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Antiviral activity of the inducible humoral immunity and its suppression by eleven BEN family members encoded in *Cotesia plutellae* bracovirus

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ABSTRACT

Upon parasitization by some endoparasitoids, polydnaviruses (PDVs) play a crucial role in inducing host immunosuppression. This study reports a novel immunosuppressive activity against humoral immune responses by BEN family genes encoded in *Cotesia plutellae* bracovirus (CpBV). A total of 11 BEN family members are encoded in 10 different CpBV DNA segments. When the CpBV segments were individually injected, specific BEN genes were expressed and suppressed the expression of antimicrobial peptide (AMP) and prophenoloxidase genes following bacterial challenge. The suppressive activities of the BEN genes were reversed by injection of the double-stranded RNA (dsRNA) specific to each BEN gene. The suppression of the AMP gene expressions by the BEN genes was also confirmed using an inhibition zone assay against Gram-positive and Gram-negative bacterial growth. The significance of the suppressive activity of BEN genes against humoral immune responses was analyzed in terms of suppression of antiviral activity by the host humoral immunity. When CpBV was incubated with the plasma obtained from the larvae challenged with bacteria, the immunized plasma severely impaired the expression activity of the viral genes. However, an expression of BEN gene significantly rescued the viral gene expression by suppressing humoral immune response. These results suggest that BEN family genes of CpBV play a crucial role in defending the antiviral response of the parasitized *Plutella xylostella* by inhibiting humoral immune responses.

1. Introduction

Polydnaviruses (PDVs) are a group of insect DNA viruses that are mutually associated with some endoparasitoid wasps (Webb et al., 2000). PDVs are classified into two genera, ichnovirus (IV) and bracovirus (BV), depending on the viral morphology and host wasp family (Webb and Strand, 2005). Both IV and BV have been regarded to be independently originated due to their difference in genome composition (Webb et al., 2000). An analysis of the ovarian transcripts of braconid wasps containing BVs indicates that BVs are originated from an ancestral nudivirus (Bézier et al., 2009a). However, a similar approach in ichneumonid wasp containing IV did not find any nudiviral rudiments supporting an independent origin of BV and IV (Volkoff et al., 2010).

The entire PDV genome is segmented and located on host wasp chromosome(s) in a proviral form (Stoltz, 1990; Bézier et al., 2009b). During viral replication at late pupal development, the ovarian calyx cells produce PDV particles and release them to the lateral oviduct lumen (Wyler and Lanzrein, 2003). The viral genome is divided into "encapsidated" and "non-encapsidated" parts during replication (Bézier et al., 2009b; Burke and Strand, 2012). The "non-encapsidated" proviral genes contribute to produce the viral particles by expressing capsid and assembly factors (Burke et al., 2013). In the meantime, the "encapsidated" proviral genes are mostly associated with the regulation of the parasitized host physiology (Beck et al., 2011; Strand et al., 2013).

An endoparasitoid wasp, *Cotesia plutellae*, parasitizes young larvae of the diamondback moth, *Plutella xylostella* (Bae and Kim, 2004). The parasitized larvae undergo an immunosuppressive state and exhibit a prolonged larval period by approximately two days at 25 °C (Ibrahim and Kim, 2006; Kwon et al., 2010). A specific PDV called *C. plutellae* bracovirus (CpBV) has been identified from *C. plutellae* and plays a crucial role in the parasitism (Kim et al., 2007). The encapsidated CpBV genome was sequenced and annotated to encode 157 genes (Chen et al., 2011). Almost half of the genes are grouped into different PDV canonical gene families proposed by Kroemer and Webb (2004), but the others remain hypothetical genes. Most canonical PDV gene families encoded in the encapsidated CpBV genome have been experimentally assessed in their functions with respect to host immunosuppression (Gad and Kim, 2008; Ibrahim and Kim, 2008; Kwon and Kim, 2008; Nalini et al., 2008; Bae and Kim, 2009; Park and Kim, 2012). In addition, some non-canonical (hypothetical) genes are also under strong positive selection process probably to meet the functional differentiation to defend various and specific host immune responses (Jancek et al., 2013).

