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SILENCING OF JUVENILE HORMONE DIOL KINASE GENE INDUCES MOULTING DIFFICULTIES IN *SPODOPTERA LITURA* (F)

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ABSTRACT

Juvenile hormone diol kinase is an important regulatory enzyme that catalyses the phosphorylation of JH diol compound into JH diol phosphate. In the present study, a JHDK complementary DNA (cDNA) was cloned from *Spodoptera litura* (F) and provisionally named as *Sljhdk1*. Initially, the *Sljhdk1* was characterized based on their stage-specific expression patterns and then the *Sljhdk1* gene was suppressed through RNAi. RNAi was induced by feeding double-stranded RNA (dsRNA) to the 3rd instar larva of *S. litura*. The dsRNA was designed to target the specific regions of the *Sljhdk1* gene sequence. Ingestion of 1µg of dsRNA by each 3rd instar larva successfully knocked down the target gene expression. Notable phenotypic aberration was observed along with deformed pupa and reduced pupal size. These findings ascertain the important regulatory roles of *Sljhdk1* in *S. litura*.

Key words: Metabolic enzyme, gene cloning, dsRNA synthesis, feeding bioassay, gene knockdown, phenotypic aberrations

Spodoptera litura (F.) is a serious pest of both field and horticultural crops. It is considered as one of the most destructive insect pests in Asia-Pacific region due to its high fecundity and sever infestation rates (Ahmad et al., 2013). In India, the yield loss of crops caused by *S. litura* is around 35-55% (Natikar and Balikai, 2017). *Spodoptera litura* has developed resistance against 36 chemical compounds including newer chemistries throughout the Asian countries. Moreover, several reports showed that *S. litura* has a greater potential to survive in the presence of Bt toxins when compared to other bollworms (Arshad and Suhail, 2011; Lalitha et al., 2012; Selvi et al., 2012). Therefore, the search for more target-specific pest management option is of utmost priority. RNA interference (RNAi) is a potent alternative (Joga et al., 2016). RNAi can be utilized for silencing of crucial genes in insects, thus causing selective killing. RNAi effects can be initiated by the ingestion of double-stranded RNAs (dsRNAs) from the environment.

Juvenile hormone diol kinase (JHDK) is an important enzyme involved in the juvenile hormone (JH) degradation pathway. JHDK is a homolog of *Drosophila melanogaster* sarcoplasmic calcium-binding protein-2 (dSCP2) (Maxwell et al., 2002b). JHDK was first identified in *Manduca sexta* (Maxwell et al., 2002a, b); *M. sexta* JHDK is specific for JH diol and requires Mg^{2}+for its activity, but the protein is inhibited by Ca^{2}+ at a low concentration (Maxwell et al., 2002b). JHDK has also been cloned from *Bombyx mori* (Li et al., 2005). *Bombyx mori* (Bommo)-JHDK cDNA (637 bp) contains an open reading frame encoding a 183-amino acid protein, which reveals a high degree of identity to the two previously reported JHDKs. *B. mori* JHDK is constitutively expressed at a high level in the gut of the silkworm, but expressed at trace levels in the Malpighian tubule and haemocytes (Yang et al., 2011). Its expression was not regulated by JH. In honeybee (*Apis mellifera*), JHDK was found to be expressed in the mushroom bodies of the brain (Uno et al., 2007). In *B. mori*, the haploid genome has only a single copy of JHDK gene, which contains only one exon (Li et al., 2005). In a recent study, a JHDK complementary DNA (cDNA) was cloned from *S. litura* and the structure and expression of the gene was characterized. The cDNA was 714 base pairs in length and encoded a protein of 183 amino acids with a molecular mass of 21 kDa and an isoelectric point of 4.55. Based on the structure, three putative calcium binding motifs and guanosine triphosphate-binding motifs were predicted in the protein. Modeling of the 3-D structure showed that the protein consisted of eight α-helixes linked with loops, with no β-sheets (Zeng et al., 2016). In another experiment HvJHDK was subsequently suppressed using RNAi to reveal its functions. Different concentrations of dsJHDK elicited the optimal interference efficiency at different life

stages of *Herotia vitessoides*. Suppression of HvJHDK decreased HvJHDK content and increased the juvenile hormone titer, thereby resulting in reduced triglyceride content, sharply declined survival rate, clearly lethal phenotypes and extended larval growth (Lyu et al., 2019). Taken together, the above findings provide molecular references for the selection of JHDK as a novel insecticidal target.

