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RESEARCH REPORT

Identification of a putative RNAse III (*dicer* homolog) gene in silkworm *Bombyx mori*

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Abstract

Like other invertebrates, silkworms also encounter a problem from microbial infection including from RNA viruses. In insects, RNA interference acts as a natural anitiviral response to RNA virus infection. Especially in *Drosophila*, it is proved that *dicer* mediated RNA interference directs innate immunity against RNA viruses. This information prompted us to identify similar RNAse III (*dicer*) gene in mulberry silkworm *Bombyx mori*. The *Drosophila dicer* gene was BLAST-searched with *B. mori* genome and single contig (GenBank accession n° AADK01001038) showed maximum homology with *dicer* gene, through which the RNAse III gene sequence was identified in the genome of silkworm *B. mori*. The RNAse III domain was present in the three regions with the length of 278 bp, 277 bp and 185 bp in the contig, possibly these three regions form exons. The primers were designed for three *B. mori* RNAse III regions and amplified through PCR. The region I was amplified only in pure Mysore silkworm strain whereas all three regions were amplified in Daizo strain. The PCR product sequences were translated and showed RNAse III domain with in the amplified product. The predicted *B. mori* RNAse III domain had phylogenetic relationship with other insect *dicer* genes. We presume that this RNAse III (*dicer*) would protect *B. mori* larvae from invading RNA viruses, which exists in the other insects.

Key words: *Bombyx mori*; RNAse III; *dicer*; domain

Introduction

Mulberry silkworm *Bombyx mori*, is highly susceptible to various diseases which adversely affects the silk production. Among silkworm diseases, viral diseases are very severe in tropical countries due to unfavorable climatic conditions coupled with improper hygiene in rearing environment (Subbarao *et al.*, 1991). *B. mori* nuclear polyhedrovirus (BmNPV) is a major viral pathogen responsible for grasserie disease followed by other viral pathogens like cytoplasmic polyhedrovirus (CPV) and infectious flacherie virus (IFV) which are known to equally contribute to silkworm crop loss (Samson *et al.*, 1990). There are many reports on *B. mori* immune response against invading microbial pathogens especially against bacterial pathogens (Ponnuvel and Yamakawa, 2002). However, the information on viral disease resistance remains scanty. The red florescence protein, lipases and serine protease are involved in antiviral response especially against BmNPV, a DNA virus (Ponnuvel *et al*., 2003). At present, the molecular immune response existing in *B. mori* against RNA viruses has not been reported.

Among silkworm viral pathogens, cytoplasmic polyhedrovirus (CPV) and infectious flacherie virus (IFV) are RNA viruses. In CPV, the genome is a segmented double strand RNA where as IFV is a positive single strand RNA virus and both viruses do not have DNA stage in their replication cycle. The IFV belongs to the picorna virus and RNA dependent RNA polymerase (RdRp) of the virus helps viral replication that converts genomic single strand RNA into a double stranded RNA (Isawa *et al*., 1997). These two viruses possess the double stranded RNA in their genome or in the replication stage.

RNAse III is a double stranded RNA-specific endonuclease. In prokaryotes, RNAse III is important in post-transcriptional control of mRNA

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stability and translational efficiency. It is involved in the processing of ribosomal RNA precursors. Prokaryotic RNAse III also plays a role in the maturation of tRNA precursors and in the processing of phage and plasmid transcripts (Kharrat *et al.*, 1995; Nicholson 1999). While, the eukaryotic RNAse III's participate (through direct cleavage) in rRNA processing, in processing of small nucleolar RNAs (snoRNAs) and snRNAs (components of the spliceosome). In eukaryotes, RNAse III enzyme such as Dicer is involved in RNAi (RNA interference) and miRNA (micro-RNA) gene silencing (Hannon, 2002).

Eukaryotes have evolved many different systems to resist virus infection. Identification of specific virus encoded molecules or recognition of nucleic acid structures that are present only in infected cells could induce antiviral responses (Lichner *et al*., 2003). As long double-stranded (ds) RNAs do not occur in the cytoplasm of eukaryotic cells, the accumulation of ds replicative intermediates of RNA viruses, like cytoplasmic polyhedrovirus (CPV) and infectious flacherie virus (IFV) could activates antiviral responses as RNA interference (RNAi) or translation inhibition and apoptosis. RNAi is an ancient defence mechanism that degrades dsRNAs and cognate mRNAs in a sequence-specific manner (Bernstein *et al*., 2001, Zamore *et al*., 2000). Viral dsRNAs are first processed by an RNAse III-like nuclease (Dicer) into 21–26 nt dsRNAs (siRNAs) that guide another nuclease complex (RISC) to cleave homologous single-stranded (ss) viral RNAs (Ding, 2000). siRNAs also serve as guides for an RNA-dependent RNA polymerase to transform the target ssRNA into dsRNA (Fire *et al*., 1998; Vance and Vauchert, 2001). RNAi was shown to act as an efficient antiviral system in plant and insect cells (Adelman *et al*., 2001; Li *et al*., 2002) and might also play an antiviral role in mammalian cells. Cell-autonomous RNAi generates an unidentified mobile signal, thereby directing sequence-specific RNA degradation in distant tissues (Baulcombe, 1999). To inhibit the antiviral effect of RNAi, plant (Anandalakshmi et al, 1998) and insect (Li *et al*., 2002) viruses express different RNAi suppressor proteins. The above findings clearly indicate existence of host virus interaction and sequence specific antiviral defence mechanism against RNA viruses in eukaryotic organisms. Numerous experiments have already proved RNAi activity in silkworm *B. mori*, whereas the characterization and sequence analysis of *dicer* gene is yet to be studied. The present paper describes identification and genomic organization of a putative RNAse III (*dicer*) gene in silkworm *B. mori*.

Materials and Methods

Silkworm strains selected

Two multivoltine silkworms namely, pure Mysore and Daizo were selected in the present work. These two strains are polyvoltine breeds and showed tolerant response to different silkworm diseases.

Identification of dicer gene homolog

In *Drosophila* the *dicer* gene was already identified and their domains were well predicted (Lee *et al*., 2004). The *dicer* gene sequence was blast (tblastn) searched with *B. mori* genomic DNA database for identification homologous sequence of *dicer* gene. The genomic DNA sequence showing homologous sequence to *Drosophila* RNAse III gene was identified and subsequently translated to determine putative amino acid sequence. The amino acid sequence was further analysed through conserved domain search for the presence of the RNAse III domain in Dicer protein.

Selection of primers

The up and down gene specific primers were designed for all three RNAse III domains existing in the *B. mori* genomic contig using the software programme of primer3 (http://frodo.wi.mit.edu/cgibin/primer3). Based on the software programme the primer binding site and the PCR product size were determined. The three different regions located in the genomic contig were amplified by PCR using genomic DNA as template. The forward primer in RNAse-region I was (5'-TGTTTGAAGGTTGGGACAGC–3') and reverse primer was (5'-CGAAAATAGGAC ACGCGAAA–3'). Similarly in RNAse III, the region II forward primer used was (5'-GGAGAAAGCACGGT TTGATAA-3') and reverse primer was (5'-CGCG TTCAAACAAACAAACT-3'), while in region III forward primer was (5'-CGAACTGTTGATGCTAAG ACCA) and reverse primer was (5'-CCTCAGCGC GTAACAGTACA-3'). The PCR products were then cloned into the TA cloning vector with M13 primer sequence in both 5' and 3' ends.

PCR and analysis of amplified product

The genomic DNA was isolated from silk moths using standard protocols and used as template DNA in PCR reactions (Nagaraja and Nagaraju 1995). The reaction was done in an MJ research thermal cycler, PTC200, using 20 μl reaction mixture containing 50-100 ng of genomic DNA as template, 2.0 μl of 10xPCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 66 ng of forward and reverse primer each and 0.3 U of *Taq* DNA polymerase (MBI Fermentas). The PCR schedule was 94 °C for 3min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min and a final extension of 7 min at 72 °C.

The PCR products were resolved on 1.5 % agarose gel in Tris-Acetic acid/EDTA buffer (1xTAE) and electrophoresis was carried out with a constant voltage of 80 V in parallel with molecular weight markers. Gel was stained with ethidium bromide (0.5 μg/ml) and photographed with gel documentation.

DNA sequencing

The PCR amplified products were purified through Gel-spin column (Bangalore Genei). In sequencing reaction, the gene specific positive sense primer was used.

Table 1 RNAse III (*dicer*) gene biological significance

Target Genes	Nature of protein	Biological activity	Expressed in	Contig GenBank accession n°
dicer	RNAse III	Development, genome organization, viral and transposon defense	Ubiquitous	AADK01001038

Results and Discussion

In invertebrates, receptors of the innate immune system recognize pathogen-associated molecular patterns in order to activate early defense mechanisms (Hoffmann, 2003). In insects, innate immune recognition plays an important role in clearing the majority of invading pathogens through activation of the Toll and immune deficiency (Imd) pathways. Although the *B. mori* immune system has been well studied in the context of antibacterial response, there is no information at the cellular or molecular level regarding the immune response directed against RNA viruses. Keene *et al.* (2004) hypothesized that RNAi may also act as an antagonist to alphavirus replication in *Anopheles gambiae* because RNA viruses form dsRNA during replication and they proved that RNAi is a mechanism to protect mosquitoes from viral infection. Silencing *AgAgo2* expression would make *A. gambiae* mosquitoes more permissive to virus infection. The findings gave direct evidence that RNAi is an antagonist of O'nyong-nyong virus replication in *A. gambiae*.

In India, flacherie disease of the silkworm *B. mori* is a major factor causing serious loss in cocoon production to sericulture farmers. Based on pathological symptoms, the causative agent of this disease is an infectious flacherie virus (IFV), which is an insect picorna virus (Isawa *et al*., 1997). The target of IFV is the goblet cells of the midgut epithelium, and the virus multiplies in the cytoplasm. The complexity of the biological properties of dsRNA *in vivo* became more apparent with the discovery of dsRNA mediated post-transcriptional gene silencing (PTGS or RNA interference [RNAi]). The phenomenon has been described in both plants and animals, including nematodes, insects, and mammals (Hannon, 2002). The sequence-specific effects of dsRNA that result in endogenous RNA degradation are widely conserved and probably present in most invertebrates including *B. mori*. Wang *et al* (2006) selected *Drosophila* model for studying the innate immunity against RNA viruses in animals. They demonstrated that a RNA interference pathway protects adult flies from infection by two evolutionarily diverse viruses. Their findings describes a molecular framework for the viral immunity, in which viral double stranded RNA produced during infection acts as the pathogen trigger, whereas *Drosophila* Dicer-2 and Argonaute-2 act as host sensor and effector respectively (Lee *et al*., 2004). Our paper reports that *B. mori* genome possess RNAse III (*dicer*) gene sequence and has maximum homology to *Drosophila dicer*. Further, the RNAse III domain of *B. mori* is conserved from plant to animal and it indicates that antiviral immunity in insects shares some of the molecular features of vertebrate antiviral responses.

Initially the *Drosophila* sequence was compared with genomic DNA database of silkworm *B. mori*. It was found that a single genomic contig (GenBank accession n° AADK01001038) possessing the *dicer* gene homologous sequence (Table 1). Further, the genomic contig was analyzed for the presence of RNAse III domain using conserved domain search. Using the program, distinct functional and structural unit of the translated protein was identified (http://www.ncbi.nlm.nih.gov/Structure/cdd/Wrpsb/cgi). The protein query sequence submitted to NCBI's protein BLAST search service was scanned for conserved domain signatures and results showed

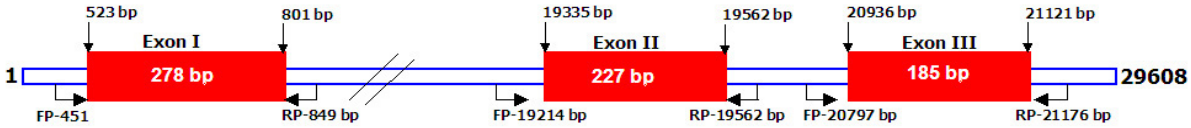

Fig. 1 Putative exons and introns of RNAse III gene present in silkworm genome (genomic contig accession no: AADK01001038). The arrow mark indicate forward and reverse primer binding site

Table 2 Details of genomic contig and amplicon size of *dicer* gene in silkworm *Bombyx mori*

Target Genes	Primer Sequence (5'-3')	Primer binding location in genomic contig.	Amplicon Size	RNAse III domain region	Corresponding DNA contig accession number
RNAse-Region I	Forward Primer TGTTTGAAGGTTGGGACAGC	451 bp	398 bp	523 bp to 801 bp	EF117689
	Reverse Primer CGAAAATAGGACACGCGAAA	859 bp			
RNAse-Region II	Forward Primer GGAGAAAGCACGGTTTGATAA	19214 bp	400 bp	19335 bp to 19562 bp	EF117690
	Reverse Primer CGCGTTCAAACAAACAAACT	19613 bp			
RNAse-Region III	Forward Primer CGAACTGTTGATGCTAAGACCA	20797 bp	358 bp	20936 bp to 21121 bp	EF117691
	Reverse Primer CCTCAGCGCGTAACAGTACA	21176 bp			

the RNAse III domain was present in the translated protein sequence. It was found that RNAse III domain was present in the contig in the three different regions, which were designated as region I, region II and region III. The first region was present from 523 bp to 801 bp whereas other two regions were at 19335 bp to 19562 bp and 20936 bp to 21121 bp (Fig. 1, Table 2). We believe that all three domains are partial and are scattered in the different exons and after splicing, it may form a Dicer enzyme with single domain of RNAse III. In order to confirm the splicing the all three domains were joined together and analyzed for the conserved domain search and it was found that it formed a single protein with RNAse III domain (Fig. 2).

