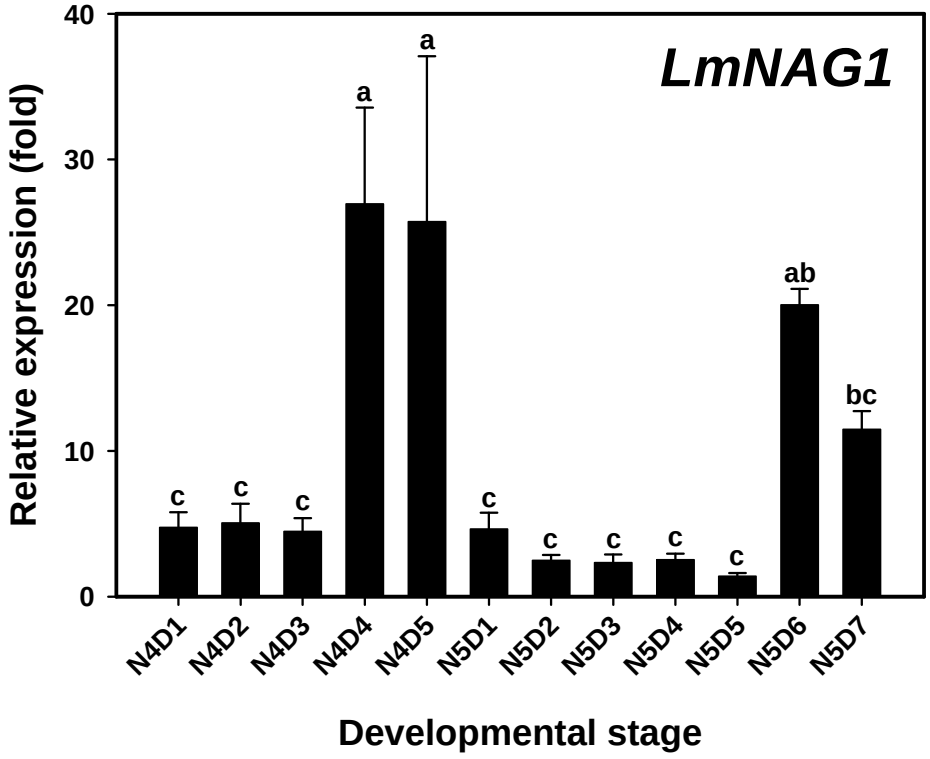
Hexosaminidases, Group IV
ENGs, Group V

Fig. 3


LmNAG1
Relative expression (fold)
40
30
20
10
0
a
a
ab
bc
c
c
c
c
c
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c
N4D1
N4D2
N4D3
N4D4
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N5D5
N5D6
N5D7
Developmental stage

