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RNAi-Mediated Silencing of Chitin Synthase 1 (CHS1) Disrupts Molting and Growth in *Monochamus alternatus*

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Abstract: Chitin synthase (CHS) plays a key role in chitin synthesis. CHS1 is ubiquitous in insects, and some studies have found that the RNA interference with CHS1 can hinder three types of molting processes (larva–larva, larva–pupa and pupa–adult). In the present study, the CHS1 of *Monochamus alternatus* was identified and characterized by a bioinformatics analysis. The developmental stage-specific expression of the *MalCHS1* (*Monochamus alternatus CHS1*) gene was obtained by a RT-qPCR, and the corresponding dsRNA was designed for functional verification. The RNA interference experiment was conducted using the microinjection method, and the injection site was selected from the abdominal segments of fifth-instar larvae. The results showed that after silencing the CHS1 gene, the larvae of *M. alternatus* showed morphological abnormalities, such as the softening of the body wall, a transparent abdomen and the swelling of somites, indicating that *MalCHS1* mediates the molting, growth and development of *M. alternatus*. RNAi-mediated *MalCHS1* gene silencing may become a promising new biological pesticide that can provide a new target gene for pest control.

Keywords: *Monochamus alternatus*; chitin synthase 1; RNA interference; molting; growth and development

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1. Introduction

Monochamus alternatus Hope is a crucial stem borer of *Pinus* plants [1,2]. It mainly feeds on *Pinus massoniana* Lamb., *P. thunbergii* Parlatore and *P. tabulaeformis* Carr. In China, *M. alternatus* also serves as a vector of the pinewood nemotade (PWN), which is responsible for pine wilt disease (PWD) [3]. This species plays a significant role in the pathogen's infection cycle by contributing to its spread within pine stands. PWD is the most serious forest disease in Japan and East Asia and it is currently considered to be one of the most serious threats to pine forests worldwide [4]. PWD is known as the cancer of pine trees [5]. Since the discovery of the PWN in the Sun Zhong Shan mausoleum in Nanjing, China in 1982, and by 2023, pine wilt disease has spread to 19 provinces (including 701 counties and cities) [6].

According to the morphological characteristics and life habits of *M. alternatus*, its life cycle consists of four stages: egg, larva, pupa and adult. The larvae are 1–5 instars (L1, L2, L3, L4 and L5). The transmission harm of *M. alternatus* is mainly in the supplementary nutrition and oviposition period of adults [2]. *M. alternatus* overwinters as larvae. In the larval stage, they mainly feed on the bast of the trunk and then gradually bore into the xylem of the trunk as it develops, disrupting water and nutrient transport, ultimately killing the tree. In the adult stage, they invade pines by feeding on the shoots and laying their eggs on the tree and at the same time carrying PWNs. The PWN feeds on the epithelial cells of the resin ducts of the tree and its proliferation impairs water transport, causing the tree to WILT and die and ultimately to the massive death of the trees [2,7,8].

Current studies have shown that chitin is a key component of the insect synthetic cuticle, digestive tract and other parts involved in growth and molting processes [9,10]. Chitinase (CHS) is the important enzyme in chitin synthesis [11,12]. Compared with the CHS of different insects, CHS has three main domains: domain A, domain B and domain C. Domain A and C are, respectively, located at the N-terminal and C-terminal and contain one transmembrane helix and seven transmembrane helixes, respectively, while domain B is the active center of CHS [13]. Currently, most insect chitinases are divided into two groups: Chitin Synthase 1 (CHS1) and chitin synthase 2 (CHS2) [14]. CHS1 mainly mediates the synthesis of chitin in the outermost cuticle of the insect epidermis, trachea and foregut of insects, while CHS2 is mainly responsible for the synthesis of chitin in the periplasmic membrane [15,16]. CHS1 is only distributed in epidermal cells derived from the ectoderm, including the epidermis, trachea, salivary glands, foregut and hindgut cells. It is involved in the synthesis of chitin in these tissues and thus plays an essential role in insect growth and molting processes [15,17,18]. Currently, RNA interference (RNAi) technology has been widely applied to the functional analysis of genes, pest management and many other aspects [19]. For example, reducing heat shock protein (hsp) expression by RNAi in *Dendroctonus* spp. resulted in greater environmental sensitivity and mortality rates [20,21]. In *Aphis gossypii* Glover, silencing the *CHS1* gene through RNAi can lead to an increased mortality, a shortened lifespan of adults and a decreased reproductive capacity [22]. In *Tribolium castaneum*, RNAi targeting CHS1 expression significantly reduced the insect resistance against fungal infestation [23]. The knockout of the *CHS1* gene in *Henosepilachna vigintioctopunctata* can lead to impaired intestinal integrity, the reduced thickness of the stratum corneum and the delayed formation of the newly generated stratum corneum during molting. In addition, the absence of *HvCHS1* inhibits the development of the tracheal system, resulting in the thinning of the tracheal band [24].

