Glucosamine-6-phosphate N-acetyltransferase gene silencing by parental RNA interference in rice leaf folder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae)

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Parental RNAi (pRNAi) is a response of RNA interference in which treated insect pests progenies showed a gene silencing phenotypes. pRNAi of *CmGNA* gene has been studied in *Cnaphalocrocis medinalis* via injection. Our results showed significant reduction in ovulation per female that was 26% and 35.26% in G1 and G2 generations, respectively. Significant reduction of hatched eggs per female were observed 23.53% and 45.26% as compared to control in G1–G2 generations, respectively. We also observed the significant variation in the sex ratio between female (40% and 53%) in G1–G2 generations, and in male (65%) in G1 generation as compared to control. Our results also demonstrated the significant larval mortality (63% and 55%) and pupal mortality (55% and 41%), and significant reduction of mRNA expression level in G1 and G2 generations. Our findings have confirmed that effectiveness of pRNAi induced silencing on the *CmGNA* target gene in G1–G2 generations of *C. medinalis*. These results suggested the potential role of pRNAi in insect pest resistance management strategies.

Rice (*Oryza sativa* L., family *Poaceae*) is the world’s largest cereal crop that is widely cultivated around the globe. Rice has been accepted as a staple food for almost one-half of the human population in the world¹². In Asia, almost 90% of people feed rice to fulfill their dietary requirements³. Rice considers as a staple food for more than 65% of Chinese population and is a subsistence crop provides income and support for rural communities⁴. Unfortunately, serious insect's pest attack reduces rice production and its quality⁵. Over 100 insect pest species of rice have been recorded worldwide⁶. Out of these, almost 20 species are considered most injurious for rice crop that include leafhoppers, mole cricket, rice bugs, rice gall midges, rice mealy bug, stem borers, and rice leaf folder⁷.

*Rice leaf folder* (*Cnaphalocrocis medinalis*) is a major destructive rice insect pest that can cause severe grain yield losses worldwide. It is widely distributed in many rice growing countries of Asia, Africa, Australia and Oceania⁸. *C. medinalis* consists of complete metamorphosis that passes four different developmental stages such as egg, larva, pupa, and adult⁹. *C. medinalis* larvae have five stadiums that can damage at all the rice stages¹⁰. However, 4th larval stadium is considered most destructive for rice leaves¹⁰. Major characteristic of this insect pest is to scroll the leaves blades and scratch chlorophyll pigment inside them¹¹. Scratched leaves become whitish, membranous, and wither that inhibits photosynthesis, and ultimately reduces crop productivity¹². *C. medinalis* larvae can reduce 30 to 80% yield during epidemic situation¹³. *C. medinalis* is a migratory insect pest that possess 1–11 generations per year¹⁴. Existence of alternative plant hosts near paddy field create suitable environment to complete its multiple generations per year¹⁵. Shady places of paddy field and high humidity with high temperature are suitable for it growth and development¹⁵. At present, *C. medinalis* population often managed with extensive use of chemical insecticides¹⁶. However, misuse of insecticides drove insecticidal resistance, insect pest resurgence, dangerous to farmer’s health, toxic to environment, polluting underground water, and poisoning of Chinese food stuffs¹⁷. Insecticidal resistance of *C. medinalis* was reported in Japan¹⁷, China¹⁸, and in India¹⁹. C.

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_S. litura_ found in three consecutive generations after silencing via dsRNA injection39,44. Therefore, RNAi in insects26. Therefore, silencing of chitin biosynthesis genes may result in abnormal growth, body deformities, inhibit molting or even cause mortality77.

RNA interference (RNAi) has been recognized as an effective gene silencing tool in eukaryotic organisms28 that have conserved intracellular mode of action used to silence the gene expression29. Firstly, RNAi was described in _Caenorhabditis elegans_30. Later on, it was found in fungi, plants, animals, and in insects31–34. In insects, RNAi has been used to silence the gene expression against different insect pests such as _Tribolium castaneum_35, _Nilapavarta lugens_36, _Anopheles gambiae_37, _Diatrobica virgifera virgifera_38, _Spodoptera exigua_39, _Gryllus bimaculatus_40, _Manduca sexta_41, _Plutella xylostella_42, and _Hensolipplachna vigintioctopunctata_ sp.47. Gene silencing has been observed in late-instar larvae and adults of the lepidopteran _S. litura_ via dsRNA injection44,44. Therefore, RNAi through dsRNA microinjection could be useful to silence the chitin biosynthesis genes in _C. medinalis_.

