Knockdown of β-N-acetylglucosaminidase 2 Impairs Molting and Wing Development in Lasioderma serricorne (Fabricius)

Wen-Jia Yang, Kang-Kang Xu, Xin Yan and Can Li *

Guizhou Provincial Key Laboratory for Rare Animal and Economic Insect of the Mountainous Region, College of Biology and Environmental Engineering, Guiyang University, Guiyang 550005, China; yangwenjia10@126.com (W.-J.Y.); kkxu1988@163.com (K.-K.X.); 13885193419@163.com (X.Y.)

* Correspondence: lican790108@163.com; Tel.: +86-851-8540-5891

Received: 8 October 2019; Accepted: 6 November 2019; Published: 8 November 2019

Abstract: β-N-acetylglucosaminidases (NAGs) are carbohydrate enzymes that degrade chitin oligosaccharides into N-acetylglucosamine monomers. This process is important for chitin degradation during insect development and metamorphosis. We identified and evaluated a β-N-acetylglucosaminidase 2 gene (LsNAG2) from the cigarette beetle, Lasioderma serricorne (Fabricius). The full-length open reading frame of LsNAG2 was 1776 bp and encoded a 591 amino acid protein. The glycoside hydrolase family 20 (GH20) catalytic domain and an additional GH20b domain of the LsNAG2 protein were highly conserved. Phylogenetic analysis revealed that LsNAG2 clustered with the group II NAGs. Quantitative real-time PCR analyses showed that LsNAG2 was expressed in all developmental stages and was most highly expressed in the late larval and late pupal stages. In the larval stage, LsNAG2 was predominantly expressed in the integument. Knockdown of LsNAG2 in fifth instar larvae disrupted larval–pupal molting and reduced the expression of four chitin synthesis genes (trehalase 1 (LsTRE1), UDP-N-acetylglucosamine pyrophosphorylase 1 and 2 (LsUAP1 and LsUAP2), and chitin synthase 1 (LsCHS1)). In late pupae, LsNAG2 depletion resulted in abnormal adult eclosion and wing deformities. The expression of five wing development-related genes (teashirt (LsTSH), vestigial (LsVG), wingless (LsWG), ventral veins lacking (LsVVL), and distal-less (LsDLL)) significantly declined in the LsNAG2-depleted beetles. These findings suggest that LsNAG2 is important for successful molting and wing development of L. serricorne.

Keywords: Lasioderma serricorne; β-N-acetylglucosaminidase; molt; wing development; RNA interference

1. Introduction

Chitin is a polymer of β-1,4-linked N-acetylglucosamine residues and an essential component of the integument, trachea, salivary gland, foregut, hindgut, and intestinal peritrophic matrix in insects [1,2]. Chitin plays major roles in maintenance of the shape, size, and protection from external forces such as mechanical injuries and infection by bacteria, fungi, and viruses [3,4]. The regulation of chitin metabolism is crucial for insect growth and development [5,6]. Among the enzymes involved in chitin metabolism, β-N-acetylglucosaminidases (NAGs, EC 3.2.1.30) are chitin-degrading enzymes that hydrolyze the chitin oligosaccharides into N-acetylglucosamine monomers [7]. NAGs (also known as β-N-acetylhexosaminidases) are exosplitting enzymes that belong to the glycoside hydrolase family 20 (GH20) [8]. Insect NAGs are divided into four subgroups: chitinolytic group I NAGs, chitinolytic group II NAGs, glycan processing group III NAGs, and hexosaminidases group IV, on the basis of their sequence similarity and functions [9–11].
Insects 2019, 10, 396 2 of 13

Insect NAGs are widely distributed and occur in many insect species, including Coleoptera, Diptera, Hemiptera, Lepidoptera, and Orthoptera [10–15]. NAGs mainly function in chitin degradation, and their roles have been studied in numerous insect species. In Aedes aegypti, the activity of NAG enzyme increased after feeding on blood or artificial diet [16]. RNA interference (RNAi)-mediated knockdown of NAGs causes molting failure in Tribolium castaneum [10], Locusta migratoria [13], Nilaparvata lugens [11], Mamestra brassicae [14], and Heortia vitessoides [17]. In Ostrinia furnacalis [17] and Lasioderma serricorne [18], NAGs are essential for the proper formation of adult wings. In addition to their central role in chitin degradation, NAGs participate in diverse physiological processes, such as post-translational modification of N-glycan proteins [19], degradation of glycoconjugates [20,21], and fertilization [22,23].

The cigarette beetle, Lasioderma serricorne (Fabricius) (Coleoptera: Anobiidae), is a serious pest of stored products worldwide [24]. The beetle causes economic losses to stored products such as tobacco, grains, traditional Chinese medicine materials, cereals, and dry foods [25,26]. Control of L. serricorne is primarily dependent on the use of insecticides, including phosphine, essential oils, pyrethrin, and organophosphates [27,28]. Excessive and frequent insecticide applications have resulted in insecticide resistance within many L. serricorne populations. In addition, insecticide residues in stored products are harmful to human health and the environment [29,30]. Therefore, new strategies are needed to effectively manage L. serricorne infestations. RNAi-based technologies have been used for the management of several insect pests [31–33]. Screening for an ideal silencing target is critical for the development of an RNAi-based control method [34,35]. In terms of practice, transgenic plants in expressing sequence-specific double-stranded RNA (dsRNA) [36] and spray-based dsRNA reagent application [37] have potential in implementation as future pest control, and a trial of Diabrotica virgifera virgifera via targeting DvSnf7 was firstly tested in filed as RNAi-based pest control incorporated with Bacillus thuringiensis Cry3Bb1 protein [38]. Chitin degrading enzymes are potential targets for novel insecticides [17,39–41]. In this study, we identified and cloned the full-length open reading frame (ORF) sequence of β-N-acetylglucosaminidase 2 gene (LsNAG2) in L. serricorne and analyzed its expression patterns in different developmental stages and tissues. We functionally characterized the knockdown effect of LsNAG2 on L. serricorne molting and the responses of genes related to wing development. The data provide significant insight into the functions of LsNAG2 in L. serricorne development.