The destruction of the diseased trees and using insecticides has controlled pine wilt disease, but the public is now demanding environmentally friendly control methods. Research is focusing on finding alternative means of control, such as biological control agents for both nematodes and vectors, insect attractants and RNA pesticides with nanocarriers [3,25,26]. Based on the *M. alternatus* genome, the *MalCHS1* (*Monochamus alternatus CHS1*) gene was identified. The expression level of the *MalCHS1* gene at key developmental stages was obtained by a quantitative reverse transcription PCR (RT-qPCR), and then the corresponding dsRNA was designed for functional verification. Afterward, dsRNA was injected into *M. alternatus* by a microinjector, and its silencing effect was verified by an RT-qPCR. In this study, we will analyze the role of CHS1 in the growth and development of *M. alternatus* through molecular biology, RNAi technology and phenotypic observation to provide new target genes for pest control and a theoretical basis for novel biorational control methods, thus reducing the spread of pine wilt disease.

2. Materials and Methods

2.1. Samples and Feeding Conditions

Experimental insects were the first generation of larvae reared in our laboratory after being trapped by lamp lure method in the field (Fuzhou, Fujian Province 119°14′ 8.3616″ E, 26°5′ 27.8658″ N). The rearing conditions within environmental chambers were as follows: GHP-300E intelligent light and temperature incubator (Sanfa, Yangzhou, China), with temperature held at 23–26 °C, light cycle set to 12 h light: 12 h dark and relative humidity held at 70%–75%. Samples for expression level: 5th-instar larvae, male and female new pupae, male and female mature pupae and sexually mature male and female adults of *M. alternatus* were collected, with three biological replicates per group. They were placed in 1.5 mL centrifuge tubes, frozen in liquid nitrogen and stored at −80 °C. Samples for real-time quantitative PCR after RNAi: 5th-instar larvae of *M. alternatus* were collected, and the intestines of the control group and the treated group larvae were dissected and separated, with three replicates for each group.

2.2. Gene Cloning

RNA of *M. alternatus* was extracted using Trizol reagent (Ambion/Invitrogen, Waltham, MA, USA). The extracted RNA was quantitatively analyzed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Science, Waltham, MA, USA). The first-strand cDNA was prepared using the Hifair® III First Strand cDNA Synthesis Kit (gDNA Eliminator Plus) (Yeasen, Gaithersburg, MD, USA). Primers for PCR amplification were designed based on the *MalCHS1* sequence (Table 1).

Table 1. Primers used in the present study.

Primer		Sequence (5′-3′)
PCR	*MalCHS1*-F	CAAAGTATGGGAATGAACAG
	MalCHS1-R	TCTTTCTGTGTCTGATCTTC
RT-qPCR	*MalCHS1*-F	CAAACTTAACGCCAACCCTG
	MalCHS1-R	TTTCTCTGGGCCACATTGTT
	β-actin-F	GTTGCCCTCGACTTCGAACA
	β-actin-R	ACGGATATCAACGTCGCACT
RNAi	T7 promoter	TAATACGACTCACTATAG

2.3. Bioinformatics Characterization of MalCHS1 in M. alternatus

The full-length *MalCHS1* gene sequence was analyzed by open reading frame analysis and protein sequence prediction (data source: https://www.ncbi.nlm.nih.gov/, accessed on 1 July 2024), identifying its coding region and potential functional domains. In NCBI, BLAST protein was used to analyze amino acid sequences, and sequences with high homology were downloaded for homology comparison. MEGA 12.0 was used to construct a phylogenetic tree, and the Neighbor-Joining Method (NJ) was selected to construct the identified CHS1 gene [27]. The gene domain was analyzed using Web CD Search Tool online tool [28], and gene protein sequence motifs were identified with MEME suite (http://meme-suite.org/tools/meme (accessed on 5 July 2024)).