RNAi technology has been divided into three categories such as, larval/ nymph/pupal RNAi (This technique has been used to study the gene expression in postembryonic stage and analyze the adult morphology on molecular basis in various organisms such as _T. castaneum_{45,46}, _Bombyx mori_{47}, _Schistocerca americana_{48}, _Blattella germanica_{49}, and _G. bimaculatus_{50–52}), embryonic RNAi (When dsRNA is incorporated into developing eggs in order to silence the target genes, the RNAi effects can observe in embryos such as _T. castaneum_{53}), and parental RNAi (Application of dsRNA into the body cavity via injection or ingestion that leads to gene silencing in offspring embryos44,54). Parental RNAi (pRNAi) effects were observed after silencing of zygotic genes in _T. castaneum_ offspring55, gap genes, and Krüppel and hunchback genes in _Oncopeltus fasciatus_{56,57}58. pRNAi effects were also recorded in _G. bimaculatus_{50–52}. In wasp, _Nasonia vitripennis_, pRNAi was also found in injected pupae with dsRNA59. Phenotypic deformities such as canine truncation and fusion of leg segments were observed in _Tetranychus urticae_ after pRNAi60. In western corn rootworm, _D. virgifera virgifera_, less hatched eggs and incomplete larval development were observed in response by targeting embryonic developmental genes61. In addition, several chromatin remodeling ATPase genes such as _iswi-1, iswi-2, mi-2_, _brahma_, and _hunchback_ genes were silenced using pRNAi in _Euschistus heros_ and _D. virgifera virgifera_62. In grain aphid, _Sitobion avenae_, pRNAi effects were observed in many generations63. In _C. medinalis_, pRNAi effects were also found in three consecutive generations after silencing _CmlHK_ gene through dsRNA injection64. However, pRNAi effects of _CmGNA_ gene has not studied in _C. medinalis_.

Glucosamine-6-phosphate N-acetyltransferase (GNA) is an essential enzyme of chitin biosynthesis pathway. Previously, GNA has been characterized in several eukaryotes such as, _human_{65}, _rat_{66,67}, pig_{68}, Saccharomyces cerevisiae_{69–71}, Candida albicans_{72}, and Aedes aegypti_{73}. It has been reported that GNA gene deletion in _S. cerevisiae_ was lethal74. Therefore, we consider that silencing of _CmGNA_ gene could be useful in pRNAi for the control of _C. medinalis_.

In RNAi assays, a dsGFP (green fluorescent protein derived dsRNA) has been used as an exogenous control for several insects, including _Spodoptera exigua_{75,76}, _Acyrthosiphon pisum_{77}, _Aedes aegypti_{78}, _Antheraea sp._80, _Locusta migratoria_{81}, Schistocerca gregaria_{42}, Bactericera cockerelli_{83}, and _Apis mellifera_{84–86}_.

In this study, we carried out series of experiments to examine the effectiveness of pRNAi. We synthesized dsRNA and injected into different groups of larvae of _C. medinalis_. We observed that pRNAi of _CmGNA_ had significant effects on eggs laying, hatched eggs, phenotypic deformities, morailties of larvae and pupae, male and female emergence rate, and reduced mRNA expression level of _CmGNA_ gene in G1-G3 generation.

**Material and methods**

**Rearing of _C. medinalis_.** _Cnaphalocrocis medinalis_ larvae were collected from rice growing field of Guizhou, China and reared at Entomological Institute of Guizhou University. The collected larvae were raised on fresh seedling of ShuHui-527 Chinese rice cultivar. The rearing chamber environment was kept at 75 ± 5% RH, 26 ± 1 °C, and 10:14 h dark: light photoperiod. Newly emerged adults were collected, paired (one male and female), and allowed them to mate for 3 to 4 days (Fig. S1). For this purpose, each adult’s pair was removed and allowed eggs to hatch. The hatched larvae were collected and used for pRNAi experiments.

