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Cloning, characterization, and expression analysis of a novel *BmGDAP1* gene from silkworm, *Bombyx mori*, involved in cytoplasmic polyhedrosis virus infection

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ABSTRACT

A novel ganglioside-induced differentiation-associated protein 1 gene (*BmGDAP1*) was first cloned and sequenced from silkworm, *Bombyx mori* using rapid amplification of cDNA ends (RACE). The full-length cDNA of *BmGDAP1* was 1514 bp, consisting of a 91 bp 5′ untranslated region (UTR), a 424 bp 3′-UTR and a 999 bp open reading frame (ORF). The ORF encoded a polypeptide of 332 amino acids, which possessed a thioredoxin (TRX)-like domain, a glutathione S-transferase-C (GST-C) family domain and a transmembrane segment. Furthermore, quantitative real-time PCR analysis revealed that *BmGDAP1* transcripts were mainly presented in the tissues of hemocytes and midgut of silkworm, and its expression level was down-regulated in the hemocytes, while up-regulated in the midgut. Therefore, it could be concluded that *BmGDAP1* plays an important role in the recognition and immune response of silkworm to BmCPV infection.

1. Introduction

Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) belongs to the genus *Cypovirus*, family Reoviridae (Hill et al., 1999), and is one of the major viral pathogens causing serious disease to silkworm and enormous damage to the sericultural industry. However, the recognition mechanism and immune response of silkworm to BmCPV infection have not been understood. In order to obtain an overall view on the molecular mechanism of BmCPV infection, a microarray system comprising 22,987 oligonucleotide 70-mer probes has been employed in a previous study in our laboratory (Wu et al., 2010). Based on the results from the microarray system, a novel ganglioside-induced differentiation-associated protein 1 gene (*BmGDAP1*) was first cloned and sequenced from silkworm, *B. mori* using rapid amplification of cDNA ends (RACE) in this study.

GDAP1 is a 358 amino acid protein, which was first identified in human (Baxter et al., 2002; Cuesta et al., 2002; Liu et al., 1999). The gene was first located on chromosome 8q21.1 (CMT4A) in Tunisian families and had a length of 23,728 bp which contains six exons and five introns (Ben Othmane et al., 1993). It was originally identified as a highly expressed gene at the differentiated stage of GD3 synthase-transfected cells (Liu et al., 1999). Subsequently, GDAP1 and related proteins were extensively studied in invertebrates and vertebrates (Marco et al., 2004). All these proteins contained characteristic domains of thioredoxin (TRX)-like domain and glutathione S-transferase-C (GST-C) family domain (http://www.ncbi.nlm.nih.gov/homologene/40713), which were linked with α4–α5 loops. Moreover, there were one or two hydrophobic stretches with potential transmembrane features at the COOH-terminal (Marco et al., 2004; Wagner et al., 2009) which is unlike cytosolic glutathione S-transferase (GST) family. In addition, GDAP1 belonged to a new subfamily of GSTs via phylogenetic and structural analysis (Cuesta et al., 2002; Marco et al., 2004). It is mainly expressed in neuronal cells and is localized in the mitochondria through its transmembrane domains but does not exhibit GST activity using standard substrates (Pedrola et al., 2005; Shield et al., 2006). More recently, mutations in GDAP1 have been reported to cause both axonal and demyelinating autosomal-recessive Charcot–Marie–Tooth (CMT) which is characterized by slow and progressive weakness and atrophy of muscles (Ammar et al., 2003; Baxter et al., 2002; Nelis et al., 2002; Nicholson and Ouvrier, 2002).

In this study, a full length cDNA of *BmGDAP1* was first cloned and sequenced from silkworm, *B. mori* on the basis of a 618 bp expressed sequence tag (EST) sequence using the RACE method. The domain, structure and evolutionary relationship of BmGDAP1 were analyzed by biology software. In addition, for better understanding the function

Abbreviations: *BmGDAP1*, Ganglioside-induced differentiation-associated protein 1 gene from *Bombyx mori*; RACE, rapid amplification of cDNA ends; ORF, open reading frame; BmCPV, *Bombyx mori* cytoplasmic polyhedrosis virus; TRX, thioredoxin; GST-C, glutathione S-transferase-C; HD1, hydrophobic domain 1; TM, transmembrane domain; EST, expressed sequence tag; qRT-PCR, quantitative real-time polymerase chain reaction; DEPC, diethylpyrocarbonate; UTR, untranslated region; GDAP1L1, ganglioside-induced differentiation associated protein 1-like 1.

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of *BmGDAP1*, quantitative real-time polymerase chain reaction (qRT-PCR) was used to study its presence and relative expression level in different silkworm tissues. All the results showed that *BmGDAP1* plays an important role in the recognition and immune response of silkworm to BmCPV infection.

2. Materials and methods

2.1. Silkworm strain

The silkworm strain *P50* used in the current study was provided by the National Center for Silkworm Genetic Resources Preservation of the Chinese Academy of Agricultural Sciences. The larvae were reared at standard temperature of 25 °C and under a photoperiod of 12 h of light and 12 h of dark up to the fourth molting for virus inoculation.