Fig. 4

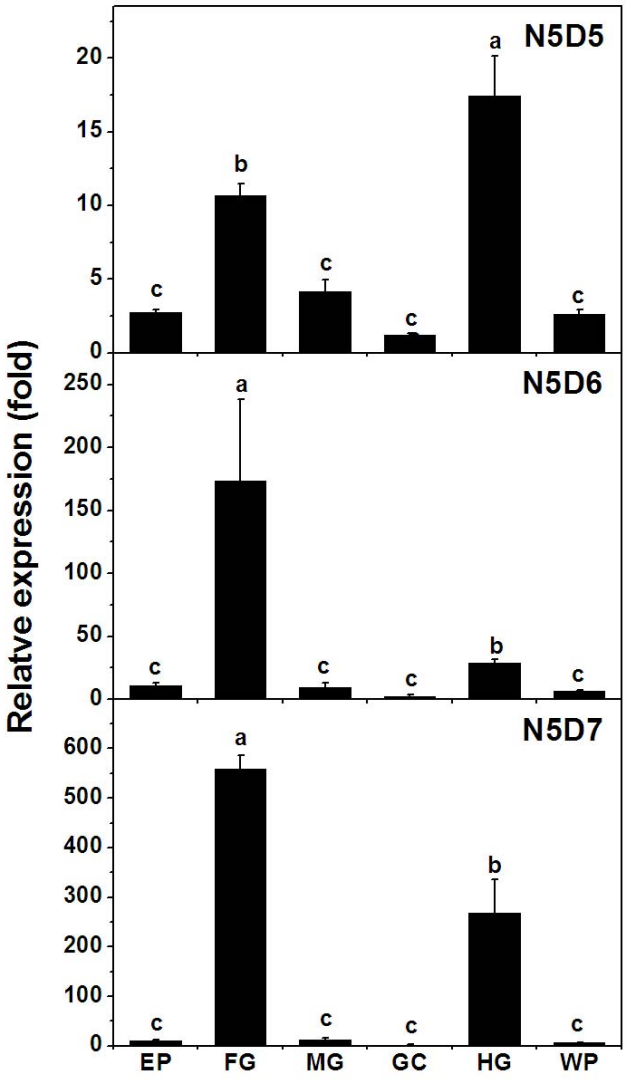
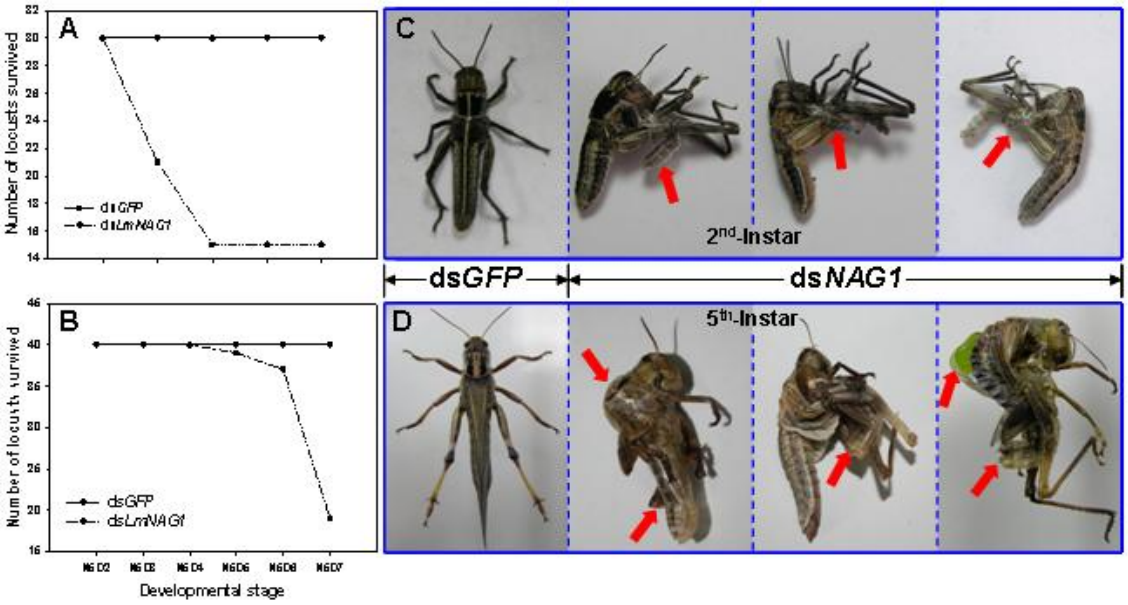


Fig. 5


A
Number of locusts survived
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B
Number of locusts survived
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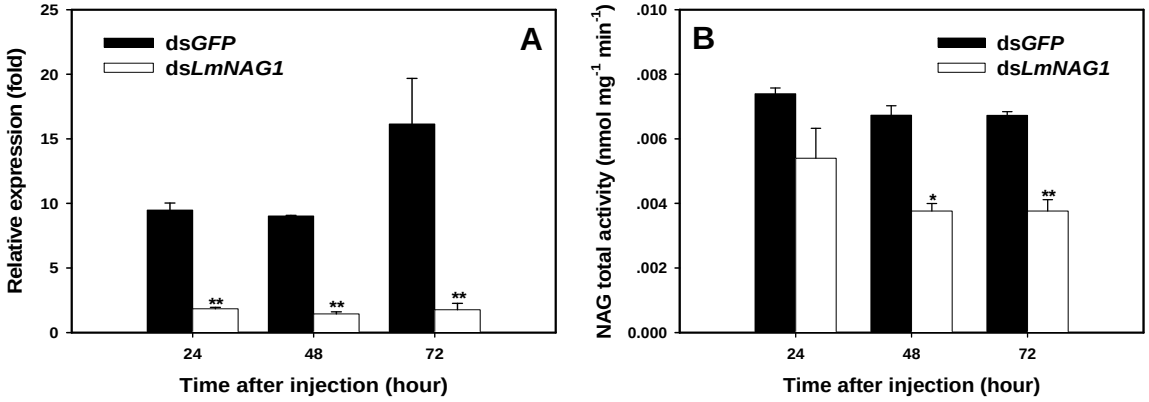
Developmental stage
C
2nd-Instar
dsGFP
dsNAG1
D
5th-Instar

Fig.6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dsGFP
dsLmNAG1
Relative expression (fold)
25
20
15
10
5
0
**
**
**
24
48
72
Time after injection (hour)
B
dsGFP
dsLmNAG1
NAG total activity (nmol mg-1 min-1)
.010
.008
.006
.004
.002
0.000
*
**
24
48
72
Time after injection (hour)