**Gene identification.** _C. medinalis_ transcriptome sequence was already described81. The _CmGNA_ GenBank accession number was MN604261.

**RNA isolation, cDNA synthesis, and RT-PCR.** RNA Isolation and cDNA Synthesis were performed as already described88. However, specific primers were designed (see list of primers in supplemental Table S1) for reverse transcription-polymerase chain reaction (RT-PCR) based on transcriptome of _C. medinalis_. RT-PCR was carried out to confirm the expected size sequence of _CmGNA_ gene. The RT-PCR system consisted of 20 μL.
reaction mixture containing 1 μL of each primer, 10 μL of 2 × Master Mix (Tsingke, Beijing, China), 1 μL of cDNA template, and 7 μL of ddH2O. The reactions conditions were as follows: initial denaturation at 94 °C for 30 s, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 5 min, and a final extension of 72 °C for 10 min. The expected size was confirmed using agarose gel electrophoresis. The final products was then stored for further experiments.

**Double standard RNA (dsRNA) preparation.** The dsRNA was synthesized in accordance with protocols as previously performed. However, dsCmGNA and dsGFP sequence amplification and its synthesis were performed using different primers (see list of primers in Supplemental Table S1). The dsGFP was used as control. The final purified product was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher, MA, USA).

**Parental RNAi (pRNAi) bioassay.** Healthy 4th instar larvae of *C. medinalis* were selected for pRNAi. Seven groups were made along with control treatment and 20 larvae pooled per replication in each group. For dsRNA injection, 8th abdominal larval segment was selected at dorsal side along with the blood flow direction. The 0.5 μL (2 μg/μL) dsCmGNA and dsGFP were prepared and injected into the selected larvae. The treated larvae raised on ShuHui-527 fresh rice seedling under laboratory conditions as described above. To analyze the effects of pRNAi, newly emerged ten adults (five male and five female) from treated larvae were selected, paired and allowed them for oviposition. After oviposition, paired adults were removed from oviposition boxes and stored them in liquid nitrogen and kept at – 80 °C. Laid eggs were counted from each of mated pairs from G1 generation. A healthy female lays about nearly 135 eggs92. In order to estimate percentage laid eggs per female in control treatments, we compared control treatment laid eggs with 135 eggs and converted them into percentage. After hatched eggs, forty larvae were selected from each group (treated and control) and checked the mortality of both larvae and pupae. Forty pupae were selected from dsCmGNA and dsGFP treated group and checked the percentage male and female emergence rate in G1, G2 and G3 generations, respectively. The mRNA transcriptional level was also measured using newly emerged adults from treatments. After measuring mRNA transcriptional level, newly emerged adults from G1 generation were used for G2, and G2 were used for G3 generation. All experiments were performed from at G1–G3 generations to verify the pRNAi effects.

**Quantitative real-time PCR (RT-qPCR).** RT-qPCR was used to measure the mRNA transcriptional level of CmGNA in G1–G3 generation adults. For this purpose, RNA isolation and cDNA synthesis from G1–G3 generation adults were carried out using protocols as mentioned above. The cDNA was used as templates to perform RT-qPCR. RT-qPCR reaction was performed using C1000 Thermal Cycler (Bio-Rad, CA, USA). The reaction system contained 20 μL reaction mixture included 1 μL cDNA, 1 μL of each primers, 10 μL 2 × iTaq Universal SYBR Green Supermix, and 7 μL ddH2O. RT-qPCR reaction was carried out under the following conditions: 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s. *C. medinalis* actin gene (GenBank number. JN029806) was taken as the internal control. RT-qPCR primers were used as listed in Supplementary Table S1. The relative expression levels of CmGNA were calculated using the 2−ΔΔCt method.

**Statistical analysis.** Statistical Analysis were carried out with ANOVA (one-way analysis of variance) followed by LSD test using SPSS 22.0 (SPSS Inc. Chicago, IL, USA).