Further, the primers were designed for the putative RNAse III regions based on the sequence available in the all three regions present in the single contig (GenBank accession n° AADK01001038). In order to amplify RNAse III domain present in the genomic contig, the PCR was performed with three sets of primers, which were binding site at region I (451F – 859R), region II (19214F – 19613R) and region III (20797F – 21176R) (Fig. 1). The primers designed in region I yield the PCR product of 398 bp, region II amplified PCR product size is 400 bp, while region III PCR product showed molecular weight of 358 bp (Table 2). The first region was amplified in pure Mysore strain, while the remaining two regions were not amplified due to lack of primer binding sequence in pure Mysore. This is due to the fact that genomic DNA database was derived from the silkworm strain of Daizo and pure Mysore strain sequence may not have homologous sequence with Daizo. Hence, the genomic DNA of Daizo was used as template and as a result all three regions were amplified in silkworm Daizo strain (Fig. 3). This finding indicates that the identified RNAse III is located in specified manner in genomic DNA, presumably in a single chromosome. Uhlirova *et al*. (2003) used a recombinant Sindbis virus as a tool to silence BR-C expression in the silkmoth *Bombyx mori*. The virus expressing a *BR-C* antisense RNA fragment reduced endogenous *BR-C* mRNA levels in infected tissues (adult wing and leg primordia) via RNA interference (RNAi). Their findings support that the *B. mori* also have the RNAse III enzyme which degrade double stranded target RNA in a sequence specific manner.

The amplified PCR fragment was further sequenced to confirm the presence of RNAse III domains. The sequence was translated and also analyzed for the presence of RNAse III domain in the translated region. It clearly indicates that RNAse III domains exist in the genome of silkworm strains of pure Mysore and Daizo. The putative amino acid sequence was further analysed for phylogenetic relationship with other eukaryotic organisms. The RNA silencing is RNA guided gene regulatory mechanism that include post transcriptional gene silencing (PGTS) and it is conserved from fission in yeast, plants to animals (Ding *et al*., 2004). Phylogenetic analysis was performed using CLUSTALW and MEGA3 programmes and results showed that the *Bombyx* RNAse III is strongly aligned with RNAse III sequences of insects, especially in *Tribolium castaneum*, a red flour beetle (Bucher *et al*., 2002). Robaino *et al.* (2004) demonstrated that in invertebrate immune system,

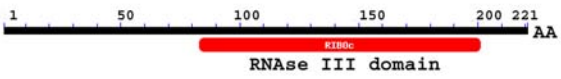

Fig. 2 PCR products amplified in all three regions were joined together, analyzed for the conserved domain in translated amino acid sequence and formed a single protein with RNAse III domain of *Bombyx mori*

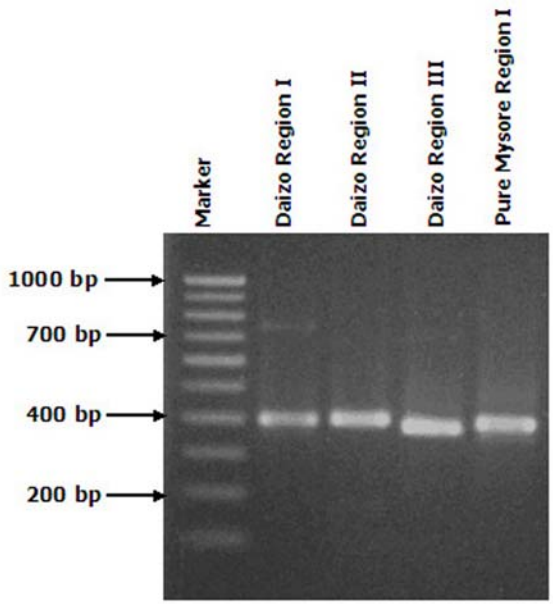

Fig. 3 PCR amplification of RNAse III gene in silkworm *Bombyx mori*

like its vertebrate counterparts, could recognize dsRNA as a virus-associated molecular pattern, resulting in the activation of an innate antiviral response. Interestingly, all the vertebrate RNAse III formed a major group and plant RNAse III gene is more close to bacterial RNAse III.