2. Materials and Methods

2.1. Insect Culture

Lasioderma serricorne were collected from a tobacco warehouse in Guiyang City, Guizhou Province, China, in 2014. A colony was established in the laboratory and maintained at 28 °C with 40% relative humidity under a scotoperiod of 24 h. Larvae were fed on dried roots of Angelica sinensis, as described previously [42].

2.2. RNA Extraction and Cloning of LsNAG2 Gene

Total RNA was extracted from entire bodies of 30 L. serricorne larvae with TRIzol reagent according to manufacturer protocol (Invitrogen, Carlsbad, CA, USA), and treated with DNase I (Promega, Madison, WI, USA). The concentration and quantity of total RNA were measured by a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was evaluated by agarose gel electrophoresis. First-strand complementary DNA was synthesized by the TransScript Synthesis Supermix (TransGen, Beijing, China) with oligo (dT)_{18} primers.

A candidate cDNA sequence of β-N-acetylglucosaminidase 2 gene was identified by Basic Local Alignment Search Tool Nucleotide (BLASTN) and Translated Basic Local Alignment Search Tool Nucleotide (TBLASTN) analyses from the transcriptome database of L. serricorne (unpublished data). The full-length ORF of the sequence was found using ORF Finder at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Two pairs of
gene-specific primers (Table S1) were designed to amplify the full-length cDNA of LsNAG2 by reverse transcription PCR (RT-PCR). The PCR amplification was carried out with an initial denaturation at 95 °C for 4 min, 34 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, and a final elongation at 72 °C for 10 min. The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced in both directions.

2.3. Sequence Analysis

Sequence similarities and domain predictions were produced using the Basic Local Alignment Search Tool program at the NCBI website (http://blast.ncbi.nlm.nih.gov). The nucleotide and deduced amino acid sequences were analyzed using DNAMAN7 (Lynnon Biosoft, Vaudreuil, Québec, Canada). The molecular weight and isoelectric point were calculated using ExPASy tools (http://www.expasy.org/tools/pi_tool.html). The N-glycosylation sites were predicted using the NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), and the signal peptide was predicted using the SignalP4.1 program (http://www.cbs.dtu.dk/services/SignalP4.1/). The phylogenetic tree was constructed by MEGA7 (MEGA, PA, USA) [43] using the neighbor-joining method with bootstrap values using 1000 replicates at the cut off of 50% similarity.

2.4. Quantitative Real-Time PCR

For the stage-specific expression analyses, samples at different developmental stages of L. serricorne were prepared as described previously [42]. To analyze tissue-specific expression, eight tissues (integument, brain, foregut, midgut, hindgut, fat body, and Malpighian tubules) were dissected from the late larvae. RNA extraction and cDNA synthesis were performed as described above. The relative expression levels of LsNAG2 were detected by quantitative real-time PCR (qPCR). The qPCR assay was performed with GoTaq qPCR Master Mix (Promega, Madison, WI, USA) using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The reaction was run at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melt curve analysis was performed to assess the specificity of the qPCR amplifications. On the basis of the evaluation, the L. serricorne elongation factor 1-alpha (EF1α, GenBank: KY549658) was used for normalization (unpublished data). All of the experiments were performed in triplicate, and the results were calculated by the 2−ΔΔCt method [44].

2.5. RNA Interference

RNAi was performed to study the effects of LsNAG2 on L. serricorne development. The primers (Table S1) used for dsRNA synthesis were designed to add the T7 polymerase promoter sequence at the 5′-ends. The dsRNAs were synthesized using a TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Wilmington, DE, USA). The quality and concentration of dsRNAs were measured with a NanoDrop 2000C spectrophotometer (Thermo Scientific), and their sizes were verified by agarose gel electrophoresis. The dsRNA of green fluorescent protein (GFP) served as the negative control. The day 2 fifth-instar larvae and day 5 pupae were used for microinjection; 50 individual insects were used for each group, and the experiment was performed in triplicate. About 300 ng dsRNA of GFP or LsNAG2 were slowly injected into the hemocoel between the second and third abdominal segments of each larva and pupa using a Nanoliter 2010 injector (World Precision Instruments, Sarasota, FL, USA). All of the injected insects were kept under the conditions mentioned above. To determine silencing efficiency, relative expression levels of LsNAG2 in both dsLsNAG2 and dsGFP group at 3 and 5 days after injection were measured by qPCR as described above. Insect survival rate and phenotype changes were observed and recorded. Photos were taken using a Keyence VHX-6000 stereomicroscope (Keyence Corporation, Osaka, Japan).
To determine the expression of chitin synthesis genes, samples were collected from the larvae treated with dsLsNAG2 and dsGFP for 5 days. The qPCR was used to analyze the expression of trehalase (LsTRE1 and LsTRE2), UDP-N-acetylglucosamine pyrophosphorylase (LsUAP1 and LsUAP2), and chitin synthase 1 (LsCHS1) as described above. We also evaluated the expressions of wing development-related genes after gene silencing in late pupae. The insect samples were collected 5 days after dsRNA injection in late pupae for expression analysis. The relative expression levels of nine wing development network genes, including miniature (LsMT), singed (LsSG), engrailed (LsEN), teashirt (LsTSH), wingless (LsWG), distal-less (LsDLL), vestigial (LsVG), ventral veins lacking (LsVVL), and apterous (LsAP), were detected by qPCR.