**Results**

**RT-PCR and dsRNA synthesis.** The cDNA sequence of CmGNA (GenBank Accession No. MN604261) consists 859 bp (Fig. S3). The dsCmGNA and dsCmGFP were clear and bright with 372 and 370 bps, respectively (Fig. S4). The dsCmGNA and dsGFP were quantified 3.1 μg/μL and 2.91 μg/μL, respectively.

**Effect of pRNAi on oviposition.** In order to examine the pRNAi effects on oviposition, treated larvae were reared until they become adults. Paired adults were laid eggs in oviposition boxes. Harvested eggs from both treatments were counted. Counted eggs were then compared with control treatments and converted them into percentage. Our results showed that G1 generation females laid eggs were 26% compared to control. In G2 and G3 generations, female laid eggs were 35.26% and 50.26%, respectively as compared to control (Fig. 1). While in G1–G3 control treatments, females were laid eggs 90%, 88%, and 90%, respectively. Therefore, these results indicated that the fecundity decreased over the generations with all being significantly lower than the control.

**Effect of pRNAi on hatched eggs.** After counting the laid eggs per female, oviposition box were placed in artificial chamber. Within in few days, 1st instar larvae were collected from hatched eggs. Newly emerged larvae were calculated and compared with previous counted eggs. Percentage hatched eggs per female were recorded as 23.53%, 45.26%, and 60.26% in G1–G3 generations, respectively as compared to control (Fig. 2). However, in control groups, hatched eggs were calculated 92%, 89%, and 98% in G1–G3 generations, respectively. Therefore, our results suggested that hatched eggs were high significantly less in G1 and G2 generations and significantly less in G3 generation as compared to control treatments.

**Effect of pRNAi on larval mortalities.** Counted 1st instar larvae were collected and placed on fresh seedling of ShuHui-527 Chinese rice cultivar. Larval mortalities were recorded on regular bases until they become pupae. Larval mortalites were 63%, 55% and 22% in G1–G3 generations, respectively (Fig. 3). While in control treatments, larval deaths were noticed as 10%, 11% and 8% in G1–G3 generations, respectively. Herein, our results showed that larval mortalities were highly significant in G1 and G2 and significant in G3 generation.
Effect of pRNAi on pupal mortalities. Survived larvae in G1–G3 generations were counted, kept in rearing chamber, and allowed them to become pupae. Pupae growth were stunted, and with lack of formation of pupation chamber which led them into delayed life span. Several pupae were died in pupation chamber and did not emerge into adults. Pupal mortalities were 55, 41, and 19%, in G1, G2 and G3 generations, respectively (Fig. 4). In control treatment, pupal mortalities were 17%, 5%, and 6%, respectively. Our results suggested that pupal mortalities were highly significant in G1 and G2, while significant in G3 group compared to their controls.
Effect of pRNAi on the rate of male emergence. In order to analyze the male emergence rate in G1–G3 dsCmGNA treated generations as compared to control, survived pupae were kept in rearing chamber until they become adults. Newly emerged adults were separated based on sexes. Male and female were counted in order to examine the rate of male emergence. In dsCmGNA treatments, emerged males in G1–G3 generations were recorded 65%, 82%, and 86%, respectively as compared to control (Fig. 5). However, male adults were emerged in dsGFP treatments were 88%, 94% and 90% in G1-G3 generations, respectively. These results indicated that male emergence was significantly different in only G1 generation as compared to control.

Effect of pRNAi on the rate of female emergence. In dsCmGNA treated G1–G3 generations, female adults were calculated as 40%, 53%, and 63% respectively as compared to control (Fig. 6), however, in control treatments, female adults were recorded as 95%, 93%, and 95%, respectively. These results indicated that female emergence was highly significant in G1 and G2, and significant in G3 generations as compared to control groups.

Phenotypic deformities of pRNAi in G1–G3 generations. pRNAi effects of CmGNA on C. medinalis have been studied in G1–G3 generations. We observed that phenotypic deformities were present in both larvae and pupae. Our results indicated that larval showed stunted growth, deformed shaped, and did not undergo in complete molting (Fig. 7). In contrast, no phenotypical deformities were examined in control treatments. Pupae did not emerged into adults and died in pupation chamber, while, significant percentage of pupae were emerged into adult individuals in control treatments (Fig. 8).

