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Research paper

dsRNA interference on expression of a RNA-dependent RNA polymerase gene of *Bombyx mori* cytoplasmic polyhedrosis virus

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ABSTRACT

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) is one of the major viral pathogens in silkworm. Its infection often results in significant losses to sericulture. Studies have demonstrated that RNAi is one of the important anti-viral mechanisms in organisms. In this study, three dsRNAs targeting the RNA-dependent RNA polymerase (RDRP) gene of BmCPV were designed and synthesized with 2′-F modification to explore their interference effects on BmCPV replication in silkworm larvae. The results showed that injecting dsRNA in the dosage of 4–6 ng per mg body weight into the 5th instar larvae can interfere with the BmCPV-RDRP expression by 93% after virus infection and by 99.9% before virus infection. In addition, the expression of two viral structural protein genes (genome RNA segments 1 and 5) was also decreased with the decrease of RDRP expression, suggesting that RNAi interference of BmCPV-RDRP expression could affect viral replication. The study provides an effective method for investigating virus replication as well as the virus–host interactions in the silkworm larvae using dsRNA.

1. Introduction

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) disease is a chronic disease greatly damaging sericulture production. It is caused by BmCPV, an RNA-containing polyhedrosis virus invading silkworm larvae per os and infecting cylindrical cells of midgut epithelium. The viruses could form polyhedra in the cytoplasm of the cylindrical cells, leading to cell rupture. Viral polyhedra were then excreted through the feces, infecting other healthy individuals and leading to a serious infection of silkworm larvae in the rearing bed. Because the normal digestive function is affected by BmCPV infection, silkworm larvae die one after another, leading to reduced cocoon yields and seriously threatening sericulture production. Currently, there is no specific therapeutic agent for BmCPV infection. The diseases are mainly prevented and controlled by the disinfection of rearing rooms and appliances before and during silkworm rearing, and the disinfection after cocoon harvest The diseased individuals can only be eliminated without other effective means (Jin et al., 2001).

BmCPV genome is composed of 10 equimolar dsRNA segments, with a total molecular weight of 20.3×10^6 Da. Studies suggested that its encoded structural proteins are mainly present in the viral particles to form the complete virions while its encoded non-structural proteins is not present in the viral particles and only exert their functions in viral replication, assembly and other processes. The whole BmCPV genome sequence has been determined and published with presumed functions of each segment (Hagiwara and Matsumoto, 2000; Hagiwara et al., 2001, 2002; Cao et al., 2012). BmCPV RNA- dependent RNA polymerase (BmCPV-RDRP) gene is located in the 2nd segment of BmCPV genome. Under normal circumstances, it plays a very important role (Velkov et al., 2014) in viral transcription and replication processes. On the one hand, it could replicate viral genes using viral RNA as a template. On the other hand, it is required to transcript genes of proteins and enzymes needed for viral replication into mRNA. In other words, BmCPV-RDRP functions as both replicase and transcriptase during the viral replication process.

RNAi is a natural and ancient antiviral mechanism of organisms (Hutvágner et al., 2001). The application of RNAi in antiviral researches has been very thorough (Hutvágner and Zamore, 2002). Researches in human diseases induced by viruses such as hepatitis B virus (Tellinghuisen and Spiess, 2014), human immunodeficiency virus (HIV) and herpes virus (Wheeler, 2014) and in animal diseases caused by Canine parvovirus (He et al., 2012) and Newcastle disease virus

Abbreviations: BmCPV, *Bombyx mori* cytoplasmic polyhedrosis virus; RNA, ribonucleic acid; RNAi, RNA interference; dsRNA, double-stranded RNA; siRNA, small interfering RNA; RDRP, RNA-dependent RNA polymerase; BmCPV-RDRP, *Bombyx mori* cytoplasmic polyhedrosis virus RNA-dependent RNA polymerase; HIV, human immunodeficiency virus; NPV, nuclear polyhedrosis virus; DEPC, diethylpyrocarbonate; OD, optical density; PCR, polymerase chain reaction; RT-qPCR, quantitative reverse transcription polymerase chain reaction; HCV, hepatitis C virus; UTR, untranslated region.

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(Yin et al., 2010) have shown that RNAi could significantly inhibit virus replication. Studies on silkworm anti-viral RNAi are focused on the screening of effective dsRNAs to inhibit *ie*-1 gene necessary to the replication of nuclear polyhedrosis virus (NPV) and obtaining NPV-resistant silkworm varieties through transgenic technology (Yang et al., 2008).

RNAi has been successfully applied in lepidoptera (Terenius et al., 2011) and achieved great progress in the studies on gene functions in the egg, larval, and pupal stages. Micro-injection, feeding and body cavity injection are commonly used techniques. Researches on larvae have found that non-invasive body cavity injection method is advantageous over feeding (Price and Gatehouse, 2008), indicating that the body cavity injection of dsRNA is suitable for more applications. Due to the lack of sensitive cell lines for BmCPV, the interfering effects of its dsRNA are unable to be studied at the cell level.

BmCPV-RDRP gene is located in the 2nd segment and has full-length of 3854 bp (GenBank sequence number GQ924586.1). It encodes an active enzyme with a key role in the process of virus replication (Rao et al., 2003). The purpose of this study is using synthetic dsRNAs targeting to BmCPV-RDRP and body cavity injection method to inhibit BmCPV replication with the hope to achieve a methodological breakthrough for studies on BmCPV.