2.6. Statistical Analysis

All the expression data are presented as mean ± standard error (SE) and evaluated using SPSS 20.0 software (IBM Corp, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by a least significant difference test was used to analyze differences among different developmental stages and tissues. Student’s t-test was used to analyze data from the RNAi-treatments after dsRNA injection. The survival rates were analyzed using the Kaplan–Meier method.

3. Results

3.1. Identification and Characterization of LsNAG2

The full-length ORF of LsNAG2 (GenBank accession number MN310607) was obtained and confirmed by RT-PCR. The LsNAG2 cDNA sequence contained an ORF of 1776 bp that encoded a protein of 591 amino acids. The predicted protein of LsNAG2 had a molecular weight of 66.6 kDa and an isoelectric point of 5.92. Signal peptides with 16 amino acids were found at the N-terminal ends of LsNAG2, which suggested it may be secreted. Two potential N-glycosylation sites at positions 150 and 549 were predicted in the N-terminal extracellular domain.

The predicted amino acid sequences contained a conserved GH20 catalytic domain (residues 213–572), and an additional GH20b domain (residues 136–186). The conserved H×GGDEV×××CW motif was considered to be the catalytic site. The conserved active site residues R224, D377, E378, W435, W459, Y486, D488, W522, and E524 were found at the N-terminal ends of LsNAG2, which suggested it may be secreted. Two potential N-glycosylation sites at positions 150 and 549 were predicted in the N-terminal extracellular domain.

The predicted amino acid sequences contained a conserved GH20 catalytic domain (residues 213–572), and an additional GH20b domain (residues 136–186). The conserved H×GGDEV×××CW motif was considered to be the catalytic site. The conserved active site residues R224, D377, E378, W435, W459, Y486, D488, W522, and E524 were found in the LsNAG2 sequence (Figure 1). Sequence alignment showed that LsNAG2 exhibited 60%, 54%, 53%, and 52% identity with NAG2 (XP_018336054) from Agrilus planipennis, NAG2 (XP_015833058) from T. castaneum, NAG (XP_023711735) from Cryptotermes secundus, and NAG (XP_022185539) from Nilaparvata lugens, respectively. A phylogenetic tree was generated using MEGA7 on the basis of the amino acid sequences of insect NAGs and related hexosaminidases. The tree showed that these proteins were classified into four major groups: NAG group I, NAG group II, N-glycan processing NAGs (group III), and hexosaminidase (group IV). The LsNAG2 protein was located on a branch with the group II NAGs of other insects (Figure 2).
Among the five tested stages, that is, early larvae (<24 h post-hatching), late larvae (older than 213–572), and an additional GH20b domain (residues 136–186). The conserved H×GGDEV×××CW (XP_018336054) from Agrilus planipennis was 60%, 54%, 53%, and 52% identity with NAG2 (XP_023711735) from Cryptotermes secundus and tissues. Student’s t-test was used to analyze differences among different developmental stages. All the expression data are presented as mean ± standard error (SE) and evaluated using SPSS 20.0 software (IBM Corp, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by a least significant difference test was used to analyze data from the RNAi-treatments after dsRNA injection. The survival rates were analyzed using the Kaplan–Meier method.

3. Results

3.1. Identification and Characterization of LsNAG2

The cDNA sequence contained an ORF of 1776 bp that encoded a predicted amino acid sequence of 591 residues. The predicted ORF of LsNAG2 had a molecular weight of 66.6 kDa and an isoelectric point of 5.92. Signal peptides with 16 amino acids were found at the N-terminal ends of LsNAG2, which suggested it may be secreted. Two potential N-glycosylation sites at positions 150 and 549 were predicted in the N-terminal extracellular domain. The predicted amino acid sequences contained a conserved GH20 catalytic domain (residues 213–572), and an additional GH20b domain (residues 136–186). The conserved H×GGDEV×××CW (N–acetylglucosaminidases used in the phylogenetic tree). The phylogenetic tree was constructed using neighbor-joining method and bootstrap support values on 1000 replicates by MEGA7. LsNAG2 is marked with black dots. Table S2 shows the protein sequences of the β-N-acetylglucosaminidases used in the phylogenetic tree.
3.2. Spatiotemporal Expression Patterns of LsNAG2

Among the five tested stages, that is, early larvae (<24 h post-hatching), late larvae (older than fourth instar but before prepupae), early pupae (>72 h post-pupation), late pupae (>144 h post-pupation), and adults (>72 h post-eclosion), LsNAG2 was continuously expressed but displayed different expression patterns. LsNAG2 was more highly expressed in the late larval and late pupal stages than in the other stages (Figure 3a). The late larval stage had the highest expression of LsNAG2 and was investigated for spatial expression patterns. The highest expression level of LsNAG2 was observed in the integument and was approximately 15.6-fold greater than in the brain (Figure 3b).

![Figure 3](image-url)

**Figure 3.** The spatiotemporal expression patterns of LsNAG2 in Lasioderma serricorne. (a) The relative expression levels of LsNAG2 in different developmental stages. EL, early larave; LL, late larvae; EP, early pupae; LP, late pupae; AD, adults. (b) The relative expression levels of LsNAG2 in different tissues including integument (IN), brain (BA), fat body (FB), foregut (FG), midgut (MG), hindgut (HG), and Malpighian tubules (MT). Elongation factor 1-alpha (EF1α) was used as the reference control. Lowercase letters above bars indicate statistical difference based on one-way ANOVA followed by a least significant difference test (p < 0.05).