2.2. Virus inoculation

BmCPV was suspended in distilled water to a concentration of 10^7 polyhedra per mL. One milliliter of viral suspension was spread evenly on 10 pieces of mulberry leaves of approximately 15 cm^2 each in size, which were fed to 25 newly molted fifth instar larvae of silkworm. The infection dose was calculated as 4×10^5 polyhedra per larva. On the other hand, the control larvae were fed with the same quantity of leaves treated with distilled water. Then, fresh mulberry leaves were used to feed to the inoculated and control larvae after the BmCPV-inoculated leaves were consumed (about 2 h).

2.3. Tissues collection

Tissues of both the BmCPV-inoculated and control larvae were excised by dissecting the larvae on ice. The isolated tissues were quickly washed with diethylpyrocarbonate (DEPC)-treated water to remove attached leaf pieces and then immediately frozen in liquid nitrogen. The hemocytes from five silkworms were pooled together as one sample, added to antioxidant phenylthiourea and immediately centrifuged at $1000\times g$ at 4 °C for 10 min to harvest the hemocytes. The samples were immediately stored at −80 °C after adding 1 mL of Trizol reagent ((Takara, Japan) for RNA extraction.

2.4. Cloning and sequencing of BmGDAP1

Total RNAs from silkworm midgut were extracted with Trizol reagent (Takara), and polyA$^+$ RNAs were purified using the PolyA Tract mRNA Isolation System II (Promega) according to the manufacturer's instructions. Full-length cDNA sequence of *BmGDAP1* was obtained using SMART™ RACE cDNA Amplification Kit (Clontech) and RT-PCR. The primers for the special 5′RACE and 3′RACE were designed according to the EST sequence of sw12355 (http://www.silkdb.org/microarray/search.php). PCR amplification was performed according to the following procedure: denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min, and then a final extension at 72 °C for 7 min. The DNA products were gel-purified and cloned into a pMD19-T simple vector (Takara, Japan). After being transformed into the competent cells of *Escherichia coli* Top10, the positive recombinants were identified via anti-Amp selection and PCR screening using the M13− and M13+ primers (Table S1). Three of the positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

The sequences were searched in GenBank using BLASTx for comparative analysis and assembled with the obtained fragments. To exclude the possibility that the composed sequence might be a misassembled artifact, we designed a pair of primers P3 and P4 designed according to the composed sequence (Table S1) and used it in PCR to obtain the full length of *BmGDAP1*. The PCR amplification was performed as follows: denaturation at 94 °C for 4 min, followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, and one final cycle of 72 °C for 7 min. And then DNA products were gel-purified, sub-cloned and sequenced as described above.

2.5. Sequence analysis, multiple sequence alignment and phylogenetic analysis

The sequences were searched in GenBank with BLASTx for comparative analysis and assembled with the obtained fragments. Furthermore, sequences were analyzed using the BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast), and the deduced amino acid sequence of BmGDAP1 was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and SMART program (http://smart.embl-heidelberg.de/). The signal peptide and the potential Asn-linked glycosylation sites were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc), respectively. Multiple protein sequences were aligned using the MegAlign program via the CLUSTAL W method in the DNASTAR software package. The phylogenetic tree was constructed using the neighbor-joining method in the MAGE 5 software package (Tamura et al., 2011). To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.6. Expression analysis of BmGDAP1

The presence and expression level of *BmGDAP1* in different tissues of silkworm were detected using the qRT-PCR method. Total RNA was isolated from hemocytes collected above, and from the midgut, testicle, vasa mucosa, head, silk gland, ovary, fat body and spiracle from five unchallenged silkworms with Trizol reagent (Takara, Japan). The RNA samples were quantified by measuring the absorbance at 260 nm. A 10 μL reaction volume containing 1 μL of DNase reaction buffer, 2 μg of each RNA sample and 1 μL of RQ1 DNase I (Promega) was incubated at 37 °C for 30 min. After inactivating the DNase at 65 °C for 10 min, 10 μL of DNase digested RNA sample was used for the synthesis of the cDNA templates using Prime Script™ RT Reagent Kit (Takara, Japan). Specific primers for *BmGDAP1* and *β-actin* were designed by Primer Premier 5.0 software (Premier). The sequences of the primers are listed in Table S1.

The SYBR Green RT-PCR assay was carried out in an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems). The PCR was carried out in a total volume of 25 μL of reaction system, containing 12.5 μL of 2×SYBR Green Master Mix (Applied Biosystems), 2 μL of the diluted cDNA, 0.5 μL of each of primers (10 μmol/L) and 10 μL of DEPC-water. A 185 bp product was amplified with RTF and RTR (Table S1) from a cDNA template, and was sequenced to verify the PCR specificity. Two *β-actin* primers, AF and AR (Table S1) were used to amplify a 135 bp fragment as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. In a 96-well plate, each sample was run in triplicate along with the internal control gene. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data was analyzed with the SDS 2.0 software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative C_t method (Livak and Schmittgen, 2001) was used to study the relative expression level of *BmGDAP1*. The C_t for the amplified target products of *BmGDAP1* and internal control *β-actin* was determined for each sample. Difference in the C_t for the target and the internal control, called ΔC_t, was calculated to normalize the differences in the amount of template and the efficiency of RT-PCR. The RNA from the larvae in a blank group were used as the reference sample, called the calibrator. The ΔC_t for each sample was subtracted from the ΔC_t of the calibrator