Effect of pRNAi on CmGNA transcriptional level in G1–G3 generations. Adult females were collected in G1–G3 generations, and used them to analyze the mRNA expression of CmGNA. We observed that mRNA expression level was high significantly decreased in G1 and G2, while, significantly reduced in G3 generations (Fig. 9). However, there was no effected on mRNA expression in G1–G3 generations of control treatments.
insects have become resistant against thuringiensis off-target insect species has driven attention for searching alternative methods of insect pest control. C. medinalis eggs which could be possible due to lack of embryonic development in eggs of Nephotettix cincticeps also present in hatched eggs of offspring embryos. Pupal mortalities were significantly observed in G1–G3 generations of C. medinalis. Previously, researcher described that pRNAi showed pupal mortality resulted in knockdown of zygotic genes the present research, our results also showed significant larval mortalities in G1–G3 generations of C. medinalis could cause significant reduction of hatched eggs in G1-G3 generations of treatments may be due to different abiotic factors in our insect rearing chamber. Recently, it was studied that pRNAi in female adults as compared to male. Same results also were observed in adults of C. medinalis single larva can damage multiple leaves and interfering with crop yield. C. medinalis larvae are a devastative for agricultural crops. At larval stage, larvae feed on plant's parts that ultimately reduces the crop yield. C. medinalis single larva can damage multiple leaves and interfering with photosynthesis. In a previous research, larval mortalities were observed in several insect pests using pRNAi. We also described that pRNAi caused a significant larval mortality in G1–G3 generations of C. medinalis. In the present research, our results also showed significant larval mortalities in G1–G3 generations of C. medinalis. Previously, researcher described that pRNAi showed pupal mortality resulted in knockdown of zygotic genes in offspring embryos. Pupal mortalities were significantly observed in G1–G3 generations of C. medinalis. Herein, pupal mortalities were significant in three generations of C. medinalis. Earlier studies have shown the highly sensitive and lethal pRNAi effects in D. virgifera virgifera were due to lack of embryonic development in eggs. pRNAi effects were also present in hatched eggs of Nephotettix cincticeps. Our result also stated the significant reduction of hatched eggs which could be possible due to lack of embryonic development in eggs of C. medinalis G1–G3 generations.

Discussion
Insect pests consider an alarming threat to globe crop production, pant biodiversity, and animal and human health. Synthetic chemicals are widely used for their control; however, insect pest resistance and resurgence, and off-target insect species has driven attention for searching alternative methods of insect pest control. Bt (Bacillus thuringiensis) crops varieties containing insecticidal proteins has been largely successful against lepidopteran insects. Multiple cases reported that insect pest have become resistant against Bt varieties. However, RNAi technology has been used against most different insect's orders, such as Diptera, Hemiptera, Coleoptera, Lepidoptera, and Orthoptera. Therefore, we used RNAi technology to target C. medinalis.

In this research work dsRNA corresponding to CmGNA gene were used to investigate the parental effect in C. medinalis. GNA is a key enzyme in insect biosynthesis pathway. We observed the pRNAi effects using dsRNA of CmGNA gene in C. medinalis. The G1–G3 generations were effected using dsCmGNA for in a long lasting manner. The experiments carried out with C. medinalis allowed us to measure the biological parameters and quantify the mRNA expression levels that continuously produces pRNAi in G1–G3 generations. Previous studies have demonstrated highly sensitive lethal pRNAi response in laid eggs of C. medinalis. In the aforementioned studies, almost similar pRNAi method was used. We observed that reduction in laid eggs were significant in dsCmGNA treatments in three generations. Abiotic factors have great influence on life cycle history of insects. Among them, temperature is the most important factor that exerts effects on the biology, reproduction, and abundance of insects. Therefore, we considered the decreased percentage biological parameters in control treatments may be due to different abiotic factors in our insect rearing chamber. Recently, it was studied that pRNAi could cause significant reduction of hatched eggs in G1–G3 generations of C. medinalis. Reduction of hatched eggs observed in D. virgifera virgifera were due to lack of embryonic development in eggs. pRNAi effects were also present in hatched eggs of Nephotettix cincticeps. Our result also stated the significant reduction of hatched eggs which could be possible due to lack of embryonic development in eggs of C. medinalis G1–G3 generations.