MATERIALS AND METHODS

Larvae of *S. litura* were collected from cabbage field at the Indian Agricultural Research Institute, New Delhi**.** The larvae were reared on semi-synthetic diet till pupation in culture room (27± 1°C, 65± 5% RH and 14:10 (L:D) photoperiod). On emergence five pairs of adults were transferred to mating jars and served with 10% honey. Paper strips folded in fan like fashion were kept inside the mating jars for egg laying. Egg masses were collected each day and kept in separate container. On hatching, neonates were again transferred to diet. The fourth larval instars were used and total RNA was extracted using the TRIzol™ reagent (Invitrogen, Waltham, MA, USA) as per manufacturer's protocol. First-strand cDNA was synthesized from 1 µg of total RNA of *S. litura* using the Revert Aid™ cDNA synthesis kit (Thermo scientific, Waltham, MA, USA) and immediately stored at −20 ◦C until further use. The cDNA sequence of a putative *S. litura* juvenile hormone diol kinase (JHDK) gene (Accession No.: KP192493.1) was obtained from NCBI database through nucleotide search and provisionally named as *Sljhdk1*. Open Reading Frame (ORF) Finder was used to acquire the sequence of *Sljhdk1* ORF which codes for the functional JHDK protein of the retrieved sequence. A pair of gene-specific primers (Sl-Jhdk 1F: CGGCGAAACAAGTATCCA; Sl-Jhdk 1R: AGCGTACACAGAGGAGAA) was designed to amplify the partial *Sljhdk1* (407 bp) to verify the sequence. Amplification of the coding region and gene product confirmation was done through colony PCR. The coding region of *Sljhdk1* was amplified by reverse transcription PCR using 1st strand cDNA template. The PCR reaction cycle was as followed viz. 2 min of initial denaturation at 95 °C; 27 cycles of 30 sec of denaturation at 95 °C and 1 min of annealing at 58 °C; then 1 min of extension at 72°C and a final extension of 10 min at 72°C. Next, the desired PCR product was gel-purified, ligated into the TA cloning vector (pGEM™-T Easy Vector System I, Promega, USA), transformed into *Escherichia coli* DH5α competent cells and positive clones were selected through blue white screening. The recovered gene fragment was confirmed through colony PCR by agarose gel electrophoresis.

Expression analysis of *Sljhdk1* gene in different life stages and tissues of *S. litura* was done using qPCR. Total RNA was extracted from different larval stages viz. one day old, first instar, second instar, third instar and fourth instar larva and different body tissues viz. head, thorax and abdomen. First-strand cDNA was synthesized from 1 µg of total RNA from each sample using Revert Aid™ cDNA synthesis kit (Thermo scientific) as per manufacturer's protocol. Specific primers were designed to conduct quantitative real time PCR (q-SlJhdk 1F: CACCTGGCAACTACATCTTC; q-SlJhdk 1R: CAGTCCAAATACAGGCGATAC). The relative transcript level of the putative gene was determined through quantitative real time PCR (qRT-PCR). For conducting RNAi, dsRNA (500 bp) was synthesized based on the cloned region of the *Sljhdk1* mRNA sequence. Specific primers tailed with T7 promoter were designed for dsRNA synthesis (ds-Jhdk 1F: TAATACGACTCACTATAGGG CGGCGAAACAAGTATCCA; ds-Jhdk 1F:AATACGACTCACTATAGGG AGCGTACACAGAGGAGAA). The purified PCR products were then used to synthesize dsRNA with the MEGAscript T7 RNAi Kit (Ambion™). Feeding bioassays with synthesized dsRNA of the cognate gene against the third instar larva of *S. litura* was carried out by diet overlay method. Each larva was served with 1µg of dsRNA. Control larva was served with nuclease free water. 30 larvae were used for RNA extraction and 30 were used for observation. In order to predict the effect of RNAi, the expression of *Sljhdk1* gene was determined after dsRNA treatment at different time point viz*.,* 24, 48 and 72 h after treatment through qPCR. For expression analysis total RNA was isolated using TRIzol™ reagent (Invitrogen, USA) from treated as well as from control. First strand cDNA synthesis was carried out by using Revert Aid™ cDNA synthesis kit (Thermo scientific) as per manufacturer's protocol. A 100 ng of cDNA template was used for RT-qPCR. The relative expression analysis was done by qPCR. A 10 µl of reaction mixture including 2X SYBR Green PCR Master mix (Thermo Scientific), 10 mM of gene specific primer, nuclease free water and 1 µl (100 ng/ µl) of template cDNA was used. Actin was used as housekeeping gene for normalization. Relative expression analysis was done by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The reaction cycle was set as 95 °C for 5 min (denaturation), followed by 40 cycles of 95 °C for 30 s and 60 °C for one min (annealing). Reaction was carried out in a C100™ Thermal Cycler (BIORAD™).