3.3. Effects of LsNAG2 RNAi on the Expressions of Chitin Synthesis Genes

To study the functions of LsNAG2 in the molting process, specific dsRNA for LsNAG2 and GFP were synthesized in vitro and injected into fifth instar larvae and late pupae. Compared to the control, the expression of LsNAG2 was significantly reduced by 78% and 87% at 3 and 5 days after injection with dsLsNAG2 in the fifth instar larvae, respectively (Figure 4a). After inhibition of LsNAG2, the survival rate of L. serricorne was reduced to 57% at 15 days in the dsLsNAG2 group (Figure 4b).

After RNAi with dsLsNAG2 or dsGFP at larvae, qPCR was used to study the expression of chitin synthesis genes. Expression levels of LsTRE1, LsUAP1, LsUAP2 and LsCHS1 were significantly decreased in larvae treated with dsLsNAG2 compared to levels in control insects (Figure 4c). This suggested that LsNAG2 is involved in the regulation of genes involved in chitin synthesis during the larval–pupal transition. The lethal phenotypes included the following: (1) 28% of the individuals were unable to molt, retained the larval form, and ultimately died; and (2) 15% of the individuals eventually shed their old cuticles and completed the larval–pupal transformation but died after molting (Figure 4d).
with an asymmetric wing pattern (Figure 5c). LsNAG2 pupae molted normally to adults during the pupa to adult transition. Pupae injected with dsLsA, contrast, the expression levels of significantly reduced in the LsNAG2 results showed that the mRNA levels of five transcripts (nine genes related to wing development after RNAi with dsLsA 44% of the individuals successfully molted but they did not complete wing development and died after treatment compared with the controls (Figure 5b). Approximately 11% of the individuals had exhibited lethal phenotypes. The accumulative survival rate of pupae was reduced to 45% at 15 days 3.4. Effects of LsNAG2 RNAi on the Expressions of Wing Development-Related Genes

Injection of late pupae with dsLsNAG2 significantly decreased the expression of LsNAG2 by 85% and 91% at 3 and 5 days, respectively, compared to the control (Figure 5a). In the control group, 96% of pupae molted normally to adults during the pupa to adult transition. Pupae injected with dsLsNAG2 exhibited lethal phenotypes. The accumulative survival rate of pupae was reduced to 45% at 15 days after treatment compared with the controls (Figure 5b). Approximately 11% of the individuals had difficulty shedding the old cuticle, failed to develop into adults, and eventually died. Interestingly, 44% of the individuals successfully molted but they did not complete wing development and died with an asymmetric wing pattern (Figure 5c).

To detect effects of dsLsNAG2 injection on wing development, we examined the expression of nine genes related to wing development after RNAi with dsLsNAG2 or dsGFP at late pupae. The qPCR results showed that the mRNA levels of five transcripts (LsTSH, LsVG, LsWG, LsVVL, and LsDLL) were significantly reduced in the LsNAG2-depleted pupae, compared to levels in the controls (Figure 5d). In contrast, the expression levels of LsAP and LsEN were significantly higher in LsNAG2 RNAi pupae.
T. castaneum [10], 6 in LsNAG1 and LsNAG2 proteins, which indicated that these two genes belong to physiological functions. For example, four expression was also seen in the last instar larvae of NAG was observed at late fourth and fifth instar larvae of LsNAG1. Similar high expression in late larvae and late pupae also occur in larvae and late pupae. This indicates its vital role in the degradation of old cuticle during molting.

In this study, we describe a second NAG, LsNAG2, in the cigarette beetle L. serricorne. The GH20 and GH20b domains are expressed in all stages of L. serricorne [11]. Several NAG genes have been characterized in many insect species. There are 4 in T. castaneum [10], 6 in Bombyx mori [45], 5 in Drosophila melanogaster [22], and 11 in N. lugens [11]. In L. serricorne, one NAG gene (LsNAG1) has been previously characterized [18]. In this study, we describe a second NAG, LsNAG2, in the cigarette beetle L. serricorne. The GH20 and GH20b domains were predicted in LsNAG1 and LsNAG2 proteins, which indicated that these two genes belong to the glycoside hydrolase family 20. Both domains were also predicted in the NAG of Exopalaemon carinicauda [46]. The phylogenetic relationships indicate that LsNAG2 belongs to the chitinolytic group II NAG [10]. All insect NAGs are related to chitin degradation, but group I and II are well characterized in chitin decomposition. The sequence characterization and phylogenies showed that TcNAG2 in T. castaneum and DmNAG2 in D. melanogaster are closely related and LsNAG2 possibly has a chitin degradation function [10,20].

NAGs are widely expressed in all developmental stages of insects because of their multiple physiological functions. For example, four NAGs are expressed in all stages of N. lugens [11]. In this study, LsNAG2 was expressed in all tested stages of L. serricorne but most highly expressed in late instar larvae and late pupae. This indicates its vital role in the degradation of old cuticle during molting. Similar high expression in late larvae and late pupae also occur in LsNAG1 [18]. High expression of NAG was observed at late fourth and fifth instar larvae of H. vitessoides [16] and O. furnacalis [47]. High expression was also seen in the last instar larvae of M. brassicae [14]. Increased expression of TcNAG1

Figure 5. Effect of LsNAG2 RNAi on the pupal–adult transition in L. serricorne. (a) Relative expression levels of LsNAG2 at 3 and 5 days after the LsNAG2 or GFP dsRNA injection at day 5 of pupae. (b) Changes in survival of L. serricorne pupae after LsNAG2 or GFP dsRNA injection. (c) Representative phenotypes of the pupae after LsNAG2 or GFP dsRNA injection. Injection of dsLsNAG2 resulted in two lethal phenotypes. P1: pupae were unable to shed off their old cuticles and died without completing adult eclosion; P2: pupae molted to adults but died with an asymmetric wing pattern. Pupae were alive and successfully molted to adults at day 3 after injection with dsGFP. (d) Effects of LsNAG2 knockdown on the expression of genes involved in wing development. The expression levels of wing development-related genes in the control is marked with a dashed line. Significant differences between treatment and control with Student’s t-test are induced by * p < 0.05, ** p < 0.01.

