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Serine proteases SP1 and SP13 mediate the melanization response of Asian corn borer, *Ostrinia furnacalis*, against entomopathogenic fungus *Beauveria bassiana*

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ABSTRACT

Exposure to entomopathogenic fungi is one approach for insect pest control. Little is known about the immune interactions between fungus and its insect host. Melanization is a prominent immune response in insects in defending against pathogens such as bacteria and fungi. Clip domain serine proteases in insect plasma have been implicated in the activation of prophenoloxidase, a key enzyme in the melanization. The relationship between host melanization and the infection by a fungus needs to be established. We report here that the injection of entomopathogenic fungus *Beauveria bassiana* induced both melanin synthesis and phenoloxidase activity in its host insect, the Asian corn borer, *Ostrinia furnacalis* (Guenée). qRT-PCR analysis showed several distinct patterns of expression of 13 clip-domain serine proteases in response to the challenge of fungi, with seven increased, two decreased, and four unchanged. Of special interest among these clip-domain serine protease genes are SP1 and SP13, the orthologs of *Manduca sexta* HP6 and PAP1 which are involved in the prophenoloxidase activation pathway. Recombinant *O. furnacalis* SP1 was found to activate proSP13 and induce the phenoloxidase activity in corn borer plasma. Additionally, SP13 was determined to directly cleave prophenoloxidase and therefore act as the prophenoloxidase activating protease. Our work thus reveals a biochemical mechanism in the melanization in corn borer associated with the challenge by *B. bassiana* injection. These insights could provide valuable information for better understanding the immune responses of Asian corn borer against *B. bassiana*.

1. Introduction

Excessive use of chemical insecticides has accumulated environmental pollution and insecticide resistance. One promising alternative for pest control is infection by entomopathogenic fungi (Fang et al., 2012). For example, *Beauveria bassiana* has been used as a biological insecticide to control a wide variety of pests including termites (Padmaja and Kaur, 2001), whiteflies (Siongers and Coosemans, 2003), aphids (Vu et al., 2007) and corn borers (Wagner and Lewis, 2000). Its use in the control of malaria-transmitting mosquitoes is also under investigation (Blanford et al., 2005). Other entomopathogenic fungus, such as *Beauveria brongniaritii*, *Isaria fumosorosea*, *Lecanicillium longisporum*, *Metarhizium anisopliae*, *Metarhizium brunneum*, and *Metarhizium flavoviride* are already commercially available (Kim et al., 2013) and successfully used to control both agriculturally harmful pests (Fang et al., 2012; Wagner and Lewis, 2000) and insect vectors which transmit infectious diseases to humans (Blanford et al., 2005; Scholte et al., 2005). Nevertheless, further development and wider adoption of these fungi as control agents may require a deeper understanding of the complex interactions between entomopathogenic fungi and their insect hosts.

Most insects are devoid of a typical adaptive immune system composing of highly specialized and systemic cells, and rely mainly on the innate immune response for defense against the infection by pathogens or parasites (Cherry and Silverman, 2006; Jiang et al., 2010; Kingsolver and Hardy, 2012). Upon bacterial or fungal challenge, insects mount multiple cellular and humoral responses including melanization, production of antimicrobial peptides, encapsulation, and nodulation (Eleftherlanos et al., 2007; Colinet et al., 2009). The injection of metabolites produced by *B. bassiana* into the hemolymph of the greater wax moth *Galleria mellonella* caused no mortality but increased antimicrobial activities and nodulations (Vilcinskas et al., 1999). The application of *B. bassiana*

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on mosquitoes *Aedes aegypti* activated anti-dengue activity and several effector genes, which are controlled through the Toll and JAK-STAT pathways (Dong et al., 2012). Injection of *B. bassiana* conidia resulted in melanization in the malaria vector *Anopheles gambiae* and this melanization response retarded *B. bassiana* growth and dissemination (Yassine et al., 2012). However, the biochemical mechanisms involved in host insect melanization against the infection of entomopathogenic fungi remain unclear (Vilcinskas et al., 1999; Dong et al., 2012; Yassine et al., 2012).

Melanization reaction, which leads to the synthesis of melanin from the activation of prophenoloxidase (PPO), is apparently a universal innate immune response in insects (Liu et al., 2007; Lu et al., 2008). It combines with other immune responses such as hemolymph coagulation, phagocytosis, and antimicrobial peptide production to encapsulate and kill invading microorganisms (Cerenius et al., 2008). During melanization, soluble pattern-recognition proteins initially recognize non-self molecular patterns from the invading micro-organisms (Jiang et al., 2004). This recognition triggers the activation of a series of serine proteases, leading to the activation of prophenoloxidase-activating protease (PAP) (An et al., 2011; Gorman et al., 2007; Kan et al., 2008; Kim et al., 2008). Activated prophenoloxidase converts inactive prophenoloxidase to phenoloxidase (An et al., 2011; Jiang et al., 2003a,b; Lee et al., 1998; Zou et al., 2010). Active phenoloxidase catalyzes the oxidation of phenols to quinones, which spontaneously polymerize to form melanin (Nappi et al., 2009).

Serine proteases (SPs), especially those with a clip domain, are actively involved in both the prophenoloxidase-activation cascade and the Toll immune signaling pathway (Jang et al., 2006; Kambris et al., 2006; Roh et al., 2009). Clip domain SP consists of the clip domain at the N-terminus terminus and a SP domain at the C-terminus (Jiang and Kanost, 2000). They are secreted into hemolymph as inactive precursors and require the specific proteolytic cleavage at the activation site for playing its role in the melanization reaction (Jang et al., 2008; Jiang and Kanost, 2000).