Threshold cycle (C_t) values and normalized gene expression were calculated using Bio-Rad CFX Manager TM software. $2^{-\Delta\Delta Ct}$ method was applied for relative expression analysis (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

The full length cDNA (714 bp) sequence of *S. litura* Juvenile Hormone Diol kinase (JHDK) gene (Accession No.: KP192493.1) was retrieved from NCBI database through nucleotide search and provisionally named as *Sljhdk*1. The ORF region was 552 bp long and encodes for a protein of 183 amino acids. Gene specific primers were used to amplify the cDNA region of *Sljhdk1* gene. Partial *Sljhdk1* (407 bp) gene was amplified from the cDNA of *S. litura* (Fig. 1A). The amplified PCR products were then gel purified and cloned into DH5ά (*Escherichia coli*) strain (Fig. 1B) and confirmed through colony PCR (Fig. 1C). Perusal of results showed that *Sljhdk1* gene is expressed in all the four larval stages. The highest level of *Sljhdk1* transcripts have been detected in third instar larvae compared to first, second and fourth instar larvae (Fig. 2) whereas in the fourth instar larva the expression was found to be lower than the other instars was done. Single dose feeding bioassay with the dsRNA specific to the cognate gene, to see the effect of dsRNA on the expression of *Sljhdk1* gene in the third instar larva. Result shows that the mRNA level of the target gene was decreased significantly after ingesting dsRNA, by a factor of 0.44, 0.21, 0.14 and 0.19, respectively, when compared with the control. However, the mRNA abundance showed an increased trend after 72 hr of post feeding (Fig. 3). In addition, the larva treated with dsRNA exhibited difficulties in moulting and eventually died. The dead larvae were found trapped in the old cuticle. Moreover, some larva turned pupa showed malformation and reduced body size (Fig. 4).

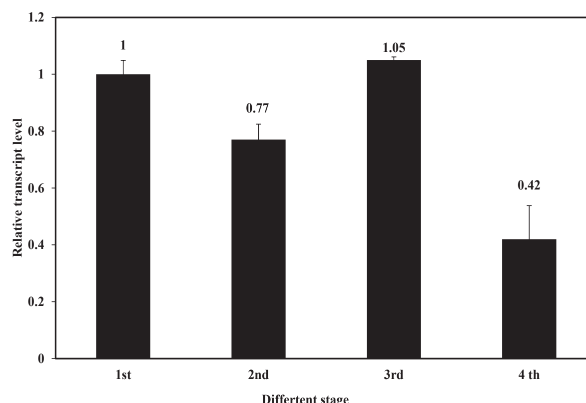

Fig. 2. Developmental expression of the Sljhdk1 gene. cDNA templates were derived from whole bodies of the first, second, third, and fourth instar larvae. The bar represents the 2^ (-ΔΔCt) value (±SE) normalized to the geometrical mean of housekeeping gene (actin) expression

JHDK is a key regulatory enzyme in the pathway of JH degradation which effectively converts JH diol into JH diol phosphate, basically a nonreactive end product, in order to maintain a precise level of JH titre in the haemolymph (Maxwell et al., 2002a, b). This regulation of the active JH titre has profound effect on insect development and metamorphosis. Till date, JHDK has been identified and cloned from lepidopteran insects, such as *M. sexta* (L.), *Bombyx mori* (F.), *Ostrinia furnacalis* (Guen.), *Plutella xylostella* (L.), *Danaus plexippus* (L.) and *Antheraea yamamai* (Guerin.) (Zheng et al., 2015) but it has been characterized only in *M. sexta* (Maxwell et al., 2002a, b) and *B. mori* (Li et al., 2005). In the present study, a fragment of JHDK cDNA (407 bp) has been cloned from *S. litura* (*Sljhdk*1) and characterized in terms of gene expression and silenced through dsRNA mediated RNAi to validate the