Insect's larvae are a devastative for agricultural crops. At larval stage, larvae feed on plant's parts that ultimately reduces the crop yield. C. medinalis single larva can damage multiple leaves and interfering with photosynthesis. In a previous research, larval mortalities were observed in several insect pests using pRNAi. We also described that pRNAi caused a significant larval mortality in G1–G3 generations of C. medinalis. In the present research, our results also showed significant larval mortalities in G1–G3 generations of C. medinalis. Previously, researcher described that pRNAi showed pupal mortality resulted in knockdown of zygotic genes in offspring embryos. Pupal mortalities were significantly observed in G1–G3 generations of C. medinalis. Herein, pupal mortalities were significant in three generations of C. medinalis. Earlier studies have shown the highly sensitive and lethal pRNAi effects in D. virgifera virgifera. However, stronger pRNAi effects were observed in female adults as compared to male. Same results also were observed in adults of C. medinalis. Our findings also suggested that pRNAi effects of CmGNA also caused significant effects in female as compared to male in G1–G3 generations. Therefore, CmGNA gene is suitable candidate for control of C. medinalis population.

DsRNA-degrading enzymes (dsRNases) have been considered as crucial factors reducing RNAi efficiency in many insect species. The presence of dsRNase have been studied in B. mori in which dsRNase is present is midgut and digestive juice. Subsequently, dsRNases are present in many insects, such as A. pisum, M. sexta, S. gregaria, Spodoptera frugiperda, P. xylostella, and Lygus lineolaris. Previously, pRNAi effects were decreased gradually in G1–G3 generations of C. medinalis. In this work, we also observed that pRNAi effects were highly significant in G1, significant in G2, and less significant in G3 generations. The mRNA expression was also decreased from G1–G3. According to our research outcomes, we considered that dsRNases may be present in C. medinalis that reduced the pRNAi efficiency. Due to presence of dsRNases, this research is not applicable at field level. Therefore, our future research direction will be analyze, characterize, and silence dsRNases efficiency that could be helpful in enhancing the effects of pRNAi and used against insecticide resistance in C. medinalis.

Figure 6. The percentage of emerged females after pupation. Each point indicates the mean ± standard error in G1–G3, and their control groups. Significant differences indicated by * (P < 0.05), ** (P < 0.01).
Figure 7. The phenotypic deformities were evaluated from larvae and pupae in G1–G3 generations. Infected larvae were observed in G1–G3 generations of treated insects using pRNAi.
Conclusion

In conclusion, we analyzed the pRNAi effects of \( \text{CmGNA} \) in the different developmental stages of \( \text{C. medinalis} \). Herein, we have described that pRNAi of \( \text{CmGNA} \) reduced the population of this notorious insect pest at any developmental stage. Our investigation led the researcher to understand the crucial role of pRNAi in insect pest management strategies. These findings provide a framework of pRNAi for testing on plants. A way leads for pRNAi as insect pest management tool which help to observe the longevity of pRNAi effects. In addition, pRNAi of \( \text{CmGNA} \) also provide a platform to better understand pRNAi in different lepidopteron insects.

Figure 8. Pupae of treated insects exhibiting deformities in G1–G3 generations.
Figure 9. Changes in mRNA transcript level of CmGNA gene in G1–G3 generations after pRNAi. Each bar indicated the mean ± SD, and significant differences indicated by *(P < 0.05), **(P < 0.01).

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**Author contributions**

Conceptualization, M.S. and S.-W.L.; methodology, M.S. and S.-W.L.; software, J.D., Y.-J.Z. and X.G.; investigation, M.S. and S.-W.L.; writing—original draft preparation, M.S.; writing—review and editing, M.S., S.-W.L. and N.S. All authors have read and agreed to the published version of the manuscript.

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**Competing interests**

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**Additional information**

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