2.4. Quantitative Real-Time PCR

The 5th-instar larvae, male and female new pupae and old mature pupa and sexually mature male and female larvae of *M. alternatus* were collected in 3 replicates per group. RNA was extracted after freezing with liquid nitrogen, and the expression levels of the *MalCHS1* gene at key developmental stages were obtained by real-time quantitative PCR (RT-qPCR). β-actin (Table 1) was selected as the internal reference gene. All RT-qPCR

primers were designed by National Online and further validated with Oligo 7 [29]. The RT-qPCR procedure is as follows: initial denaturation at 95 °C for 5 min, followed by 10 s at 95 °C and 30 s at 60 °C, for a total of 40 denaturation cycles.

2.5. RNAi of MalCHS1

To synthesize dsRNA via in vitro transcription, T7 promoter sequences were added to the 5′ ends of PCR primers during target gene cloning (Table 1). Synthesize the target gene dsRNA using T7 RNAi Transcription Kit TR 102 (Vazyme, Nanjing, China). Afterward, RNAi treatment was performed on the 5th-instar larvae using dsRNA-*MalCHS1*, with an injection volume of 2000 ng/uL. RNase-free ddH2O was injected as a negative control group, and dsGFP was injected as a positive control group. The syringe was washed three times with anhydrous ethanol and RNase-free ddH_2O, with three replicates for each treatment. Observe the morphological changes in larvae after injection and record the time and quantity of death and pupation. To maintain the effectiveness of RNAi throughout the phenotypic observation period, test insects were re-injected with dsRNA every 48–72 h. Based on the close relationship between CHS1 and intestinal chitin synthesis, this study will dissect and isolate the gut of 5th-instar larvae 24 h after injection and detect the expression level of *MalCHS1* gene in the gut of *M. alternatus* larvae after injection of dsRNA through RT-qPCR experiment.

$$\text{Mortality} = \frac{\text{The number of dead larvae}}{\text{Total number of larvae}} \times 100\% \quad (1)$$

$$\text{Pupation rate} = \frac{\text{The number of pupae}}{\text{Total number of larvae}} \times 100\% \quad (2)$$

2.6. Data Analysis

Each group of experiments was repeated three times independently, and the data were expressed as the mean ± standard deviation of three independent experiments. Data were analyzed using SPSS statistical software (IBM SPSS Statistics 27 software). Differences in expression at key developmental stages were analyzed by one-way ANOVA test, and differences between treatment and control groups in RNAi experiments were plotted by independent samples *t*-test and then plotted using Graphpad (https://www.graphpad.com/) and SPSS software [30,31].

3. Results

3.1. Bioinformatics Analysis of MalCHS1 in M. alternatus

The phylogenetic analysis shows that the *MalCHS1* is clustered with *Anthonomus grandis* Boheman and closely related to *H. vigintioctopunctata*, suggesting its function may be conserved (Figure 1A). To further analyze the conservative features of *MalCHS1*, 10 conserved motifs (motifs 1–10, Figure 1B) were identified using the MEME online tool. The multiple sequence alignment shows that the CHS1 in most species has a highly consistent motif tissue pattern. The structural domain prediction analysis shows that the *MalCHS1* contains the following functional domains (Figure 1C): the GH18_chitinase-like superfamily, which mediates physiological processes such as insect molting, embryonic development and allergic inflammation and the Glyco_tranf_GTA_type superfamily, which catalyzes glycosyltransfer reactions and mediates the biosynthesis of oligosaccharides, polysaccharides and sugar conjugates.

