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Inductive expression patterns of genes related to Toll signaling pathway in silkworm (*Bombyx mori*) upon *Beauveria bassiana* infection

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ABSTRACT

Innate immunity is critical to insects and plays an important role in pathogen elimination and wound healing. Toll signaling pathway is the major signaling pathway associated with insect innate immunity mediating synthesis of anti-fungal/bacterial peptides. To better understand the Toll signaling pathway mediated immune response in *Bombyx mori* against *Beauveria bassiana* infection, expression patterns of genes encoding sixteen putative components of Toll signaling pathway in the silkworm larvae challenged with *B. bassiana*, including four pattern recognition receptors (PRRs, i.e. *BmβGRP 1*, *2*, *3*, *4*), eight Toll-like receptors (TLRs, i.e. *Bm18w*, *BmToll 1*, *3*, *6*, *9*, *7*, *10*, *11*) and four effectors (*BmMoricin 1*, *BmGloverin 2*, *BmDefensin 1* and *BmLysozyme 1*), were analyzed using quantitative real-time PCR. At the same time, the changes in their expression by RNAi knock-down of the four PRRs were also detected. Moreover, the effects of Toll signaling pathway inhibitors on antifungal activity in larvae hemolymph were also analyzed. The results showed that the expression levels of genes encoding sixteen putative components of Toll signaling pathway were obviously altered by the challenge with *B. bassiana*, but their temporal regulation mode was significantly different. Based on the expression patterns of the genes related to Toll signaling pathway, two sub-paths of immune signal recognition and transduction might be proposed in the response of silkworm larvae against *B. bassiana* infection. Besides, Toll signaling pathway inhibitor could significantly inhibit the antifungal activity in hemolymph and resulted in increased sensitivity of silkworm larvae to the *B. bassiana* infection, while the treatment with heat-inactivated *B. bassiana* could induce antifungal activity in the hemolymph and led to stronger resistance of the silkworm. These results implied that Toll signaling pathway played important roles in the antifungal immune response of the silkworm larvae, in which different components of Toll signaling pathway might play a specific regulatory function. These findings yield insights into the innate immune mechanisms underlying Toll signaling pathway in silkworm.

Introduction

Bombyx mori is a model insect for *Lepidoptera* and has contributed enormously to the study of insect immunology especially the humoral immunity (Goldsmith et al., 2005). Insect innate immune response is initiated from the rapid recognition of non-self antigens and the amplification of the infected signals (Takahiro et al., 2007). Then, signal transduction pathway is triggered and effector molecules, especially antimicrobial peptide (AMPs), are activated and highly expressed generally in fat body and secreted into the hemolymph (Luna et al., 2002). So far, more than 200 AMPs have been identified and isolated from insects (Lamberty et al., 2001). They are divided into antibacterial peptides and antifungal peptides. The expression of antibacterial peptide genes is mainly regulated at transcriptional level, but the antifungal peptide genes is regulated at both the transcriptional and translational levels (Dimopoulos, 1997).

Two signaling pathways, Toll signaling pathway and Imd signaling pathway, are involved in mediating the synthesis of different AMPs for different pathogens. Generally, Toll signaling pathway quickly mediates the synthesis of antimicrobial peptides against the pathogenic fungi and gram-positive bacteria. Toll families are first discovered as a class of transmembrane protein receptors from *Drosophila*, with a typical leucine-rich repeat (LRR) in extracellular membrane domain and an intramembranous Toll-interleukin homolog domain (TIR) (Carl et al., 1988). Toll signaling pathway consists of three processes: pathogen recognition, signal transduction and expression of functional genes (Naitza and Ligoxygakis, 2004). For the Toll signaling pathway, the pathogens carrying pathogen associated molecular patterns (PAMPs) such as LPS and β-l,3-glucan (the main component of fungal cell wall) could be recognized by PRRs (Carl et al., 1988; Yoshida et al., 1986). Spaetzle protein was activated by the serine protease cascades downstream of pattern recognition receptors (PRRs) and that the mature

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Spaetzle was combined to LRR of TLRs, resulting in the allosteric regulation of TIR domain (Ao et al., 2008). The allosteric TIR could bind to the death domain (DD) of adapters such as MyD88, Pelle and Tube (Servane et al., 2002). MyD88, Tube and Pelle exit mostly as MyD88-Pelle-Tube complex and Pelle contains a serine-threonine kinase domain, similar to the interleukin receptor-associated kinase (IRAK) in mammals (Jean and Jules, 2001). TIR-MyD88-Pelle-Tube complex could trigger ubiquitination, phosphorylation and degradation of Cactus (IkB-like protein), and released the NF-kB-like protein (DIF or Dorsal) into the nucleus and regulate the expression of the downstream AMP genes (Uvell and Engstrm, 2007). Cheng and colleagues had identified *spatzle*, *toll*, *myd88*, *pelle*, *tube*, *traf2*, *cactus*, *moricin1*, *gloverin2*, *defensin1*, *lysozyme1* and other putative components of Toll signaling pathway and pointed out that Toll signaling pathway could be up-induced by *Staphylococcus aureus*, *Escherichia coli* and *B. bassiana* (Cheng et al., 2008). The transcriptional level of the *Bmspatzle 1* gene in hemolymph was clearly up-regulated after *B. bassiana* infection, but was not obviously increased after *B. mori* nucleopolyhedrovirus (BmNPV) infection, and in the midgut after *B. mori* cypovirus (BmCPV), *B. mori* bidensovirus (BmBDV) and *Nosema bombycis* (Nb) infections, suggesting that the Toll signaling pathway was mainly activated by challenge with the fungus *B. bassiana* (Liu et al., 2015). Transcriptome analysis of silkworm during early response to *B. bassiana* challenges found that total 89 differentially expressed genes, including 44 up-regulated and 45 down-regulated ones were involved in 5 significantly enriched KEGG pathways (Qvalue ≦ 0.05), including β-1,3-glucan recognition protein, Toll-like receptors, Cactus, Myd88, Moricin 1, Lysozyme precursor and other components of Toll signaling pathway (Hou et al., 2014). However, the molecular mechanism of Toll signaling pathway mediated immune response in silkworm against fungal infection is largely unknown.

In the present study, the inductive temporal expression levels of genes encoding sixteen putative components of Toll signaling pathway in silkworm larvae after challenging with *B. bassiana* were measured using quantitative real-time PCR (qRT-PCR), and the changes in their expression levels by RNAi knock-down of the four PRRs were also detected. Meanwhile, the regulation of Toll signaling pathway inhibitors on antifungal activity in silkworm larvae hemolymph were analyzed. All the efforts were made to better understand the molecular mechanisms of silkworm antifungal immune response.

Material and methods

B. bassiana and silkworm

Domestic silkworm strain p50 (Dazao) (provided by Sericultural Research Institute, Chinese Academy of Agricultural Sciences) was used as target insect in this study. Silkworm larvae were reared with fresh mulberry leaves under a standard condition (90% humidity at 26 °C) until experimentation. *B. bassiana*, named as HN-6 strain, was originally isolated from the naturally occurred white muscardine silkworm larvae, was preserved in Sericultural Research Institute, Chinese Academy of Agricultural Sciences.

Preparation of B. bassiana conidia suspension

B. bassiana was cultured on potato dextrose agar (PDA) medium for 1 week in tubes at 27 ± 1 °C until the conidia generated. The conidia were washed down with 0.15 M NaCl solution and transferred into a 50 mL centrifuge tube with 20 μL Tween-80, vortex shaked for 10 min and filtered with sterilized absorbent cotton. The conidia suspension was diluted with 0.15 M NaCl solution to the concentration of 1×10^8 spores/mL. 1 mL of the prepared conidia suspension was added into a 50 mL flask containing 10 mL potato dextrose (PD) medium and cultured at 30 ± 1 °C, 180 rpm for 3 days. The mixture of hyphae and blastospores were autoclaved at 121 °C for 20 min. The resulted heat-inactivated suspension of *B. bassiana* hyphae and blastospores was used for immune induction in silkworm.

Infection of silkworm larvae with B. bassiana

The second instar silkworm larvae were submerged in *B. bassiana* conidia suspension (10^8 spores/mL, with 1% penicillin-streptomycin) for 5 s, and for the control group in 0.15 M NaCl solution for the same period, two repetitions for each treatment. Then the larvae were fed with fresh mulberry leaves under a standard condition. The tests for every treatment were conducted in three replicates. 5 larvae were collected and put into a cryotube every 3 h till all the inoculated larvae died (about 48 h after infection). The cryotubes were frozen in liquid nitrogen and stored immediately at −80 °C for the preparation of total RNA.

Synthesis of cDNA and qRT-PCR

Total RNA was extracted using RNApure ultrapure total RNA extraction kit (BLKW Biotechnology, RN0302) and the residual genomic DNA was digested using RNase-free DNase I (Takara) according to the manufacturer's instructions. 2 μg total RNA was reverse-transcribed with TUREscript 1st Strand cDNA Synthesis Kit (BLKW Biotechnology, PC1802). Expression patterns of genes encoding sixteen putative component of Toll signaling pathway were detected using qPCR. The *B. mori* housekeeping gene *Actin 3* was used as an internal control for normalization. The primers were designed using Premier 5.0 and the sequences were shown in Table 1.

qRT-PCR was carried out using Applied Biosystems 7300 real-time PCR system with SYBR green dye. The 20 μL reaction mixture contained 2 μL cDNA, 0.5 μL each primer (10 mM), 10 μL 2 × qRT-PCR mix (BLKW Biotechnology, Beijing, China), and 6 μL ddH_2O. Thermal cycling

Table 1
Primers for qRT-PCR.

Gene		Sequence	GenBank accession NO.
BmActin	F	CCGTATGCGAAAGGAAATCA	NM_001126254.1
	R	TTGGAAGGTAGAGAGGGAGG	
BmβGRP 1	F	CCTTGGAGTCAAAGTATGGC	NM_001043375.1
	R	CAGTTATGATGGGTGGCAAA	
BmβGRP 2	F	AGCAATTTGATTCCCTGGAC	NM_001043985.1
	R	TAGGACACGAAGGGGTATTC	
BmβGRP 3	F	AAAGATTTGACGAGTTGGGG	NM_001135200.1
	R	TTTACTCTCCAAAAGCACCG	
BmβGRP 4	F	TCGAATCCAGAGGCAACA	NM_001166142.1
	R	TTCCCATCCGCTTAGTCC	
	R	GTTCCATTTTCAGTTCGGGA	
Bm18w	F	TGGGAGCGTCTCAGGTAT	NM_001123349.1
	R	CAGGCGACTTATCAGGAA	
BmToll 1	F	AGACAACATGGGCGTCACTA	XM_012691451.1
	R	TCAACAAAAGCGTCGTCTGG	
BmToll 3	F	TTGGAAGAGCTCAGACTGGG	XM_004927259.2
	R	TCACGGTCAGCTCTTTGGAT	
BmToll 6	F	CGGCAGTTGGAAAAGTTGGA	XM_012697885.1
	R	AACTGCGCGTTCTCTGAATC	
BmToll 7	F	CGTCGCGACTCTCATTCAAG	XM_004921675.1
	R	CATTGCACTCCACCATACCG	
BmToll 9	F	ACCCGTTATCAAGCACCAGA	XM_004921685.2
	R	CGGGAGCTAACGAACTTTGG	
BmToll 10	F	TAAAAGGTTCGGAGGCGAGT	XM_012688476.1
	R	TCGGACGAAGGTGAACTCTC	
BmToll 11	F	AGACAACATGGGCGTCACTA	XM_004925618.2
	R	TCAACAAAAGCGTCGTCTGG	
	R	ACTGTCTCTTCGCTCT	
BmMoricin 1,	F	AAAAACAGTAAACCGCGCA	NM_001043364.2
	R	TTTACCGACTGCCTTTCCTA	
BmGloverin 2,	F	GAAGATTACTCGATCAGCGG	AB190864.1
	R	CTTTTCCAAAGAGGCCATCA	
BmDefensin 1	F	CACGGTGCTCGTGTTTGT	DQ118523.1
	R	ATCCGCCCAGGAATCTTA	
BmLysozyme 1	F	TTCTGAAGCCAAAACGTTCA	NM_001043983.1
	R	TGGAACAATCCGTAGTCCTT	