The Asian corn borer, *Ostrinia furnacalis* (Guenée), is an important insect pest in Asia, causing serious damage on corn, sorghum, millet and other crops (Afidchao et al., 2013). Control of this pest with chemical insecticides is currently hindered by the cryptic nature of larval behavior. The strategy suppressing the corn borer larvae by *B. bassiana* has been proposed (Wagner and Lewis, 2000). However, the molecular and biochemical mechanisms involved in interaction between corn borer and *B. bassiana* are largely unknown. In previous work, we obtained an Asian corn borer transcriptome dataset using high-throughput Illumina sequencing, and identified 13 potential clip domain SP transcripts (Liu et al., 2014). Here, we further investigated the expression profiles of these 13 clip domain SPs under the challenge of *B. bassiana*. In particular, we discovered that two clip-domain serine proteases, SP1 and SP13, are involved in prophenoloxidase activation in *O. furnacalis* by forming a proteolytic cascade. SP1 activates recombinant proSP13 by cleaving its zymogen, and then the activated SP13 directly converts prophenoloxidase to produce phenoloxidase.

2. Material and methods

2.1. Insect rearing

Asian corn borer (*O. furnacalis* (Guenée)) larvae were reared on an artificial diet at 28 °C under a relative humidity of 70–90% and a photoperiod of 16 h light and 8 h darkness (Liu et al., 2014).

2.2. B. bassiana culture and conidia suspension preparation

B. Bassiana strain Vuillemin (a kind gift from Dr. Yongdan Li of China Agricultural University) were cultured on potato dextrose agar plates at 25 °C and 80% humidity. Conidia (spores) used for *O. furnacalis* infection were harvested from 3 to 4 week old cultures by scraping conidia off the surface and suspended into sterile deionized water containing 0.1% Tween-80. Conidia were separated from other mycelial structures over a sterile funnel packed with autoclaved glass wool, washed two times with ddH_2O by centrifugation at 4000 rpm, counted and diluted to 2×10^5 conidia/μl. Freshly prepared conidial suspensions were used for all experiments.

2.3. Induction of the melanin synthesis and PO activity in O. furnacalis by B. bassiana conidia

To check whether *B. bassiana* has the ability to induce the melanization response in the Asian corn borer, we injected 3 μl of *B. bassiana* conidial suspensions (2×10^5 conidia/μl) into *O. furnacalis* fifth instar day 0 larvae. Injection of sterile PBS was used as a control. Pictures were taken to document melanin synthesis on the body of injected larvae in several 1 h intervals. In addition, hemolymph samples were collected from fifth instar day 0 larvae and centrifuged to remove hemocytes as described previously (Qu et al., 2011). The obtained plasma (cell-free hemolymph) was pooled. Aliquots (0.5 μl) of plasma were incubated with 1.0, 5.0, 10, 20, and 30×10^4 *B. bassiana* conidia in a total volume of 10 μl at room temperature for 10 min. PO activity in the reaction mixtures was measured using dopamine as substrate (An et al., 2009). One unit of PO activity was defined as the amount of enzyme producing an increase in absorbance (ΔA_{490}) of 0.001 per min.

2.4. Sequence analysis of 13 potential clip domain SPs in O. furnacalis

We have identified 13 potential clip domain SP transcripts from the transcriptome of *B. bassiana* – injected *O. furnacalis* larvae (Liu et al., 2014). Now, we report detailed sequence analysis for these 13 clip domain SPs. Analysis of deduced amino acid sequences, including prediction of molecular weight, isoelectric point, and glycosylation sites, was carried out in the EXPASY (Expert Protein Analysis System) proteomics server (http://www.expasy.org). Multiple sequence alignment was performed by using the CLUSTALW program, along with the same region in the other invertebrate SPs with defined functions. The sequences (with GenBank accession number) used for the alignment were: *A. gambiae* CLIPB9 (HM070255); *Bombyx mori* BAEEase (ABB58762); PPAE (NP_001036832); *Drosophila melanogaster* Easter (NP_524362), Grass (NP_733197), Persephone (NP_573297), SPE (NP_651168), Spirit (NP_727276), Snake (NP_524338); *Holotrichia diomphalia* PPAF1 (BAA34642); *Tenebrio molitor* SAE (AB363979), SPE (AB363980). Phylogenetic trees were constructed by the neighbor-joining method using MEGA Version 5 software (Tamura et al., 2007). For neighbor-joining method, gaps were treated as characters, and statistical analysis was performed using the bootstrap method with 1000 replicates.

2.5. Quantitative RT-PCR (qRT-PCR) analysis of the induced expression profiles of 13 clip domain SPs

To investigate the transcriptional changes of 13 clip domain SP genes upon challenge with *B. bassiana*, Asian corn borer fifth instar day 0 larvae from the same batch were injected into the haemocoele with 3 μl of *B. bassiana* suspension (2×10^5 conidia/μl) or sterile water as a control. After 10 h, five larvae from challenged or control group were collected, and total RNA samples were individually prepared using TRizol Reagent (TIANGEN). One μg of total RNA samples equally from 5 larvae in each group was converted into first-strand cDNA using TIANScript RT Kit (TIANGEN). The