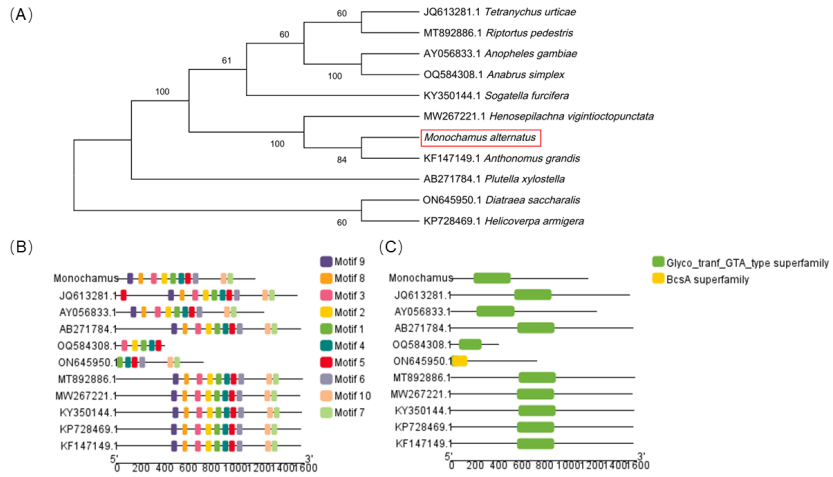


Figure 1. Phylogenetic analysis, conserved motif analysis and domain analysis of chitin synthase gene (CHS) in *Monochamus alternatus* and related species. (**A**) Neighbor-Joining phylogenetic tree of CHS protein sequences from *M. alternatus* (highlighted in red) and other arthropods. Bootstrap values (>50%) are shown at nodes. GenBank accession numbers are listed for each sequence. (**B**) Motif architecture predicted by MEME, showing 10 conserved motifs (color-coded boxes) across 11 arthropod species. Motif positions are scaled to protein length (bottom axis: 0–1600 aa). (**C**) Schematic representation of conserved domains in CHS proteins. Glyco_transf_CTA_type (chitin synthase catalytic domain) and BcsA superfamily (transmembrane domain) are annotated. Scale bar indicates amino acid position (0–1600).

3.2. The Developmental Stage-Specific Expression of the MalCHS1 Gene

The developmental stage-specific expression analysis revealed that the *MalCHS1* gene was expressed in all key stages of *M. alternatus*, with significantly lower expression levels in the fifth-instar larval stage than in other developmental stages. However, in the new male and female pupae stage, the expression level of the *MalCHS1* gene was significantly upregulated (Figure 2). This stage-specific high expression pattern suggests that *MalCHS1* may be involved in the degradation and synthesis of chitin from fifth-instar larvae to the pupal stage of *M. alternatus*, particularly in developmental processes such as new epidermal synthesis and old epidermal degradation.

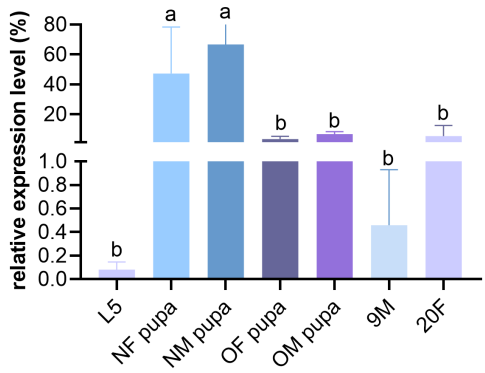


Figure 2. Expression patterns of *CHS1* at different developmental stages in *Monochamus alternatus*. L5: fifth-instar larvae; NF: new female pupae; NM: new male pupae; OF: mature female pupae; OM: mature male pupae; 9M: sexually mature male insect; and 20F: sexually mature female. Standardized as β-actin (one-way ANOVA with Tukey's post hoc test); a and b marked with different letters indicate statistically significant differences.

3.3. Changes in MalCHS1 Gene Expression in M. alternatus After RNAi

The RT-qPCR results showed that RNAi could effectively reduce the expression level of the CHS1 gene in the fifth-instar larvae of *M. alternatus*. As shown in Figure 3, the relative

expression level of *MalCHS1* in the intestine of larvae in the dsRNA-*MalCHS1* treatment group was significantly reduced compared to the control group (injected with RNase-free water). This significant inhibitory effect not only indicates that RNAi is highly effective in targeting *MalCHS1* transcripts but also provides a theoretical basis for the CHS1 gene as a feasible target for controlling *M. alternatus*.