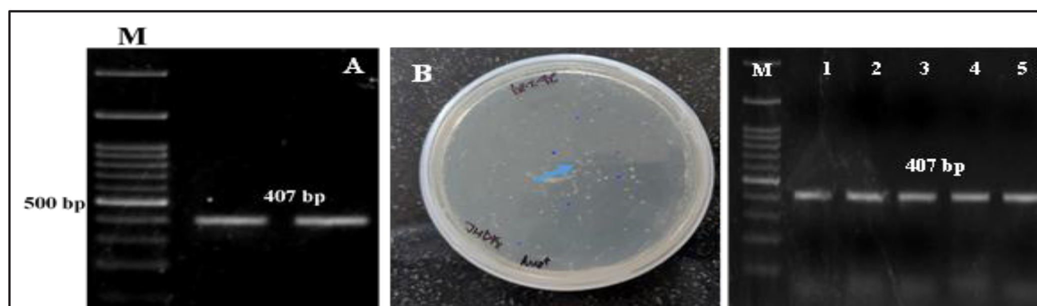

Fig. 1. (A) PCR amplification of partial *Sljhdk1* gene from the cDNA of *Spodoptera litura*. (B) Cloned PCR product of *Sljhdk1* gene in DH5ά (*Escherichia coli*) strain. White colonies are the positively transformed colony with our gene of interest. (C) Amplification of positive clones from the transformed colony of *Sljhdk1* genecDNA templates derived from whole bodies of the fourth instar larvae after 24, 48, 72 and 96 hr of treatment. The bar represents the 2^ (-ΔΔCt) value (±SE) normalized to the geometrical mean of housekeeping gene (actin) expression.

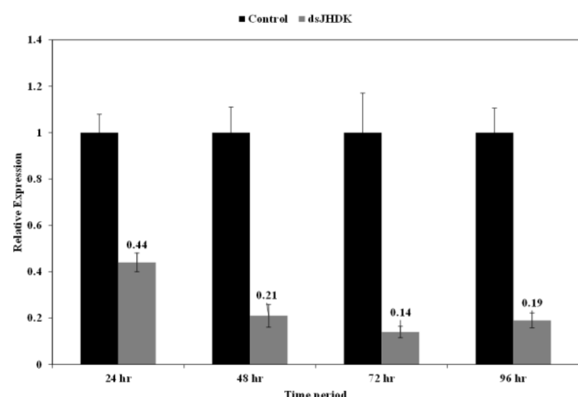

Fig. 3. Effects of dietary ingestion of dsRNA on the expression of Sljhdk1 gene. cDNA templates derived from whole bodies of the fourth instar larvae after 24, 48, 72 and 96 hr of treatment. The bar represents the 2^ (-ΔΔCt) value (±SE) normalized to the geometrical mean of housekeeping gene (actin) expression

function of *Jhdk* gene in regulating JH titer in insect. The results indicated that the *Sljhdk1* gene was expressed in all the developmental stages with higher levels in the early instars larva. Our result can be compared with the previous study conducted by Zeng et al. (2016) where they have seen the higher expression level of JHDK gene in early days of larval development than late days.

In the present study, it has been found that ingestion of dsRNA by the larva of *S.litura* successfully silenced the putative *Sljhdk1* gene which invariably resulted in inter-moulting difficulties, pupal deformities in the test insect. We found optimal interference of the target gene upon ingestion of 1µg of dsRNA by a 4th instar larva. We have found that the expression of *Sljhdk1* gene was steadily declined up to 72 hr of ingestion and suppression of *Sljhdk1* gene invariably increased the JH titre which further affected the moulting process in the *S. litura* larva. In a recent study on JHDK showed similar kind of result where abnormal moulting and extended larval duration was resulted upon suppression of JHDK gene through dsRNA incorporation (Lyu et al., 2019). Lastly, being the most crucial regulatory enzyme in the insect body, genes related to these enzymes can be a potential target for RNAi mediated pest management. JHDK is a metabolically important enzyme have profound effect on the maintenance of the JH titre for successful metamorphosis in insects. Our study showed the molecular inference of selecting these gene as potential target for managing *S. litura*.

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AUTHOR CONTRIBUTION STATEMENT

Both authors contributed equally.

CONFLICT OF INTEREST

No conflict of interest.

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Fig. 4. Effect of Sljhdk1 dsRNA ingestion on the survival of the Spodoptera litura larva. (A) Inter-molt difficulties in the treated larva due to silencing of *Sljhdk1* gene resulted due to dsRNA ingestion by the larva. (B) Pupal deformities in the dsRNA treated larva (C) control larva

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