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Silencing of Chitin Synthase A gene in *Spodoptera litura* (Fabricius) Using RNAi Approach

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Tobacco cutworm, *Spodoptera litura* (Fabricius) is the major constraint for the production and productivity of several agricultural and horticultural crops. The most common and effective method for the management of *S. litura* is the use of insecticides. However, the extensive use of insecticides leads to environmental pollution, contamination of food materials and development of resistance in insect pests. Hence, there is an urgent need for the development of effective and alternative pest management strategy. Post-transcriptional gene silencing or RNA interference (RNAi) approach was found as an environmentally safe and effective method for the management of several lepidopteran and coleopteran insect pests. Thus, the present study was focused on the design of double-stranded RNA (dsRNA) mediated knockdown strategy for the management of *S. litura* targeting *chitin synthase* (*SlCHSA*) gene. In this study, the *SlCHSA* gene specific dsRNA was synthesized and assessed their effect on growth and development of *S. litura* under *in vitro* conditions. Comparatively lower larval body weight and moulting rate was observed on the larvae fed with *dsSlCHSA* treated leaves than in untreated leaves. The moulting rate was recorded as 26.67 per cent where, the larvae fed with *dsSlCHSA* treated leaves during 4th moult. The result of the RT-qPCR analysis showed less expression level (6.25 fold) of *SlCHSA* on larvae fed with *dsSlCHSA* compared to control. The results suggested that *SlCHSA* can be used as a good candidate gene for RNAi based insect pest control. The enhancement of expression level of *dsSlCHSA* can be studied through plant mediated RNAi for developing an effective RNAi strategy against *S. litura*.

Key words: *Spodoptera litura*, *Chitin synthase A* (*CHSA*), dsRNA and RNAi approach.

Tobacco cutworm, *Spodoptera litura* (Fabricius) is one of the most destructive pests of various agricultural and horticultural crops and more or less of universal occurrence except in regions where extremes of climate prevails. It has been reported to feed on 112 cultivated plants all over the world of which 40 are grown in India (Muthukrishnan *et al*., 2005) including tobacco, tomato, cotton, chillies, okra, cauliflower, castor, groundnut, soybean, maize and black gram. Severe outbreaks of *S. litura* on cotton, tobacco, chilli and groundnut in Tamil Nadu (Santharam, 1986) with severe yield losses have been reported. The most common method for the management of *S. litura* is the use of insecticides. However, overuse of pesticides leads to environmental pollution, contamination of food materials, development of resistance in insect pests, toxicity to natural enemies and human beings (Xiao *et al*., 1994). These problems necessitate finding an alternate pest control strategy to supplement or even to supplant the present pest management methods.

Application of gene silencing, which may lead to be an effective and alternative management techniques of several lepidopteran insect pests through naturally occurring biological process of living organism (Terenius *et al*., 2011). Post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) is the phenomenon whereby double-stranded RNA (dsRNA) blocks the expression of its homologous gene, and has been reported in prokaryotes, nematodes, and other invertebrate animals (Lundgren and Duan, 2013). In general, the lepidopteran genome encodes for basic components of the RNAi pathway including dicer and enzymes and structural proteins that use 'diced' RNAs to hunt down and degrade matching RNA (Aronstein *et al*., 2011). Thus, the RNAi mediated gene knockdown has been studied in economically important lepidopteran insects including *Helicoverpa armigera* (Hubner) (Xiong *et al*., 2013) and others. Importantly, these studies have demonstrated that the RNAi approach is functional in lepidopteran insect. However, the most challenging task in RNAi approach for pest management is the identification or selection of target site and standardization of effective dose of dsRNA.

Chitin metabolism is one of the more interesting targets to provide an alternative management, which has the advantage of exerting less selective pressure and being relatively nontoxic to higher animals or plants which lack chitin. Chitin is an insoluble linear β-1, 4 linked polymer of N-acetyl glucasamine (GlcNAc) is one of the most abundant polysaccharide in nature (Deshpande, 1986) and it

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is synthesized by the polymerizing enzyme, chitin synthase. The gene *chitin synthase A* (*CHSA*) is responsible for the production of chitin synthase. Thus the knockdown of *CHSA* gene resulted the reduced growth and development of several insect pests including *Tribolium castaneum* (Hubner) (Arakane *et al*., 2005), *Spodoptera exigua* (Hubner) (Chen *et al*., 2008; Tian *et al*., 2009), *Locusta migratoria* (Meyen) (Liu *et al*., 2012), *Aphis glycines* (Matsumura) (Bansal *et al.,* 2012) and *Nilaparvata lugens* (Stal.) (Wang *et al*., 2012). However, very limited work has been done on the knockdown of *CHSA* gene in *S. litura*. Hence, the present study was focused on the knockdown of *CHSA* gene for the management of *S. litura*.

