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Construction and preliminary analysis of a normalized cDNA library from *Locusta migratoria manilensis* topically infected with *Metarhizium anisopliae* var. *acridum*

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ABSTRACT

The insect immune response to fungal infection is poorly understood at the molecular level. To explore the molecular basis of this process, a novel method to analyze the gene transcripts of insects in response to pathogenic fungus was established. A normalized cDNA library based on the SMART method combined with DSN (duplex-specific nuclease) treatment was constructed using mRNA extracted from the fat body and hemocytes of *Locusta migratoria manilensis* 6–24 h after being topically infected with *Metarhizium anisopliae* var. *acridum*. Analysis of 259 unigenes out of 303 sequenced inserts from the cDNA library revealed that the cDNA library was not contaminated with *M. anisopliae* transcripts and validated the presence of the immune-related genes characterized here. These results suggest that this method overcame the difficulties of contamination from a fungal source in constructing the host cDNA library from mycosed insects and proved that this method is reliable and feasible for investigation of host genes in response to fungal infection. Further studies of the expressed sequence tags from this library will provide insights into the molecular basis of insect immune response to fungal infection.

1. Introduction

Insect innate immunity is the first line of defense against invading pathogens, and it involves cellular and humoral reactions (Lavine and Strand, 2002; Lavine et al., 2005). Cellular defense refers to a hemocyte-mediated immune response such as phagocytosis, nodulation, and encapsulation. In the humoral defense response, the antimicrobial peptides (AMPs) or proteins are mainly synthesized in the fat body (equivalent of liver in mammals) and secreted into the hemolymph to eliminate foreign invaders (Gillespie et al., 1997). The transcription of these genes will reach a maximum at 12–48 h after immune stimulation (Hoffmann, 1995), but the immunodeficient insects cannot transcribe the mRNA of AMPs or express them at very low levels (Lowenberger et al., 1999). Insects respond to microbial infection by the rapid and transient expression of several genes encoding potent AMPs. Once immune activated, transcripts can be detected by 6 h, resulting in 1–100 mM concentrations of specific peptides in the hemolymph within 24 h (Hetru et al., 1998). The induction of AMPs is fast in response to infection, and the peptides are detectable in the hemolymph 2–4 h after infection (Uttenweiler-Joseph et al., 1998). Pattern-recognition, the interactions between conserved molecular structures present on the surface of pathogens and host proteins, is at the beginning of the innate immunity (Medzhitov and Janeway, 1997).

The insect immune response to fungal infection has been of great interest (Pendland and Boucias, 1993; Bidochka et al., 1997; Gillespie et al., 2000a,b). The majority of studies of the host insect immune response to infection have focused on injury via the alimentary canal or factitious injection. There are many advantages to these infection methods, such as the rapid appearance and easy detection of the pathological immune response. A number of studies on the interactions between insects and fungi have involved the injection of conidia or *in vitro* production of blastospores (Hung et al., 1993; Boucias et al., 1994; Mazet et al., 1994; Ursic-Bedoya and Lowenberger, 2007). However, this inoculation method may cause microbial contamination (Ursic-Bedoya and Lowenberger, 2007) and does not accurately mimic the natural immune response because it bypasses the natural route of infection and involves a form of the fungus that is not normally present in the hemolymph. Entomopathogenic fungi have the ability to penetrate the cuticle directly to establish infection. It is to be confirmed that signal exchange between insect and fungus during penetration of the cuticle plays a significant role in the immune response when the insects were inoculated topically with conidia (Gillespie et al., 2000a,b).

Transcript expression profiles of insects responding to fungal infection will provide invaluable information on the molecular

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immunology of insects. The identification of insect defense genes will help establish new strategies in biological control (St Leger and Bidochka, 1996). The oriental migratory locust *Locusta migratoria manilensis* is an orthopteran pest and a representative member of hemimetabolous insects (Ma et al., 2006). *Metarhizium anisopliae* var. *acridum* is an important entomopathogenic fungus used for the biocontrol of pests, including locusts and grasshoppers (Lomer et al., 2001; Elliott et al., 2002). Oil-based formulations of *M. anisopliae* have been field-tested successfully against locusts and grasshoppers in West and South Africa (Scherer et al., 1992; Douro-Kpindou et al., 1995), and China (Peng et al., 2008). The slow speed of killing is perceived as the drawback of mycoinsecticides. Because of the genome sequencing and EST projects, information on the transcripts expressed during the immune reaction of several insects has been obtained (Khush and Lemaitre, 2000; Ursic-Bedoya and Lowenberger, 2007; Dimopoulos et al., 2000; Holt et al., 2002; Cheng et al., 2008). However, in Orthoptera, particularly in locusts, the mechanisms that regulate insect immune responses are largely unknown. The migratory locust has an unusually large genome size of 8500 Mb, which is 70 times larger that of the *Drosophila melanogaster* genome (Kang et al., 2004). Construction of a cDNA library is an efficient way of intensively studying the immune response to fungal infection; however, fungal contamination is a major obstacle in the construction of such cDNA libraries. Therefore, no attempt has been made to construct cDNA libraries from mycosed insects.

In this study, a new method was developed for the construction of a normalized cDNA library from the fat body and hemocytes of the host insect *L. migratoria* 6–24 h after topical infection with the entomopathogenic fungus *M. anisopliae*. Analysis of the cDNA library demonstrated that the library contained immune-related genes without any *M. anisopliae* transcripts. Thus, this method is reliable and feasible for constructing host cDNA libraries from mycosed insects.