4. Discussion

Chitin synthesis and chitin degradation are both important in insect development [5]. Chitinases and NAGs are critical enzymes for insect chitin metabolism. Chitinases hydrolyze chitin into oligosaccharides, whereas NAGs further degrade the small oligomers into N-acetylglucosamine monomers [7]. Several NAG genes have been characterized in many insect species. There are 4 in T. castaneum [10], 6 in Bombyx mori [45], 5 in Drosophila melanogaster [22], and 11 in N. lugens [11]. In L. serricorne, one NAG gene (LsNAG1) has been previously characterized [18]. In this study, we describe a second NAG, LsNAG2, in the cigarette beetle L. serricorne. The GH20 and GH20b domains were predicted in LsNAG1 and LsNAG2 proteins, which indicated that these two genes belong to the glycoside hydrolase family 20. Both domains were also predicted in the NAG of Exopalaemon carinicauda [46]. The phylogenetic relationships indicate that LsNAG2 belongs to the chitinolytic group II NAG [10]. All insect NAGs are related to chitin degradation, but group I and II are well characterized in chitin decomposition. The sequence characterization and phylogenies showed that TcNAG2 in T. castaneum and DmNAG2 in D. melanogaster are closely related and LsNAG2 possibly has a chitin degradation function [10,20].

NAGs are widely expressed in all developmental stages of insects because of their multiple physiological functions. For example, four NAGs are expressed in all stages of N. lugens [11]. In this study, LsNAG2 was expressed in all tested stages of L. serricorne but most highly expressed in late instar larvae and late pupae. This indicates its vital role in the degradation of old cuticle during molting. Similar high expression in late larvae and late pupae also occur in LsNAG1 [18]. High expression of NAG was observed at late fourth and fifth instar larvae of H. vitessoides [16] and O. furnacalis [47]. High expression was also seen in the last instar larvae of M. brassicae [14]. Increased expression of TcNAG1
and TcNAG3 was investigated in late instar larvae and pupae of *T. castaneum*, respectively [10]. Several NAGs were also highly expressed in the early pupal stage in other insects [10,14].

Insect NAGs are widely expressed in multiple tissues [11,45]. In *N. lugens*, a group II NAG (also defined as β-N-acetylhexosaminidases) gene, *NlHex3*, was stably expressed in all tissues, whereas the others showed a tissue-biased pattern, such as the gut, Malpighian tubules, salivary gland, and reproductive organ [11]. The spatial expression indicated tissue-specific functions of NAGs in insects. In *B. mori*, ovary- and testis-specific expressions were observed [45]. A fertilization-related function was demonstrated in *D. melanogaster*, in which three NAG genes were expressed in the male germ line [22]. In this study, *LsNAG2* was prominently expressed in integument. A similar expression pattern was also demonstrated in *NlHex4* of *N. lugens* [11]. The high expression of NAGs in integument may be associated with chitin metabolism during the molting process. In *Choristoneura fumiferana*, an immunohistochemical staining assay revealed that CNAG occurred in the degrading exocuticle and molting fluid during late ecdysis [48]. Ecdysone is a hormone involved in the insect molting process, and it helps to regulate NAG activity during development. In *H. vitessoides* and *M. brassicae*, the NAG gene was upregulated by exogenous 20-hydroxyecdysones [14,16]. This induced expression can be suppressed by juvenile hormone [7]. The 20E-induced expression was studied in *LsNAG1* and shown to be involved in the larval–pupal transition [18].

The suppressed *LsNAG1* disrupted the metamorphic transition from larva to pupa and pupa to adult [18]. The expression profiling also showed a similar role in the transition. When the *LsNAG2* was successfully suppressed by exogenous dsRNA micro-injection, almost 43% of the larvae exhibited a lethal phenotype (failure to successfully molt). A similar disruption was observed in *N. lugens* when *NlHex4* was suppressed, but it did not reveal any abnormal phenotypes in the remaining 10 NAG homologs [11]. In *L. serricorne*, suppression of *LsNAG2* led to molting failure of *LsNAG1* and chitin deacetylase 1 gene [18,49]. The function of NAGs in molting was also studied in *L. migratoria* [13], *M. brassicae* [14], and *O. furnacalis* [18,47]. NAGs were present in the molting fluid and showed a positive synergistic effect on chitin degradation together with chitinases [50,51]. Gene expression of the chitin synthesis pathway was studied by qPCR, and four out of the selected five genes were downregulated by silencing *LsNAG2*. This indicated that the disrupted chitin degradation also inhibited new chitin biosynthesis when the chitin metabolism-related *LsNAG2* was suppressed at the transcriptional level. However, the relative expression of the chitin synthesis-related genes was not influenced when NAG was knockdown by dsRNA in *H. vitessoides* [16]. In addition, the genes involved with chitin degradation showed no change when *HoNAG1* was silenced. Group I NAGs are all secreted proteins that have been isolated from molting fluid, integument, and gut. They are functionally responsible for chitin degradation in the cuticle [51]. In this study, *LsNAG2* had a role similar to other group I NAGs and was involved in chitin degradation within the cuticle. The phenotypes produced in *T. castaneum* by knocking down the other NAGs was studied, and the efficiency was not as strong as that produced by the group I NAG (TcNAG1) [10].

In *M. brassica*, suppression of NAG expression in larvae also increased mortality at the pupal-adult transition [14]. The continuous suppression was also reflected in the following stages [13,16]. We studied the role of *LsNAG2* in the pupa-adult transition and observed high mortality when *LsNAG2* was silenced. The higher mortality of *LsNAG1* indicated a stringent efficiency during pupal–adult molting, which can also be concluded by the comparison of accumulative mortality of NAG1 and NAG2 knocked phenotype in *L. serricorne* [10,18]. Similar to the *LsNAG1* RNAi bioassay, asymmetric wing development was also observed in the *LsNAG2* RNAi, indicating a critical role in the wing development. Suppressed *HoNAG1* in *H. vitessoides* also led to an asymmetric wing pattern in adults [16]. In *O. furnacalis*, suppressed *OfHex2* resulted in 23.3% of the adults having abnormal wings [17]. The relationship of chitin-degrading enzymes and the upstream chitinase 7 with wing development was also documented in *T. castaneum* [52]. However, the mechanism of this abnormality in wing development is unclear. Therefore, we studied the gene expression of several wing development-related genes after RNAi of *LsNAG2*. Five out of the nine candidate genes showed downregulated expression. These genes are
involved in wing development. In *N. lugens*, downregulated expression of *apterous* and *teashirt* genes induced by the suppressed *trehalase* gene resulted in wing deformity [53]. The vital roles of wingless and vestigial involved in wing development have been studied in *Drosophila* [54]. In *T. castaneum*, RNAi analysis showed that the vestigial gene is essential for normal wing formation [55]. Downregulation of such genes indicates that chitin metabolism may be functionally related with wing development and formation. Given that NAG is crucial for insect molting and wing development, it may be possible to serve as a candidate insecticide target. Further studies are needed for developing transgenic plants expressing specific NAG dsRNA or spray-based dsRNA reagents to control insect pests.

5. Conclusions

We identified and cloned a NAG gene (*LsNAG2*) in *L. serricorne*, which belongs to the group II NAG. Quantitative expression analysis showed that *LsNAG2* was predominantly expressed during the larval–pupal and pupal–adult molting transitions, especially in the integument tissue. An RNAi bioassay showed that *LsNAG2* is functionally involved in metamorphic transitions by regulating the chitin degradation of the old cuticle. *LsNAG2* was also involved in the wing development of *L. serricorne*. These results demonstrated that *LsNAG2* is a promising RNAi target for *L. serricorne* control, and this gene might be useful for developing novel pest control strategies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/10/11/396/s1: Table S1: Primers used in this study. Table S2: Details of β-N-acetylglucosaminidase protein sequences used for phylogenetic analysis.

Author Contributions: W.-J.Y. and C.L. conceived and designed the experiments; K.-K.X. and X.Y. contributed materials and performed the experiments; W.-J.Y. analyzed the data; W.-J.Y. and C.L. wrote the paper.

Funding: This work was supported in part by the National Natural Science Foundation of China (31501649), the Program for Science & Technology Outstanding talents in Higher Learning Institution of Guizhou Province (2017083), the Program for Science and Technology Youth Talents in Department of Education in Guizhou Province (2018298), the Discipline and Master’s Site Construction Project of Guizhou University by Guizhou City Financial Support Guizhou University (SH-2019), and the Program for First-class Discipline Construction in Guizhou Province (201785).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Kramer, K.J.; Koga, D. Insect chitin: Physical state, synthesis, degradation and metabolic regulation. *Insect Biochem.* 1986, 16, 851–877. [CrossRef]
2. Moussian, B. The apical plasma membrane of chitin-synthesizing epithelia. *Insect Sci.* 2013, 20, 139–146. [CrossRef] [PubMed]
3. Merzendorfer, H.; Zimoch, L. Chitin metabolism in insects: Structure, function and regulation of chitin synthases and chitinases. *J. Exp. Biol.* 2003, 206, 4393–4412. [CrossRef] [PubMed]
4. Moussian, B.; Schwarz, H.; Bartoszewski, S.; Nusslein-Volhard, C. Involvement of chitin in exoskeleton morphogenesis in *Drosophila melanogaster*. *J. Morphol.* 2005, 264, 117–130. [CrossRef] [PubMed]
5. Kramer, K.; Muthukrishnan, S. Chitin metabolism in insects. *Compr. Mol. Insect Sci.* 2005, 4, 111–144.
6. Nation, J.L. *Insect Physiology and Biochemistry*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2008.
7. Zen, K.C.; Choi, H.K.; Krishnamachary, N.; Muthukrishnan, S.; Kramer, K.J. Cloning, expression, and hormonal regulation of an insect β-N-acetylhexosaminidase gene. *Insect Biochem. Mol. Biol.* 1996, 26, 435–444. [CrossRef]
8. Slámová, K.; Bojarová, P.; Petrášková, L.; Kren, V. beta-N-acetylhexosaminidase: What’s in a name? *Biotechnol. Adv.* 2010, 28, 682–693. [CrossRef]
9. Qu, M.B.; Liu, T.; Chen, L.; Chen, Q.; Yang, Q. Research progress in insect glycosyl hydrolyase family 20 β-N-acetylhexosaminidase. *Sci. Agric. Sin.* 2014, 47, 1303–1312.
10. Hogenkamp, D.G.; Arakane, Y.; Kramer, K.J.; Muthukrishnan, S.; Beeman, R.W. Characterization and expression of the beta-N-acetylhexosaminidase gene family of Tribolium castaneum. Insect Biochem. Mol. Biol. 2008, 38, 478–489. [CrossRef] [PubMed]