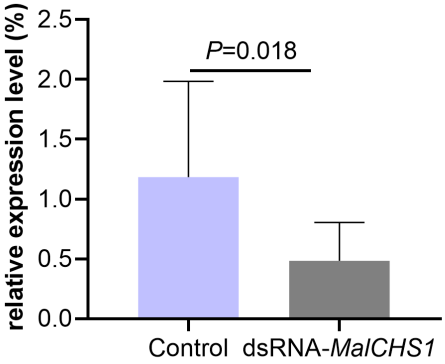


Figure 3. The effect of the dsRNA injection on the mRNA expression level of the *CHS1* gene in *Monochamus alternatus*. Error bars represent SEM; asterisks indicate significance (the independent samples *t*-test).

3.4. Phenotypic Changes in M. alternatus Larvae After RNAi

After the RNAi treatment, the pupation rate of *M. alternatus* larvae was 0% (Formula (2)), and the mortality was as high as 65% (Formula (1)), while the control group had a pupation rate of 40% (Formula (2)) and a mortality of 30% (Formula (1)) (Figure 4A,B). The dsRNA-*MalCHS1* treatment group of larvae showed the phenomenon of larval death, with the body becoming soft, the abdomen becoming transparent or the tail turning black. The abdomen or chest appears swollen (Figure 4C), indicating that this gene may be involved in synthesizing the new epidermis and degrading the old epidermis in *M. alternatus*.

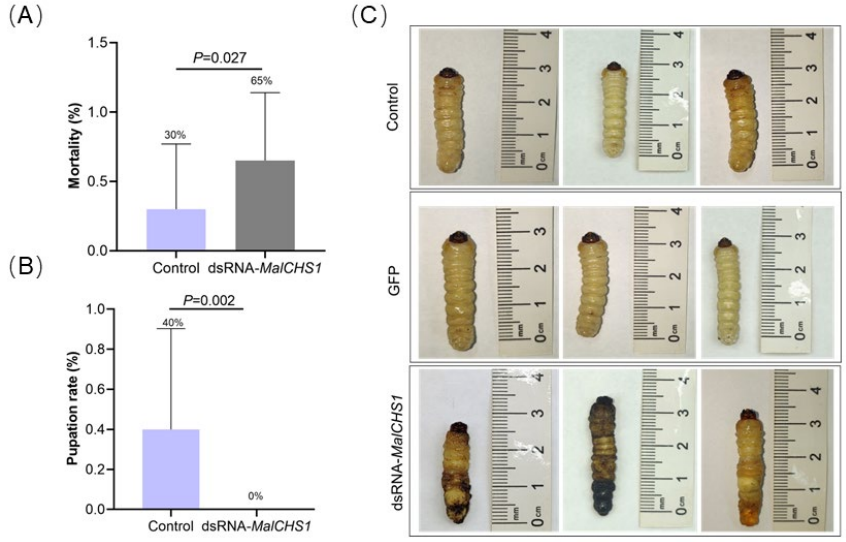


Figure 4. The effect of the RNAi on the mortality, pupation rate and phenotypic changes in Monochamus *alternatus* larvae. (**A**) The mortality of the larvae after the injection of dsRNA-*MalCHS1*. (**B**) The pupation rate after the injection of dsRNA-*MalCHS1*. (**C**) Phenotypic changes in larvae after injections. Unlike the control and GFP groups, the treatment group showed morphological abnormalities, such as the softening of the body wall, the transparency of the abdomen and the swelling of the body segments. Asterisks indicate significance (independent samples *t*-test).

4. Discussion

In this study, we determined the role of CHS1 in the growth and development of *M. alternatus* through molecular biology and RNAi technology, combined with phenotypic observations. This study not only reveals the function of CHS1 in the *M. alternatus*, but also provides target genes for the subsequent control of *M. alternatus* using RNAi technology and provides a theoretical basis for novel biorational prevention and control tools [32].