2. Materials and methods

2.1. Silkworm strain

Domestic silkworm strain Qing-Song × Hao-Yue (Provided by *Sericultural Research Institute, Chinese Academy of Agricultural Sciences*) was used in this study. They were reared at standard temperature under a photoperiod of 12 h of light and 12 h of dark up to the fourth molting.

2.2. dsRNA design and synthesis

Three dsRNAs (Table 1) were designed using the online tool (http://bioinfo.clontech.com/rnaidesigner/sirnaSequenceDesignInit.do) based on BmCPV-RDRP gene nucleotide sequence (http://www.ncbi.nlm.gov.cn) targeting respectively to three different regions as shown in Fig. 1 and synthesized by Shanghai Gemma Biological Pharmaceutical Co., Ltd. with 2′-F modification (Pallan et al., 2011; Chen et al., 2012).

2.3. Virus inoculation, dsRNA injection and midgut collection

BmCPV inoculation was performed as previously reported (Wu et al., 2011). In brief, BmCPV polyhedra were prepared in sterile water as 1×10^8/mL suspension. 160 μL of the virus suspension was spread to each round mulberry leaf pieces with an area of 3 cm^2. After slightly dried, the virus-containing leaves were used to feed two 5th instar larvae. The dose of infection was calculated as 8×10^6 polyhedra per larva. The control uninfected larvae were fed with the same amount of mulberry leaves with sterilized water spread on them.

dsRNA solution was prepared as reported previously (Brass et al., 2010). In brief, about 167.5 ng dsRNA (about 5 OD) was dissolved in 25 μL diethylpyrocarbonate (DEPC) water and mixed with 25 μL DEPC water containing 1 μL liposome (2% liposome solution) to prepare as about 3000 ng/μL dsRNA-liposome solution. After incubated at room temperature for 20 min, 2 μL of the mixture (equivalents of 4000–6000 ng dsRNA) was subcutaneously injected into each silkworm larva from its intersegmental membrane using a 10 μL micro-injector as reported previously (Hamamoto et al., 2011), in which the injector needle was sustained in the silkworm body for 3 s and then pulled out. The control silkworm was injected with similar mixture containing no dsRNA.

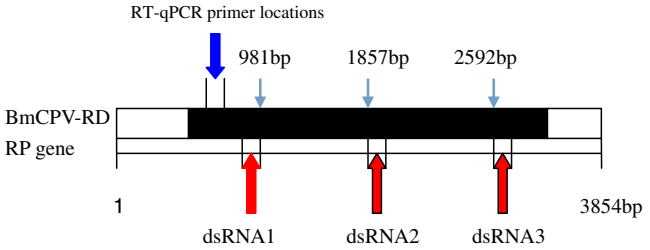

Fig. 1. Schematic representation of BmCPV-RDRP gene, showing dsRNA target sites and primer pairs. Red arrows indicate dsRNA target sites and blue arrows indicate PCR primers.

The midguts of both BmCPV-infected and control larvae were collected on ice and quickly rinsed in 0.9% DEPC-treated NaC1 to remove the attached leaf pieces before being stored in liquid nitrogen. The midguts of 5 larvae were pooled as a sample for RNA extraction and following experiments. All the experiments were carried out in three biological repetitions.

2.4. Viral RNA extraction

Total RNA was isolated from the midguts of CPV infected and control larvae using the Viral RNA extraction kit (Purchased from Beijing BLKW Biotechnology Co., Ltd) according to the manufacturer's protocol. Following purification, total RNA were quantified using a spectrophotometer and stored at -80 °C.

2.5. Detection of RNA interference effects

2.5.1. Features of RDRP gene expression in silkworm larvae infected with BmCPV

The 5th instar larvae were assigned into virus-infected and non-infected groups with 30 individuals in each group. Silkworms in the virus-infected group were fed with leaves spread with about 8×10^5 polyhedra of BmCPV. The larvae in both infected and non-infected groups were sampled at 5 h, 12 h, 24 h, 48 h, 72 h and 96 h post-infection by randomly picking up five silkworms separately from each group. The midguts of the larvae at each time point were anatomically collected and pooled into one sample, then stored at −80 °C for extracting total RNA followed by RT-qPCR.

2.5.2. Examination of the interference effects of dsRNA on the expression of BmCPV-RDRP gene

The interference effects of dsRNA on the expression of BmCPV-RDRP gene were examined on the two aspects: prevention and therapeutics. The prevention experiment was performed referring to the method previously reported by Maffioli (Maffioli et al., 2012) and Novina (Novina et al., 2002). In brief, 25 of 5th instar larvae in each group were injected with dsRNA and then fed with BmCPV-containing leaves.

Table 1
Sequences of dsRNAs.

Name	Target	Passenger strand (5′-3′)	Guide strand (5′-3′)
dsRNA1	RDRP-981	GCGAGCGGAACUAUUAUAUTT	AUAUAAUAGUUCCGCUCGCTT
dsRNA2	RDRP-1857	GCGUCAGUCACAGGUUAAATT	UUUAACCUGUGACUGACGCTT
dsRNA3	RDRP-2592	GCCAAGAGGAGGAGAAUAUTT	AUAUUCUCCUCCUCUUGGCTT
R-control	control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT