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TIL-type protease inhibitors may be used as targeted resistance factors to enhance silkworm defenses against invasive fungi

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ABSTRACT

Entomopathogenic fungi penetrate the insect cuticle using their abundant hydrolases. These hydrolases, which include cuticle-degrading proteases and chitinases, are important virulence factors. Our recent findings suggest that many serine protease inhibitors, especially TIL-type protease inhibitors, are involved in insect resistance to pathogenic microorganisms. To clarify the molecular mechanism underlying this resistance to entomopathogenic fungi and identify novel genes to improve the silkworm antifungal capacity, we conducted an in-depth study of serine protease inhibitors. Here, we cloned and expressed a novel silkworm TIL-type protease inhibitor, BmSPI39. In activity assays, BmSPI39 potently inhibited the virulence protease CDEP-1 of *Beauveria bassiana*, suggesting that it might suppress the fungal penetration of the silkworm integument by inhibiting the cuticle-degrading proteases secreted by the fungus. Phenol oxidase activation studies showed that melanization is involved in the insect immune response to fungal invasion, and that fungus-induced excessive melanization is suppressed by BmSPI39 by inhibiting the fungal cuticle-degrading proteases. To better understand the mechanism involved in the inhibition of fungal virulence by protease inhibitors, their effects on the germination of *B. bassiana* conidia was examined. BmSPI38 and BmSPI39 significantly inhibited the germination of *B. bassiana* conidia. Survival assays showed that BmSPI38 and BmSPI39 markedly improved the survival rates of silkworms, and can therefore be used as targeted resistance proteins in the silkworm. These results provided new insight into the molecular mechanisms whereby insect protease inhibitors confer resistance against entomopathogenic fungi, suggesting their potential application in medicinal or agricultural fields.

1. Introduction

Entomopathogenic fungi invade insects by penetrating their cuticles, using a combination of mechanical pressure and enzymatic degradation (Charnley, 1984; Goettel et al., 1989; Xiong et al., 2013). Cuticle-degrading proteases are important virulence factors. They are usually secreted onto the insect integument at the time of conidial germination and participate in the penetration of the host cuticle (Charnley, 2003; St. Leger, 1995). The overexpression of toxic proteases can significantly enhance the virulence of pathogenic fungi (St. Leger et al., 1996; Yang et al., 2005, 2011; Zhang et al., 2008). Although a basic model of fungal penetration of the insect integument has been proposed, how insects protect themselves against fungal invasion and the specific interactions between insects and fungi have yet to be clarified.

Unlike mammals, insects have no lymphocytes or immunoglobulin, so serine protease inhibitors play an important role in insect immunity (Cerenius et al., 2010; Fullaondo et al., 2011; Kanost, 1999; Tang et al., 2008). Studies have shown that

Abbreviations: FPI-F, fungal protease inhibitor-F; TIL, trypsin inhibitor-like; RACE, rapid amplification of cDNA ends; NTA, nitrilotriacetic acid; CDEP-1, cuticle-degrading protease 1; PPO, prophenol oxidase; rPPO, recombinant prophenol oxidase; ddH_2O, double-distilled water; hpi, hours postinfection; dpi, days postinfection.

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abundant extracellular proteases are secreted by pathogens during the invasion process, helping them to penetrate the host's physical barrier (St. Leger, 1995; Travis et al., 1995). To resist the invasion of pathogenic microorganism, the host produces large amounts of protease inhibitors in response to microbial proteases (Augustin et al., 2009; Clermont et al., 2004; Donpudsa et al., 2009; Wang et al., 2009).

A large number of protease inhibitors exist in the insect integument and hemolymph, and these are closely related to the insect's resistance to pathogenic microorganisms. After preinjection with a zymosan or inactivated yeast cells, *Galleria mellonella* larvae survived for a prolonged time after infection with *Beauveria bassiana*, and their cell-free hemolymph exhibited enhanced inhibitory activity against exocellular *B. bassiana* proteases, indicating the presence of an inducible inhibitor in the hemolymph (Vilcinskas and Wedde, 1997). These results also suggest that the capacity of insects to release fungal protease inhibitors is related to their antifungal capacity. Serine protease inhibitors in the silkworm hemolymph play important roles in the defense against microbial invasion (Eguchi, 1982; Kanost, 1999). Fungal protease inhibitor-F (FPI-F) of the silkworm inhibits the conidial germination and germ-tube development of *B. bassiana* (Yoshida et al., 1990), and also significantly suppresses the subtilisin produced by *Aspergillus melleus* and the protease activity in the culture filtrate of *B. bassiana* (Eguchi et al., 1993). BmSI-7 of *Rhipicephalus microplus* exerts inhibitory activity against subtilisin A and Pr1 protease from *Metarhizium anisopliae* (Sasaki et al., 2008). However, only small amounts of these protease inhibitors have been obtained, insufficient to perform further biological experiments.

In our previous study, we used a microarray to screen for genes with altered expression after microbial infection (Zhao et al., 2012). At least 19 serine protease inhibitors were upregulated or downregulated after oral microbial infection. It is noteworthy that the expression levels of various trypsin inhibitor-like (TIL)-type protease inhibitors were significantly upregulated after *B. bassiana* infection. We also identified a structurally unique TIL-type inhibitor in the silkworm, designated “BmSPI38”, and demonstrated its inhibitory activities against microbial proteases (Li et al., 2012b). BmSPI38 also inhibits the harmful melanization caused by the fungal proteases produced by entomopathogenic fungi. Our preliminary results suggested that these serine protease inhibitors are involved in the silkworm's resistance to pathogenic microorganisms.

To clarify the molecular mechanism underlying the resistance of insects to fungal invasion and to identify novel target compounds with which to improve the silkworm's antifungal capacity, we also cloned and expressed the gene encoding another silkworm TIL-type protease inhibitor, BmSPI39, measured its activities, and investigated the molecular mechanism of BmSPI39 action against fungal invasion. Our results show that BmSPI38 and BmSPI39 both significantly enhanced the antifungal capacity of the silkworm, and could be used as targeted resistance factors in the silkworm. This study provides valuable information about the molecular mechanism of immune antifungal defenses and the genetic modification of this family of protease inhibitors.

2. Materials and methods

2.1. Silkworms, other organisms, and reagents

The Dazao strain of *Bombyx mori* was maintained at the Gene Resource Library of Domesticated Silkworm, Southwest University, China. The silkworms were reared on fresh mulberry leaves under standard conditions. Two-day-old pupae were collected for RACE (rapid amplification of cDNA ends)-Ready cDNA preparation. The middle silk glands from day 3 fifth instar larvae were collected for RNA isolation. Three-day-old pupae and wandering silkworms were prepared for protein injection and *B. bassiana* infection, respectively. The *B. bassiana* used was *B. bassiana* (Bals.) Vuill (Bb1), which was obtained from the silkworm cadavers (Dazao strain of *B. mori*), and was maintained at the State Key Laboratory of Silkworm Genome Biology, Southwest University, China. The fungus was cultured at 25 °C on potato dextrose agar and harvested after two weeks. *Escherichia coli* strains BL21(DE3) and Origami 2(DE3) were purchased from Invitrogen (USA), and were cultured in 2 × YT medium at 37 °C. Trypsin and α-chymotrypsin from the bovine pancreas, elastase from the porcine pancreas, subtilisin A from *Bacillus licheniformis*, protease K from *Engyodontium album*, papain from *Carica papaya*, and protease from *A. melleus* were purchased from Sigma–Aldrich (USA). Cuticle-degrading protease 1 (CDEP-1) comes from the pathogenic fungus, *B. bassiana*, and was a gift from our collaborator as listed in the acknowledgments.

2.2. cDNA cloning and sequencing analysis

RACE-Ready cDNA preparation and 3′- and 5′-RACE PCR were performed according to the GeneRacer™ Kit manual. Based on the EST sequences of BmSPI39, we designed four gene-specific primers, including a forward primer (5′-GGCTCTCATAGCCAGTAACAGTTCGT-3′), a forward nested primer (5′-GAGTGAGTGCGTCAACTGTTTCCAT-3′), a reverse primer (5′-ATGGAAACA GTTGACGCACTCACTC-3′), and a reverse nested primer (5′-ACGAACTGTTACTGGCTATGAGAGC-3′). The PCR products were separated on agarose gel, purified with a Gel Extraction Mini Kit (Watson Biotech, China), cloned into the pEASY-T1-simple Vector (TransGen Biotech, China), and sequenced. The protein properties, including its molecular weight, isoelectric point, signal peptide, and domains, were predicted with these online services: http://Web.ExPASy.Org/compute_pi/, http://www.cbs.dtu.dk/services/SignalP/, and http://smart.EMBL-Heidelberg.de/. The nucleotide and deduced amino acid sequences of BmSPI39 were analyzed with the BioEdit program.

2.3. Protein expression and purification

Total RNA was extracted from the middle silk glands of day 3 fifth instar larvae using TRIzol Reagent (Invitrogen, USA). Based on the full-length cDNA sequence of BmSPI39, we designed two primers for the PCR amplification of the coding region, without the signal sequence. The PCR was conducted using the forward primer (5′-CGCCATATGTTTGAAAAAGATTGTCCTG-3′) containing an *Nde*I site (underlined) and the reverse primer (5′-ATTTGCGGCCGCT-TATGACTGTTGTTTATGG-3′) containing a *Not*I site (underlined). The purified PCR product was cloned into a derivative expression plasmid of pET28a (designated p28), which was provided by Congzhao Zhou, University of Science and Technology, China. The resultant plasmid was transformed into *E. coli* strain BL21(DE3) cells or Rosetta(DE3) cells, and expressed as a recombinant protein with 9 residues of a polyhistidine tag at the N-terminus, after it expression was induced with 0.2 mM IPTG at 16 °C for 20 h or at 37 °C for 4 h. The *E. coli* cells were lysed by sonication in binding buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.9). After centrifugation (16,000 × *g*, 4 °C, 30 min), the separated lysates were resolved with 15% reduced SDS-PAGE. The recombinant protein from the BL21(DE3) cells was purified with Ni^{2+}-NTA (nitrilotriacetic acid) affinity chromatography (Merck). The protein concentrations were measured as described by Bradford (Tiangen, China) (Bradford, 1976).