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A novel PDV gene family, BEN (BANP, E_5R and NAC_1), has been proposed in the CpBV genome, which contains eleven members encoded in 10 CpBV segments (Chen et al., 2011; Ali and Kim, 2012). The transient expression of BEN family genes significantly suppressed hemocyte nodule formation in response to bacterial challenge and played a crucial role in inducing a significant suppression in cellular immunity (Ali and Kim, 2012). Furthermore, a BEN family member (CpBV-ORF301) suppresses both cellular and humoral immune responses of host insects by inhibiting specific mRNA expression probably with nuclease activity of its RNase T_2 domain (Park and Kim, 2010, 2012). The inhibition of cellular immune response helps to protect the wasp egg and larva from a fatal encapsulation behavior of host hemocytes. However, the inhibition of humoral immune response by CpBV has been poorly understood in the biological significance. In the meantime, some inducible humoral immune responses have been suggested to be associated with antiviral responses of insects (Imler and Eleftherianos, 2009). These led us to impose a hypothesis that BEN family members of CpBV inhibit host humoral immune response to suppress the host antiviral activity.

In this study, we analyzed the suppressive effect of BEN family members on expression of antimicrobial peptide (AMP) and prophenoloxidase (PPO) genes of *P. xylostella*. To test the hypothesis, changes of gene expressions of four AMPs and PPO in response to bacterial challenge were monitored after transient expression of BEN genes. Furthermore, their specific immunosuppressive activities were tested by individual RNA interference (RNAi) of BEN family members. Finally, we analyzed the antiviral responses of the induced humoral immunity on the CpBV infection to understand the physiological significance of the suppressive activity of BEN family genes.

2. Materials and methods

2.1. Insect rearing and parasitization

Larvae of *P. xylostella* were reared on cabbage leaves at 25 ± 1 °C and 16:8 (L:D) h photoperiod. Adults were fed 10% sucrose. Young larvae (2 days after hatch) of *P. xylostella* were parasitized with *C. plutellae* adults at 2:1 (host:wasp) ratio. The parasitized larvae were then allowed to feed cabbage leaves at the same conditions until the end of parasitoid larval development. Adults emerged from the cocoons (11 days after parasitization at 25 ± 1 °C) were collected and allowed to mate for 24 h. The mated adults were used for the parasitization.

2.2. Bacterial culture

Bacillus subtilis ATCC6633 and *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) bacteria were cultured on Luria-Bertani (LB) agar plates and LB liquid broth at 37 °C. After overnight culture with LB broth, the bacterial cells were collected by centrifugation at 5000 ×*g* for 10 min and were resuspended with 100 mM phosphate-buffered saline (PBS, pH 7.4) for injection. Bacteria to be injected were counted using a hemocytometer (Superior, Marienfeld, Germany) with the help of a tally counter.

2.3. cDNA preparation of P. xylostella transcripts

Total RNAs of *P. xylostella* larvae were extracted using Trizol (Invitrogen) according to manufacturer's instruction and digested with DNase (Promega, Madison, WI, USA). After the inactivation of DNase at 65 °C for 15 min, first strand cDNA was synthesized by reverse-transcription of RNAs (1 μg) using RT-Premix oligo-dT (5′-CCAGTGAGCAGAGTGACGA GGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT-3′) kit (Intron Biotechnology, Daejeon, Korea) according to the manufacturer's instruction and subsequently treated with RNase H (Promega). The synthesized cDNA was used as a template for PCR amplification. No DNA contamination was confirmed by PCRs using the RNA templates.