2. Materials and methods

2.1. Fungus culture and insect maintenance

M. anisopliae (isolate CQMa102) was cultured on 1/4 strength Sabourauds Dextrose agar at 28 °C for 7–10 days under constant light. The suspension of fungal spores was prepared according to Mullen and Goldsworthy's method (Mullen and Goldsworthy, 2006).

Immune response to infection may be affected by physical activities, such as oviposition, of the adult females. Therefore, mature adult male locusts from the gregarious population were used in all experiments and were reared according to Gillespie's description (Gillespie et al., 2000a,b).

2.2. Immune activation

Locusts were chilled on ice for 30 min prior to natural inoculation with an LD_{50} dose, viz, 5 μl of cotton seed oil containing 7.5×10^4 conidia (Scherer et al., 1992) applied under the pronotum using a micropipette. Treated locusts were housed individually in an incubator at 28 ± 2 °C with a 16 h light:8 h dark photoperiod and were fed wheat seedlings and water.

2.3. Tissue collection and mRNA isolation

Hemolymph was collected from the arthrodial membrane of the locust hind leg. The membrane was first swabbed with 70% ethanol, allowed to air dry and then pierced with a sterile needle. The hemolymph at 6 h, 12 h, and 24 h after inoculation of *M. anisopliae* conidia were collected and immediately diluted 10-fold in ice-cold *Locusta* Ringer (Clements and May, 1974) to prevent coagulation. The collected hemolymph was centrifuged at 600 × *g* for 5 min at 4 °C, the supernatant was removed, and the pellet was used for mRNA isolation. Fat bodies were dissected at 6 h, 12 h, and 24 h after inoculation of *M. anisopliae* conidia and thoroughly rinsed in ice-cold PBS to wash any contaminants. Total RNA extraction was performed using Trizol reagent (Invitrogen, USA) according to the manufacturer's specifications. mRNA was obtained from total RNA with Quick Prep™ Micro mRNA Purification Kit (Pharmacia, UK) according to the manufacturer's protocol. mRNA was quantified by measuring the absorbance at 260 nm and the quality of mRNA was assessed by measuring the ratio of absorbance at 260 nm/280 nm in a UV-Spectrometer (Beckman, USA).

2.4. Synthesis of cDNA

First-strand cDNA was synthesized from the mixture of 500 ng mRNA from fat body and 500 ng mRNA from hemocytes using Superscript II reverse transcriptase (Invitrogen, CA, USA) in a reaction mixture containing SMART IV oligonucleotides and CDS-3M adaptor. The resulting first-strand cDNA was used as a template for PCR amplification of double-stranded (ds) cDNA in a 100 μl reaction mixture containing 2 μl first-strand cDNA, 2 μl dNTP mix (10 mM each), 4 μl 5′ PCR primer (10 μM), 10 μl 10 × PCR buffer, 10 μl $MgCl_2$ (25 mM), 1 μl LA Taq (5 U/μl, TAKARA, Japan), and 71 μl MilliQ water. The PCR was carried out with the following program: 94 °C for 4 min and 21 cycles of 94 °C for 25 s and 68 °C for 6 min. The amplified ds cDNA products were electrophoresed on a 1.2% agarose/EtBr gel in 1 × Tris-Acetate–EDTA buffer and smeared bands, ranging from 500 bp to about 4000 bp, were purified and recovered using the Gel Extraction Kit (Bioflux, Japan) for normalization.

Oligonucleotides (5′–3′) for cDNA synthesis were: SMART IV oligonucleotide, AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG; CDS-3M PCR primer, AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCC$(T)_{20}$VN, where (N = A, C, G or T; V = A, G or C); 5′ PCR primer: AAGCAGTGGTATCAACGCAGAGT.

2.5. Construction of normalized cDNA library

Normalized cDNA library was constructed using Creator SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA) combined with DSN (Evrogen, Moscow, Russia).

Approximately 300 ng of ds cDNA was normalized by DSN according to the manufacturer's recommendations. The ds cDNA was denaturalized at 98 °C for 2 min, followed by incubation at 68 °C for 5 h; then, 1 μl of 1/4 DSN (1 μl of DSN solution diluted with 3 μl of storage buffer) was used to degrade the reassociated ds cDNA for 20 min. This normalized ds cDNA was amplified by PCR in a 50 μl reaction mixture consisting of 1 μl normalized ds cDNA, 4 μl dNTP mix (2.5 mM each), 2 μl 5′ PCR primer (10 μM), 5 μl 10 × PCR buffer, 5 μl $MgCl_2$ (25 mM), 0.5 μl LA Taq, and 32.5 μl MilliQ water. PCR was conducted under the following conditions: 94 °C for 4 min and 20 cycles of 94 °C for 25 s and 68 °C for 6 min. The PCR product was purified with a PCR extraction kit (Qiagen, Netherlands) and then digested by *Sfi*I restriction enzyme, followed by cDNA size fractionation using CHROMA SPIN-400 (Clontech, Palo Alto, CA, USA). Approximately 150 ng of digested and fractionated cDNA was ligated into 100 ng of *Sfi*I-digested, dephosphorylated pDNR-LIB vector that was provided with the kit. Finally, the recombinant plasmids were transformed into *Escherichia coli* XL1-Blue competent cells and the transformed cells were recovered in 1 ml of LB by shaking at 180 rpm at 37 °C for 1 h.