Down regulation of *dicer* makes insects vulnerable to get viral infection (Wang *et al*., 2006). All these findings clearly indicate that Dicer enzymes are involved in disease resistant mechanism of insects, especially against RNA viruses. Silkworms are also known to get infection from RNA viruses like CPV and IFV and it is reasonable to expect RNAi mediated disease resistant mechanism may also prevail in *B. mori.* In future studies, the disease resistant mechanism of *B. mori* against the RNA viruses will be deciphered by designing dsRNA against *B. mori dicer* and other proteins involved in RISC complex.

Nucleotide sequence accession number

The nucleotide sequences of RNAse III regions have been deposited with NCBI, DDBJ and EMBL libraries under accession n° EF117689, EF117690 and EF117691.

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References

Adelman ZN, Blair CD, Carlson JO, Beaty BJ, Olson KE. Sindbis virus induced silencing of dengue viruses in mosquitoes. Insect Mol. Biol. 10: 265-273, 2001.

Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, *et al*. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA 95: 13079-13084, 1998.

Baulcombe D. Viruses and gene silencing in plants. Arch. Virol. Suppl. 15: 189- 201,1999.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363-366, 2001.

Bucher G, Scholten J, Klingler M. Parental RNAi in *Tribolium* (Coleoptera). Curr. Biol. 12: 85-86, 2002.

Ding S W. RNA silencing. Curr. Opin. Biotechnol. 1: 152-156, 2000.

Ding SW, Li H, Lu R, Li F, Li WX. RNA silencing: a conserved antiviral immunity of plants and animals. Science 102: 109-115, 2004.

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811, 1998.

Hannon GJ, RNA interference. Nature, 418: 224-251, 2002.

Hoffmann JA. The immune response of *Drosophila*. Nature 426: 33-38, 2003.

Isawa H, Asano S, Sahara K, Iizuka T, Bando H. Analysis of genetic information of an insect picorna-like virus, infectious flacherie virus of silkworm: evidence for evolutionary relationships among insect, mammalian and plant picorna (-like) viruses. Arch. Virol.143: 127-143, 1997.

Keene KM, Foy BD, Vargas IS, Beaty BJ, Blair CD, Olson KE. RNA interference acts as a natural antiviral response to O' nyong-nyong virus infection of *Anopheles gambiae*. Proc. Natl. Acad. Sci. 101: 17240-17245, 2004.

Kharrat A, Macias MJ, Gibson TJ, Nilges M, Pastore A. Structure of the dsRNA binding domain of *E. coli* RNase III. EMBO J. 14: 3572-3584 1995

Lee YS, Nakahara K, Pham JW, Kim K, Zhengying HE, Sontheimer EJ, *et al*. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117: 69-81, 2004.

Li HW, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. Science 296: 1319-1321, 2002.

Lichner Z, Daniel S, Burgyan J. Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences. J. Gen. Virol. 84: 975-980, 2003.

Nagaraja GM, Nagaraju J. Genome fingerprinting of the silkworm *Bombyx mori* using random arbitrary primers. Electrophoresis 16: 1633-1638, 1995.

Nicholson AW. Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol. Rev. 23: 371-390,1999.

Ponnuvel KM, Nakazawa H, Furukawa S, Asaoka A, Ishibashi J, Tanaka H, *et al*. A lipase isolated from the Silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. J. Virol. 77: 10725-10728, 2003

Ponnuvel KM, Yamakawa M. Immune resposes against bacterial infection in *Bombyx mori* and regulation of host gene expression. Curr. Sci. 83: 447-454, 2002.

Samson MV, Baig M, Sharma SD, Balvenkatasubbaiah TO, Jolly MS. Survey on the relative incidence of silkworm diseases in Karnataka. Indian J. Sericult. 29: 248-254, 1990.

Subbarao G, Chandra AK, Bhattacharya J. Incidence of crop loss from adopted rearers level in West Bengal due to silkworm diseases. Indian J. Sericult. 34: 167-170, 1991.

Robaino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, *et al*. Induction of antiviral immunity by double-stranded RNAi in a marine invertebrate. J. Gen. Virol. 78: 10442-10448, 2004.

Uhlirova M, Foy BD, Beaty BJ, Olson KE, Riddiford LM, Jindra M. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of broad-complex in insect metamorphosis. Proc. Natl. Acad. Sci, USA 100: 15607-15612, 2003.

Vance V, Vaucheret H. RNA silencing in plants - defense and counter defense. Science 292: 2277-2280, 2001.

Wang XH, Aliyari R, Li WX, LI HW, Kim K, Carthew R, *et al*. RNA interference directs innate immunity against viruses in adult *Drosophila*. Science 312: 452-454, 2006.

Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101: 25-33, 2000.