2.4. RT-PCR

Eleven CpBV-BEN genes, four AMP genes, PPO gene, and other CpBV genes were amplified using gene-specific primers (Table S1) under the following conditions: a pre-denaturation step (94 °C, 3 min), followed by 35 amplification cycles (denaturation at 94 °C for 1 min, annealing for 1 min at temperatures as described in Table S1, chain extension at 72 °C for 1 min) and final extension at 72 °C for 10 min. To confirm the cDNA preparation, β-actin expression was analyzed with primers (5′-ATGTACCCTGGTATTGCTCA-3′ and 5′-GGACGATAGAGGGGCCAG AC-3′) by RT-PCR.

2.5. Preparation of individual viral segments containing different CpBV-BEN genes and microinjection

BEN domain-containing DNA segments of CpBV were cloned using a plasmid capture system (Choi et al., 2005). For transient expression, each viral DNA segment was mixed with Metafectene PRO transfection reagent (Biontex, Planegg, Germany) according to manufacturer's instruction. Briefly, DNA segment (≈200 ng/μL) was mixed with the transfection reagent at 1:1 (v/v) ratio and incubated for 20 min at room temperature to allow DNA-lipid complexes to be formed before injection. Glass capillary injection needles were prepared using a Micropipette puller (PN-30, Narishige, Tokyo, Japan). The DNA-lipid complex was injected into larval hemocoel of *P. xylostella* through dorsal intersegmental membrane with 0.5 μL volume at a rate of 50 nL/s using a Ultra Micropump (Four) with SYS-microcontroller (World Precision Instruments, Sarasota, FL, USA). Microinjection was performed under a

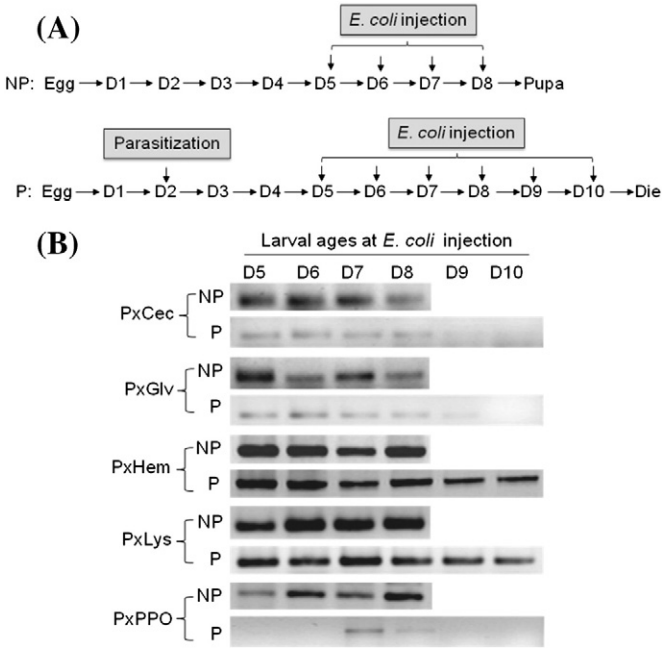

Fig. 1. Influence of the parasitism by *Cotesia plutellae* on expression of humoral factors of *Plutella xylostella* larvae. (A) Diagram of experimental trials. Nonparasitized (NP) individuals spent 8 days ('D8') for larval period, while parasitized (P) individuals spent 10 days ('D10') and died. Parasitization occurred at the second day ('D2'). All bacterial challenges began at the third instar at the fifth day ('D5'). Each bacterial challenge used 5×10^4 cells of *Escherichia coli*. (B) Expression of five humoral factors at 8 h after the bacterial challenge. The immune genes analyzed in this RT-PCR are cecropin ('PxCec'), gloverin ('PxGlv'), hemolin ('PxHem'), lysozyme ('PxLys'), and prophenoloxidase ('PxPPO'). The expressions of different periods in the parasitized larvae are individually compared with those of the corresponding periods in the nonparasitized larvae. All cDNA templates were confirmed by expression of β-actin.