Materials and Methods

dsRNA synthesis of SlCHSA from S. litura

RNA isolation

Total RNA was isolated from single third instar larvae of *S. litura* using TRIzol method. The insect sample was homogenized with liquid nitrogen using a sterile pestle and mortar and the homogenized insect tissues were lysed with 1 ml of TRIzol reagent and were transferred to 2 ml microfuge tubes. The tubes were centrifuged at 10,000 rpm for 10 minutes. The supernatant was added with 0.2 ml of chloroform and vortexed vigorously for 10 minutes and incubated at room temperature for 10-15 min. The samples were again centrifuged at 13,000 rpm for 15 min at 4°C. Subsequently, the mixture separates into lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase.

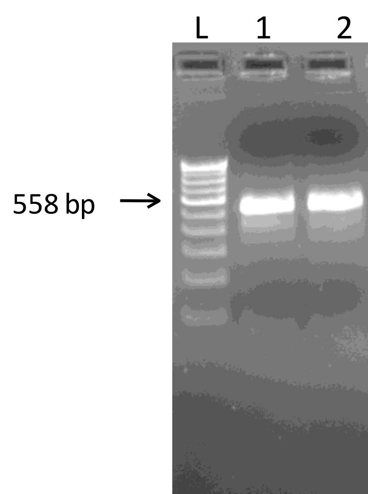

Figure 1. DNA template for in vitro transcription. Lane L-100 bp DNA ladder, Lane 1- 2: SlCHSA gene amplification in S. litura

The RNA in upper aqueous phase was transferred to a new tube without disturbing the inter phase and precipitated using ice cold isopropyl alcohol. The samples were incubated at room temperature for 10-15 min and centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, RNA gets precipitated at the sides and bottom of the tube. The supernatant was completely removed and pellet was washed with 75 per cent ethanol. Samples were mixed by vortexing and centrifuged at 7,000 rpm for 5 min. The pellets were dried to remove the ethanol. RNA was dissolved in DEPC-treated water.

The quality of total RNA was checked with 0.8 per cent agarose gel (0.8 g dissolved in 100ml of 1X TBE buffer) and the quantification of RNA was done by using Nanodrop Spectrophotometer (ND-1000). Based on the Nanodrop readings, RNA dilutions were made to make a final concentration of 50 ng/ µl and stored at -80°C for further use.

Synthesis of cDNA and dsRNA

The total RNA isolated from *S. litura* was converted into cDNA using ThermoFisher's Revert Aid First Strand cDNA synthesis kit. A synthesized cDNA fragment containing a *SlCHSA* gene specific partial sequence was amplified with T7 RNA polymerase binding site using forward and reverse primers incorporated with T7 promoter sequence (Table 1). The amplified PCR products of ~558 bp (Figure 1) was purified by column purification and then used as a template for synthesis of dsRNA with MEGAscript® RNAi kit (Ambion, Cat # AM1626) as per the manufacturer's instructions. The dsRNAs were annealed by incubating at 37ºC for 3.5 h, followed by slow cooling to room temperature. The annealed dsRNAs were treated with DNase I and RNase at 37ºC for one hour and then purified. The quality of purified dsRNAs was analyzed by agarose gel (1.0 %) (Figure 2).

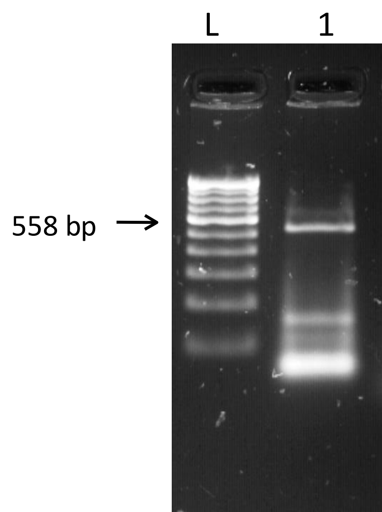

Figure 2. Synthesis of dsRNA.
Lane L-100 bp DNA ladder, Lane 1: dsRNA for CHSA from S. litura

Bioassay study

Mass culturing of *S. litura* was carried out with a laboratory stock, which was supplemented with field collected larvae from castor, periodically. The culture was maintained under prevailing room temperature and mass rearing was done according to the methodologies of Britto (1980). The uniform sized third instar larvae were used for bioassay.

For assessing the effect of dsRNA of *SlCHSA* on growth and development of *S. litura,* the concentration of 300 ng /10 µl dsRNA was used. The treatments include, untreated castor leaf, DEPC water treated castor leaf, sugar solution treated castor leaf and dsRNA of *SlCHSA* and sugar solution (1:1) treated castor leaf. The treatments were overlaid on individual castor leaves and the treated castor leaves were provided to the starved third instar larvae of *S. litura*. Each treatment was replicated three times and ten larvae were released per treatment. Untreated control was maintained simultaneously with DEPC treated water. The larvae were carefully transferred and reared in the containers by providing fresh food daily.

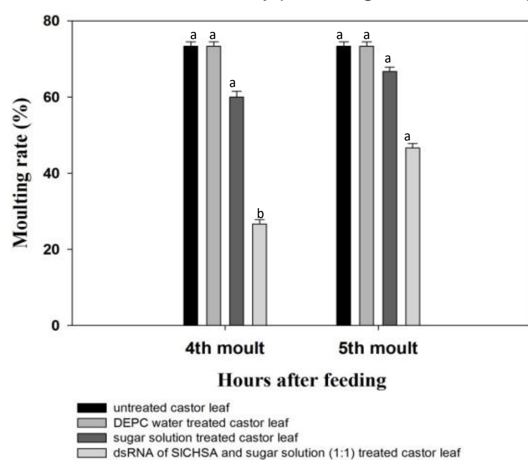

Figure 3. Effects of ingested dsSlCHSA on S. litura moulting rate. Moulting rate significantly reduced during 4th and 5th moulting after ingestion of dsSlCHSA in treatment *viz.,* dsRNA of SlCHSA and sugar solution (1:1) treated castor leaf than in untreated castor leaf, DEPC water treated castor leaf and sugar solution treated castor leaf. Error bars indicate standard error of mean. Statistical significance of difference were analyzed with ANOVA (P=0.05). Bars labelled with the same letter are not significantly different.

The growth and development were recorded at 72 hours after treatment. This study was conducted at room temperature of 26 ºC ± 2 ºC and RH of 91 to 95%. The data was analyzed statistically by one-way analysis of variance (ANOVA) at significance level (0.05) using the STAR software.

Expression analysis using RT-qPCR

Total RNA was isolated from bio-assayed larvae at 72 h using TRizol method. Subsequently, the cDNA was synthesized using ThermoFisher's Revert Aid First Strand cDNA synthesis kit. One hundred ng of cDNA product was used to perform the RT-qPCR amplification on CFX Connect™ Real-Time PCR Systems (Bio-Rad®) using primers specific to SlCHSA. 20 µl RT-qPCR reaction mixture included 1 µl of cDNA, 10 µl of 2X iQTM SYBR® Green supermix (Bio-Rad®), 100 nM of primers and nuclease free water to make up the total volume. The relative quantification was performed on CFX ConnectTM systems (Bio-Rad®) with the initial denaturation for 3 min at 95°C followed by 40 cycles at 95°C for 15 sec and 55°C for 1 min. The results were normalized with constitutively expressing SlActin gene of S. litura. All the primer sequences are provided in the Table 1. RT-qPCR data was analyzed by using CFX Manager 2.1 (Bio-Rad®) software and was further verified using standard delta-delta-Ct (ddCt) method.

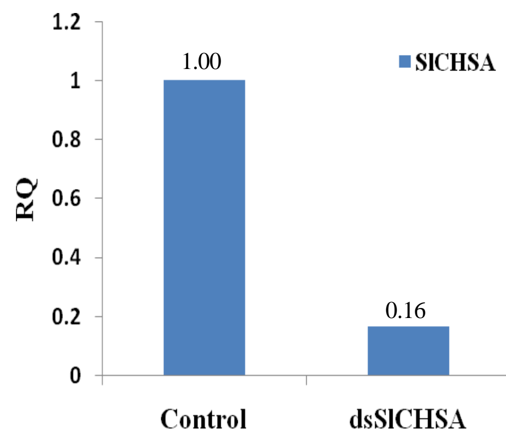

Figure 4. Effect of double-stranded RNA (dsSlCHSA) on mRNA level of SlCHSA gene in the S. litura larvae. SlCHSA mRNA level in S. litura were significantly decreased after 72 h of feeding. Five larvae were pooled for RNA extraction and were carried out for analysis. The RTq-PCR data were analyzed using the delta-delta Ct method. Housekeeping gene, SlActin, used as internal control. The mRNA level in the treated group were relative to control group at the same time point.

Results and Discussion

The effect of dsRNA *SlCHSA* on larval body weight and moulting of *S. litura* was recorded and presented in Table 2 and Figure 3. Larval body weight of *S. litura* was not significantly different in all the treatments at 72 h after treatment. Among the treatments, the treatment with dsRNA *SlCHSA* recorded low larval body weight of 0.23 g followed by treatment with sugar solution (0.24 g) (Table 2). The result of the present study was in agreement with the earlier report Tian *et al.*, (2009) who also found reduction of larval body weight on *S. exigua* larvae fed with dsRNA treated leaves. The constant increase of larval body weight was recorded throughout the study period in all the treatments of present study. However, the treatment with dsRNA *SlCHSA* recorded decreasing trend of larval body weight at 72 h after treatment and it demonstrated the efficiency of dsRNA begin at 72 h. This was in concordance with Sarath Chandra (2014) who found the similar trend of decreasing body weight of *H. armigera* fed with dsRNA treated leaves at 72 h after treatment. Similar reduction of larval body weight was recorded on *H. armigera* treated with dsRNA of many target genes (Asokan *et al.*, 2014). However, Mao *et al.*, (2011) conducted the similar experiment with transgenic tobacco plants targeting P450 gene (CYP6AE14) and found significant amount of larval body weight reduction in *H. armigera*.

The down regulated effect of *dsRNA SlCHSA* on moulting of *S. litura* was recorded in the present study (Figure 3). A significant variation in the larval moulting of *S. litura* was observed at 4th and not in 5th moult after treatment. Among the treatment, the treatment with dsRNA *SlCHSA* recorded lower moulting of 26.67 and 46.67 per cent larva during 4th and 5th moulting after treatment, respectively and malformation of few larvae was also observed. Successive moulting was observed in all other treatments. The result of the present study was in accordance with the report of Arakane *et al.*, (2008) who also found significant reduction of moulting rate in insects treated with moulting related dsRNA. Yang and Han (2014) also reported the partial moulting and absence of pupal stages in the larvae of *H. armigera* treated with *dsRNA* of chitinase gene.

Table 1. Primers used for amplification of partial *SlCHSA* gene and RT-qPCR assays

Primer Name	Sequence (5'-3')	Amplicon Size (bp)
SlCHSA AF	taatacgactcactataggg GTGATGATGATTCGCAAGTGA	538
SlCHSA AR	taatacgactcactataggg AGGATGAATACGACCGCAAG	538
Actin	F- AATCGTGCGTGACATCAA R- TGTAAGTGGTCTCGTGGAT	218
Chitin synthase A	F- GACTCTGGACGGAGACAT R- GCCTACAGGATGAATACGAC	112

In order to assess the effect of dsRNA mediated knockdown of *CHSA* gene in *S. litura*, RT-qPCR was carried by using dsRNA treated and control insects. The results showed that the *SlCHSA* gene expression level was approximately 6.25 times less in dsRNA treated larvae as compared to the control group (Figure 4). This was in consistence with the earlier report on *S. exigua*, where significant lower level of transcript was detected after dsRNA mediated silencing of *SlCHSA* gene (Chen *et al.*, 2008; Tian *et al.*, 2009).

Table 2. Effect of ingested *dsSlCHSA* on *S. litura* larval body weight

Treatment	Larval body weight (g) ± SE at 72 h*
Untreated castor leaf	0.27 ± 0.05
DEPC water treated castor leaf	0.25 ± 0.07
Sugar solution treated castor leaf	0.24 ± 0.07
dsRNA of *SlCHSA* and sugar solution (1:1) treated castor leaf	0.23 ± 0.04

* Mean of three replications; Treatments are not significantly different according to ANOVA at p=0.05

However, *N. lugens* showed 98 per cent reduction in transcript level of *NlCHS1* after injection of dsRNA (Wang *et al.*, 2012), suggesting RNAi knockdown varied from species to species (Yang and Han, 2014).

Conclusion

Although, the results of the present study showed decrease in larval body weight and expression level of *SlCHSA* gene, it was not 100 per cent efficient, still larvae were forming cuticle stating the presence of *SlCHSA* gene. Also, significant moulting variation was recorded only at 4th moult, not at 5th moult. Thus, there is need to enhance the expression level of dsRNA, which can be done through plant mediated RNAi to develop an effective RNAi strategy against *S. litura.*

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