11. Xi, Y.; Pan, P.L.; Zhang, C.X. The beta-N-acetylhexosaminidase gene family in the brown planthopper, Nilaparvata lugens. Insect Mol. Biol. 2015, 24, 601–610. [CrossRef]

12. Cattaneo, F.; Ogiso, M.; Hoshi, M.; Perotti, M.E.; Pasini, M.E. Purification and characterization of the plasma membrane glycosidases of Drosophila melanogaster spermatozoa. Insect Biochem. Mol. Biol. 2002, 32, 929–941. [CrossRef]

13. Rong, S.; Li, D.Q.; Zhang, X.Y.; Li, S.; Zhu, K.Y.; Guo, Y.P.; Ma, E.B.; Zhang, J.Z. RNA interference to reveal roles of beta-N-acetylglucosaminidase gene during molting process in Locusta migratoria. Insect Sci. 2013, 20, 109–119. [CrossRef] [PubMed]

14. Zhang, H.; Zhao, K.J.; Fan, D. Molecular cloning and RNA interference analysis of beta-N-acetylglucosaminidase in Mamestra brassicae L. Asia Pac. Entomol. 2016, 19, 721–728. [CrossRef]

15. Filho, B.P.; Lemos, F.J.; Secundino, N.F.; Pascoa, V.; Pereira, S.T.; Pimenta, P.F. Presence of chitinase and beta-N-acetylglucosaminidase in the Aedes aegypti: A chitinolytic system involving peritrophic matrix formation and degradation. Insect Biochem. Mol. Biol. 2002, 32, 1723–1729. [CrossRef]

16. Lyu, Z.H.; Chen, J.X.; Li, Z.X.; Cheng, J.; Wang, C.Y.; Lin, T. Knockdown of beta-N-acetylglucosaminidase gene disrupts molting process in Heortia vitessoides Moore. Arch. Insect Biochem. Physiol. 2019, 101, e21561. [CrossRef]

17. Liu, F.Y.; Yang, Q. Investigation of as a potential target with RNA interference in Ostrinia furnacalis. Chin. J. Pestic. Sci. 2013, 15, 145–152.

18. Chen, X.Y.L.; Xu, K.K.; Yan, X.; Chen, C.X.; Cao, Y.; Wang, Y.W.; Li, C.; Yang, W.J. Characterization of a beta-N-acetylglucosaminidase gene and its involvement in the development of Lasioderma serricorne (Fabricius). J. Stored Prod. Res. 2018, 77, 156–165. [CrossRef]

19. Liu, F.Y.; Liu, T.; Qu, M.B.; Yang, Q. Molecular and biochemical characterization of a novel beta-N-acetyl-D-hexosaminidase with broad substrate-spectrum from the Asian corn borer, Ostrinia furnacalis. Int. J. Biol. Sci. 2012, 8, 1085–1096. [CrossRef]

20. Leonard, R.; Rendic, D.; Rabouille, C.; Wilson, I.B.H.; Preat, T.; Altmann, F. The Drosophila fused lobes gene encodes an N-acetylglucosaminidase involved in N-glycan processing. J. Biol. Chem. 2006, 281, 4867–4875. [CrossRef]

21. Luo, Y.M.; Chen, L.; Qu, M.B.; Chen, Q.; Yang, Q. Biochemical characterization of a novel beta-N-acetyl-D-hexosaminidase from the insect Ostrinia furnacalis. Arch. Insect Biochem. Physiol. 2013, 83, 115–126. [CrossRef]

22. Cattaneo, F.; Pasini, M.E.; Intra, J.; Matsumoto, M.; Briani, F.; Hoshi, M.; Perotti, M.E. Identification and expression analysis of Drosophila melanogaster genes encoding beta-hexosaminidases of the sperm plasma membrane. Glycobiochemistry 2006, 16, 786–800. [CrossRef] [PubMed]

23. Pasini, M.E.; Intra, J.; Gomulski, L.M.; Calvenzani, V.; Petroni, K.; Briani, F.; Perotti, M.E. Identification and expression profiling of Ceratitis capitata genes coding for beta-hexosaminidases. Gene 2011, 473, 44–56. [CrossRef] [PubMed]

24. Ashworth, J.R. The biology of Lasioderma serricorne. J. Stored Prod. Res. 1993, 29, 291–303. [CrossRef]

25. Mahroof, R.M.; Phillips, T.W. Life history parameters of Lasioderma serricorne (F.) as influenced by food sources. J. Stored Prod. Res. 2008, 44, 219–226. [CrossRef]

26. Li, C.; Li, Z.Z.; Cao, Y.; Zhou, B.; Zheng, X.W. Partial characterization of stress-induced carboxylesterase from adults of Stegobium panicum and Lasioderma serricorne (Coleoptera: Anobiidae) subjected to CO2-enriched atmosphere. J. Pest Sci. 2009, 82, 7–11. [CrossRef]

27. Kim, S.; Park, C.; Oh, M.H.; Cho, H.C.; Ahn, Y.J. Contact and fumigant activity of aromatic plant extracts and essential oils against Lasioderma serricorne (Coleoptera: Anobiidae). J. Stored Prod. Res. 2003, 39, 11–19. [CrossRef]

28. Allahvaisi, S. Controlling Lasioderma serricorne F. (Col.: Anobiidae) by fumigation and packaging. World Appl. Sci. J. 2013, 28, 1983–1988.

