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 antiviral 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.
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 rRNA 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 (snRNA)s 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 RNAs in the cytoplasm and the presence of RNA in the cytoplasm as a result of the virus replication is an indication of the existence of host virus interaction and sequence specific virus recognition system. RNAi is a specific antiviral defence mechanism against RNA viruses in eukaryotic organisms (Adelman et al., 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 (Anandakshmi 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/cgi-bin/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-I region I was (5’-TGGTTGAAAGGTGGGACACG-3’) and reverse primer was (5’-CGAAATAGGAGCGGCAA-3’). Similarly in RNase-II, the region II forward primer used was (5’-GGAGAAAGCACGGTGTTGATAA-3’) and reverse primer was (5’-CGCGGTTCAAACAAACACA-3’). Between region II and III forward primer was used was (5’-GGAGAAAGCACGGGTGTTGATAA-3’) and reverse primer was (5’-CGCGGTTCAAACAAACACA-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 MgCl2, 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 3 min 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.
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

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Nature of protein</th>
<th>Biological activity</th>
<th>Expressed in</th>
<th>Contig GenBank accession n°</th>
</tr>
</thead>
<tbody>
<tr>
<td>dicer</td>
<td>RNAse III</td>
<td>Development, genome organization, viral and transposon defense</td>
<td>Ubiquitous</td>
<td>AADK01001038</td>
</tr>
</tbody>
</table>

![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](image-url)
Table 2 Details of genomic contig and amplicon size of dicer gene in silkworm Bombyx mori

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Primer Sequence (5'-3')</th>
<th>Primer binding location in genomic contig.</th>
<th>Amplicon Size</th>
<th>RNase III domain region</th>
<th>Corresponding DNA contig accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAse-Region I</td>
<td>Forward Primer TGGTTGAGGTTGGGACAGC</td>
<td>451 bp</td>
<td>398 bp</td>
<td>523 bp to 801 bp</td>
<td>EF117689</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer CGAAATAGGACACCGCAGAA</td>
<td>859 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAse-Region II</td>
<td>Forward Primer GGAGAAACGGATGTGATTAA</td>
<td>19214 bp</td>
<td>400 bp</td>
<td>19335 bp to 19562 bp</td>
<td>EF117690</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer CGCGTTCAAAACAAACTAACT</td>
<td>19613 bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNAse-Region III</td>
<td>Forward Primer CGAATCCTGTTGATGTGTAAGCCA</td>
<td>20797 bp</td>
<td>358 bp</td>
<td>20936 bp to 21121 bp</td>
<td>EF117691</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer CCTAAGCCGTAAACAGTACA</td>
<td>21176 bp</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

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. Uhlirrova 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,
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