Chitin Synthase 1 is an important enzyme in the formation of chitin in insects and widely regulates the development of the insect epidermis, trachea and other tissues [15]. Recent studies have shown that the silencing of this gene can significantly affect the physiological function of pests. For example, using RNAi against *Panonychus citri* McGregor also showed that dsRNA significantly inhibited the oviposition ability and egg hatching rate of *P. citri* [33]. The RNAi-mediated CHS1 gene knockout increased the formation of the cuticular appressorium in *Nilaparvata lugens* (Stal), thereby enhancing the sensitivity of *N. lugens* to *Cordyceps sinensis*, nitenpyram and nitenpyram [34]. Therefore, RNAi-mediated CHS1 gene silencing can provide theoretical support for the development of biorational and safe biopesticides and pest control target genes.

The experimental results showed that after the *MalCHS1* gene silencing, the larva mortality during the critical period of pupation was greatly increased and the pupation rate was significantly reduced. At the same time, the larvae showed typical phenomena of chitin synthesis disorders, such as the softening of the body wall, a transparent abdomen, the localized swelling of the body and other morphological abnormalities. Similarly, after the RNAi treatment of the CHS1 gene of *Culex pipiens*, the larval survival rate decreased and molting disorders occurred [35]. In addition, the plant-mediated RNAi targeting of the CHS1 gene of *M. avenae* also resulted in significantly higher mortality and malformation rates [36]. Therefore, *MalCHS1* may play an important role in the pupation process of *M. alternatus*, and its mechanism may be related to the synthesis and degradation of the epidermis. It may hinder larval growth and development by disrupting the metabolic processes of *M. alternatus* chitin, which in turn hinders larval growth and development and even leads to larval death.

Conventional chemical pesticides are prone to inducing pest resistance and causing environmental pollution [37]. Currently, dsRNA is becoming a new control method, which can efficiently and specifically interfere with the expression of target genes and, therefore, can specifically control pests [38]. Studies have shown that dsRNA pesticides for forest pests, including nanocarrier-based formulations, have broad applications and can be applied by foliar spraying or trunk injections, which is an environmentally friendly, residue-free and highly targeted insecticide compared with traditional chemical pesticides [39,40]. It not only improves the efficiency of insecticides but also conforms to the direction of the development of biorational prevention and control [25,41,42]. However, there are still challenges in developing RNA pesticides, including ensuring the effectiveness of the dsRNA under field conditions, determining the optimal dsRNA concentration for the target pest and avoiding off-target effects [40]. Therefore, this study not only provides target genes for pest control but also lays the theoretical foundation for the development of new biorational pesticides, such as nanocarrier RNA insecticides.

5. Conclusions

This study comprehensively investigated the functional role and expression dynamics of *MalCHS1* in *M. alternatus*. The RNAi-mediated silencing of *MalCHS1* induced severe developmental impairments, manifesting as a significantly reduced larval viability and the complete suppression of pupal transition. These phenotypic alterations unequivocally demonstrate the enzyme's pivotal involvement in chitin biosynthesis and exoskeletal

formation. The results of this study not only revealed the role of the CHS1 gene in *M. alternatus* but also that the current control methods for *M. alternatus* have problems such as poor targeting and environmental pollution, so the results of this study can provide new target genes for the control of *M. alternatus* and provide theoretical support for the biorational and safe prevention and control of pests.

Author Contributions: Conceptualization, W.Y. (Wanlin Ye) and M.W.; methodology, W.Y. (Wanlin Ye) and T.L.; software, T.L. and W.G.; validation, T.L., W.Y. (Wei Yu) and F.X.; formal analysis, M.W. and W.Y. (Wanlin Ye); investigation, T.L. and W.G.; data curation, W.Y. (Wanlin Ye); writing—original draft preparation, W.Y. (Wanlin Ye) and M.W.; writing—review and editing, T.L. and M.W.; visualization, W.Y. (Wanlin Ye); supervision, Y.G.; project administration, S.W. and Y.G.; funding acquisition, S.W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Conflicts of Interest: The authors declare no conflicts of interest.

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