29. Rajendran, S.; Narasimhan, K.S. Phosphine resistance in the cigarette beetle Lasioderma serricorne (Coleoptera: Anobiidae) and overcoming control failures during fumigation of stored tobacco. Int. J. Pest Manag. 1994, 40, 207–210. [CrossRef]
30. Sağlam, Ö.; Edde, P.A.; Phillips, T.W. Resistance of Lasioderma serricorne (Coleoptera: Anobiidae) to fumigation with phosphine. J. Econ. Entomol. 2015, 108, 2489–2495. [CrossRef]

31. Huvenne, H.; Smagghe, G. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. J. Insect Physiol. 2010, 56, 227–235. [CrossRef]

32. Sağlam, Ö.; Edde, P.A.; Phillips, T.W. Resistance of Ostrinia furnacalis (Coleoptera: Anobiidae) to fumigation with phosphine. J. Econ. Entomol. 2015, 108, 2489–2495. [CrossRef]

33. Khan, A.M.; Ashfaq, M.; Khan, A.A.; Naseem, M.T.; Mansoor, S. Evaluation of potential RNA-interference-target genes to control cotton mealybug, Phenacoccus solenopsis (Hemiptera: Pseudococcidae). Insect Sci. 2018, 25, 778–786. [CrossRef] [PubMed]

34. Zhang, J.; Khan, S.A.; Hasse, C.; Ruf, S.; Heckel, D.G.; Bock, R. Full crop protection from an insect pest by RNA interference. Insect Sci. 2019, 10, 396–412. [CrossRef] [PubMed]

35. Li, H.; Jiang, W.H.; Zhang, Z.; Xing, Y.R.; Li, F. Transcriptome analysis and screening for potential target genes for RNAi-mediated pest control of the beetle armyworm, Spodoptera exigua. PLoS ONE 2013, 8, e65931. [CrossRef] [PubMed]

36. Zhao, X.X.; Situ, G.M.; He, K.; Xiao, H.M.; Su, C.C.; Li, F. Function analysis of eight chitinase genes in rice stem borer and their potential application in pest control. Arch. Insect Biochem. Mol. Biol. 2001, 41, 6667–6678. [CrossRef] [PubMed]

37. Kramer, K.J.; Muthukrishnan, S. Insect chitinases: Molecular biology and potential use as biopesticides. Insect Biochem. Mol. Biol. 1997, 27, 887–900. [CrossRef]

38. Zhao, X.X.; Situ, G.M.; He, K.; Xiao, H.M.; Su, C.C.; Li, F. Function analysis of eight chitinase genes in rice stem borer and their potential application in pest control. Insect Biochem. Mol. Biol. 2001, 25, 402–408. [CrossRef]

39. Zhai, Y.F.; Huang, M.X.; Wu, Y.; Zhao, G.D.; Du, J.; Li, B.; Shen, W.D.; Wei, Z.G. The expression profile and β-N-acetylglucosaminidase in the silkworm, Bombyx mori. J. Pest Sci. 2019, 1–11. [CrossRef]

40. Kramer, K.J.; Muthukrishnan, S. Insect chitinases: Molecular biology and potential use as biopesticides. Insect Biochem. Mol. Biol. 1997, 27, 887–900. [CrossRef]

41. Zheng, Y.P.; Krell, P.J.; Doucet, D.; Arif, B.M.; Feng, Q.L. Cloning, expression, and localization of a molt-related β-N-acetylglucosaminidase in the spruce budworm, Choristoneura fumiferana. Arch. Insect Biochem. Physiol. 2008, 68, 49–59. [CrossRef]

42. Yang, W.J.; Xu, K.K.; Yan, X.; Chen, C.X.; Cao, Y.; Meng, Y.L.; Li, C. Functional characterization of chitin deacetylase 1 gene disrupting larval–pupal transition in the drugstore beetle using RNA interference. Comp. Biochem. Physiol. Part B 2018, 219, 10–16. [CrossRef]

43. Wu, Q.; Liu, T.; Yang, Q. Cloning, expression and biocharacterization of OfCht5, the chitinase from the insect Ostrinia furnacalis. Insect Sci. 2013, 20, 147–157. [CrossRef]
51. Zhu, K.Y.; Merzendorfer, H.; Zhang, W.; Zhang, J.Z.; Muthukrishnan, S. Biosynthesis, turnover, and functions of chitin in insects. *Annu. Rev. Entomol.* **2016**, *61*, 177–196. [CrossRef]

52. Zhu, Q.; Arakane, Y.; Beeman, R.W.; Kramer, K.J.; Muthukrishnan, S. Functional specialization among insect chitinase family genes revealed by RNA interference. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6650–6655. [CrossRef] [PubMed]

53. Zhang, L.; Qiu, L.Y.; Yang, H.L.; Wang, H.J.; Zhou, M.; Wang, S.G.; Tang, B. Study on the effect of wing bud chitin metabolism and its developmental network genes in the brown planthopper, *Nilaparvata lugens*, by knockdown of TRE gene. *Front. Physiol.* **2017**, *8*, 750. [CrossRef] [PubMed]

54. Brook, W.J.; Sm, D.B.F. Organizing spatial pattern in limb development. *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 161–180. [CrossRef] [PubMed]

55. Clark-Hachtel, C.M.; Linz, D.M.; Tomoyasu, Y. Insights into insect wing origin provided by functional analysis of vestigial in the red flour beetle, *Tribolium castaneum*. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 16951–16956. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).